School of Pharmacy and Biomedical Sciences

Investigation of Novel Biomarkers for Pancreatic Cancer

Aikaterini Emmanouilidi

This thesis is presented for the Degree of

Doctor of Philosophy

of

Curtin University

October 2019

Declaration

To the best of my knowledge and belief, this thesis contains no material previously published by any other person except where due acknowledgement has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council National Statement on Ethical Conduct in Human Research (2007). The research study received human research ethics approval from the Curtin University Human Research Ethics Committee, Approval Number HR54/2014.

Signature: 

Date: October 24th, 2019

Acknowledgements

First and foremost, I would like to thank my supervisor Professor Marco Falasca for giving me the opportunity to come to Australia and undertake this PhD project. It has been a long and challenging journey and I am deeply grateful for his support, for believing in me, teaching me valuable life lessons, encouraging me to set high goals and be the best version of myself. Thank you Professor for all the long and constructive talks which helped me evolve as a scientist and as a person. I consider myself lucky to have been part of your group.

I would also like to extend my gratitude to my co-supervisor, Dr Pat Metharom, for her support throughout these years, as well as our collaborators Dr Connie Jackaman, Dr David Greening and Prof Peter Meikle. A big thank you to the staff of the Curtin Health Innovation Research Institute for their kindness and support. My special thanks also goes to Mrs Jeanne Edmands, Senior Technical Officer in CHIRI, for her invaluable help with the flow cytometry part of my project.

I thank my fellow lab-mates, past and present, for all the encouragement, the laughs and the beautiful moments we shared and I will always cherish. I also want to express my gratitude to Mrs Ilaria Casari, for always being willing to lend me an ear and give me advice.

Thank you also to all my dear friends who despite being in completely different time zones, they were always there for me to discuss and lift my spirit.

Finally, I am eternally grateful to my mother and father, who have always supported my choices no matter how big of an impact that would have on their lives. Had it not been for their endless love, this achievement would not have been possible.

Contents

Acknowledgements 2

List of primary publications 5

This thesis contains 4 first author peer-reviewed publications listed below, with author contributions and copyrights permissions being included in the Appendix section. 5

Abbreviations 6

Abstract 9

Chapter 1: Literature Review 12

1.1 Introduction 12

1.2 The pancreas – physiology and structure 12

1.3 Pancreatic ductal adenocarcinoma – pancreatic intraepithelial neoplasia and genetics 13

1.4 Epidemiology 17

1.4.1 Established factors 17

1.4.2 Putative risk factors 18

1.4.3 Dietary factors 19

1.5 Biomarkers 19

1.5.1 Lewis antigens and CA19.9 20

1.6 Exosomes 23

1.6.1 Biology of Exosomes 23

1.6.2 Current methods on EV and exosome research 26

1.6.3 Physiological roles of exosomes 32

1.6.4 Exosomes in cancer and pancreatic cancer 36

1.7 Metabolic reprogramming – Lipid metabolism 39

1.7.1 Lipid metabolism in cancer 39

1.7.2 Lipid metabolism in pancreatic cancer 41

1.8 Study Aims 43

Chapter 2: Characterization of the proteome of pancreatic cancer-derived exosomes as potential biomarkers for PDAC 45

2.1 Article synopsis 45

2.1.1 Background 45

2.1.2 Experimental design in brief 46

2.1.3 Results in brief 46

2.1.4 Concluding remarks in brief 46

Publication 1 48

Chapter 3: Lipidomic analysis of PDAC exosomes and corresponding cells 62

3.1 Introduction 62

3.2 Study aims 65
3.3 Materials and Methods 66
3.3.1 Cell lines 66
3.3.2 Samples collection for lipidomic analysis with mass-spectrometry 67
3.3.3 Mass spectrometry 67
3.3.4 Lymphocytes isolation from healthy donors' whole blood 68
3.3.5 T cell proliferation assay 69
3.3.6 Cytokine expression assay 70
3.4 Results 70
3.4.1 Lipidomic analysis of pancreatic, prostate and ovarian epithelial and cancer cells and their respective exosomes 70
3.4.2 The effect of CE depletion on T cells proliferation and subsequent cytokine expression 79
3.5 Discussion 89
Chapter 4: Targeting PDK1 in pancreatic cancer 98
4.1 Articles synopsis 98
4.1.1 Background 98
4.1.2 Experimental design in brief 99
4.1.3 Results in brief 99
4.1.4 Concluding remarks in brief 99
Publication 2 100
Publication 3 105
Publication 4 131
Chapter 5: General discussion, limitations and future directions 145
Bibliography 149
Appendix 170

List of primary publications

This thesis contains 4 first author peer-reviewed publications listed below, with author contributions and copyrights permissions being included in the Appendix section.

1. **Emmanouilidi, A.**, Paladin, D., Greening, D. W., & Falasca, M. (2019). Oncogenic and Non-Malignant Pancreatic Exosome Cargo Reveal Distinct Expression of Oncogenic and Prognostic Factors Involved in Tumor Invasion and Metastasis. *Proteomics*, *19*(8), 1800158.

2. **Emmanouilidi, A**., & Falasca, M. (2017). 3-Phosphoinositide-Dependent Kinase 1 (PDK1). *Encyclopedia of Signaling Molecules*, 1-4.

3. **Emmanouilidi, A**., & Falasca, M. (2017). Targeting PDK1 for chemosensitization of cancer cells. *Cancers*, *9*(10), 140.

4. **Emmanouilidi, A**., Fyffe, C. A., Ferro, R., Edling, C. E., Capone, E., Sestito, S., ... & Maffucci, T. (2019). Preclinical validation of 3-phosphoinositide-dependent protein kinase 1 inhibition in pancreatic cancer. *Journal of Experimental & Clinical Cancer Research*, *38*(1), 191.

Abbreviations

ACAT-1 acyl coenzyme A-cholesterol acyltransferase 1

ADM acinar-to-ductal metaplasia

AFM atomic force microscopy

APCs antigen presenting cells

BMI body mass index

CAFs cancer-associated fibroblasts

CE cholesteryl ester

CNS central nervous system

COH cholesterol

DC dendritic cell

DG diacylglycerol

DLS dynamic light scattering

EGFR epidermal growth factor receptor

EM electron microscopy

ESCRT endosomal sorting complexes required for transport

EVs extracellular vesicles

FA fatty acid

FSC forward-scattered light

FUT fucosyltransferase

IFN interferon

IL interleukin

ILV intraluminal vesicle

IPMNs intraductal papillary mucinous neoplasms

ISEV International Society of Extracellular Vesicles

LBPA lysobisphosphatidic acid

LDLR low-density lipoproteins receptor

LPA lysophosphatidic acid

MAPK Mitogen-Activated Protein Kinase

MCNs mucinous cystic neoplasms

MHC major histocompatibility complex

MISEV Minimal Information for Studies of Extracellular Vesicles

MMP matrix metalloproteinase

MSCs mesenchymal stem cells

mtDNA mitochondrial DNA

MVB multivesicular body

MVEs multivesicular endosomes

NK natural killer

NSCLC non-small cell lung cancer

NTA Nanoparticle Tracking Analysis

ODC oligodendrocyte

PanINs pancreatic intraepithelial neoplasias

PBMCs peripheral blood mononuclear cells

PC phosphatidylcholine

PDAC Pancreatic ductal adenocarcinoma

PDK1 3-phosphoinositide-dependent protein kinase 1

PE phosphatidylethanolamine

PG phosphatidylglycerol

PI phosphatidylinositol

PI(4,5)P2 phosphatidylinositol 4,5-bisphosphate

PI3K phosphoinositide 3-kinase

PL phospholipid

PS phosphatidylserine

PSC pancreatic stellate cells

PSGL-1 P-selectin glycoprotein ligand-1

RAF Rapidly Accelerated Fibrosarcoma

SEC size exclusion chromatography

SEM scanning electron microscopy

SEM Standard error of the mean

SL sphingolipid

SM sphingomyelin

SREBP1 sterol regulatory element-binding protein 1

SSC side-scattered light

STB synctiotrophoblast

TEM transmission electron microscopy

TG triacylglycerol

TGF-β transforming growth factor beta

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a disease with a dismal prognosis and a mere 8-11% survival margin within 5 years post-diagnosis. It arises from the exocrine part of the pancreas and comprises more than 85% of pancreatic cancer cases. Its progression is rapid and by the time of diagnosis most patients present with metastasis, therefore being rendered unsuitable for surgical resection of the tumour. The current golden standard for disease diagnosis and patients monitoring is the carbohydrate antigen sialyl-Lewis α biomarker, also known as carbohydrate antigen 19.9 (CA19.9). However, CA19.9 levels are not only dependent on the Lewis status of the individual / patient, but also from the tumour size, as tumours < 3cm cannot be detected with the use of this biomarker and give false negative results. At the same time, CA19.9 levels can be affected by other pathologic conditions of the gastrointestinal tract, therefore this biomarker exhibits neither the desirable sensitivity nor specificity.

In recent years the potential of exosomes as biomarkers has emerged. Exosomes are part of the bigger Extracellular Vesicles (EVs) family and are lipid bilayer vesicles 30-150nm in diameter, shed by almost all cells and implicated in physiological and pathological conditions. They are derived from the late endosomes and shed in the intercellular space following fusion of multivesicular bodies with the plasma membrane. When they were first discovered in 1983, they were seen as a “garbage bin” relieving the cell from abundant or faulty molecules, however this notion soon changed. It is now established that exosomes are key mediators in intercellular communication and can greatly impact the behaviour of neighbouring cells as well as cells residing in distal sites. Great efforts have been and are being made in order to thoroughly characterize their cargo (comprising of DNA, RNA, proteins and lipids) and exploit is as a rich source of diagnostic and prognostic biomarkers.

This thesis mainly explores the potential of exosomes derived from pancreatic cancer cell lines as source of biomarkers for PDAC. In Chapter 1 an in–depth literature review covers topics ranging from the physiology of the pancreas and PDAC occurrence and epidemiology, to the biology of exosomes, current isolation and characterization methods used in exosome research and their roles in physiological conditions and also in cancer and pancreatic cancer. Metabolic reprogramming in pancreatic cancer is also discussed, as it affects many aspects of the disease.

In Chapter 2, an extensive characterization of the proteomic cargo of PDAC and pancreatic epithelial cells-derived exosomes as well as their donor cells is being presented. A total of 1725 and 271 proteins have been uniquely detected in cells and exosomes respectively, and 1023 were identified in both sample types. Malignant exosomes were enriched in proteins involved in hallmarks of cancer such as proliferation, invasion and metastasis, and also known PDAC biomarkers were confirmed to be present in our exosomal preparations.

In Chapter 3, the lipidome of pancreatic, ovarian and prostate cancer cells was analysed together with their respective exosomes. Corresponding non-malignant

epithelial cells and their respective exosomes were also analysed and used as points of reference. The outstanding finding of this study was the very high levels of cholesteryl esters (CE) specifically in the PDAC exosomes compared to all other exosome types – malignant and non-malignant. Taking into account the highly immunosuppressive environment of PDAC, and to further examine the biological relevance of this CE accumulation, we treated two PDAC cell lines with an acyl coenzyme A-cholesterol acyltransferase (ACAT)-1 inhibitor and we co-incubated the respective exosomes with CD3 CD28 stimulated $CD4^+$ and $CD8^+$ T cells. Consequent cell proliferation and cytokine expression was analysed, and treatment with avasimibe seemed to relieve the anti-proliferative effect of the untreated cells-derived exosomes, especially in $CD8^+$ cells. Moreover, cytokines including IFN-γ, IL-8, IL-23 and MCP-1 were downregulated upon incubation with exosomes derived from treated cells, compared to untreated controls, and the potential biological relevance is discussed.

In Chapter 4, the pharmacological targeting of the 3-phosphoinositide-dependent kinase-1 (PDK1) protein in PDAC is explored. More than 90% of PDAC cases bear *KRAS* mutations and this results in instigation of downstream pathways mediating cancer progression. One of these pathways is the PI3K/PDK1 pathway, and since *KRAS* itself is an undruggable cancer target, the possibility of targeting alternative major proteins is tempting. PDK1 is also involved in lipid metabolism and generation of lipids acting as second messengers, therefore its pharmacological attenuation could have many implications. Experimental outcomes depicted SGK3 and NDRG1 as the downstream effectors of PDK1 in pancreatic cancer signalling and indicated possible Akt independence. Moreover, *in vitro* combinatorial pharmacological inhibition of PDK1 and PI3K p110γ showed to have better outcome in cell growth than administration of each treatment alone, opening routes to dual targeting options towards hampering the progression of the disease.

Finally, in Chapter 5 limitations of the current study and future directions are discussed.

CHAPTER 1

Chapter 1: Literature Review

1.1 Introduction

The first historical report on the pancreas is dated back in the 4th century B.C. when Aristotle defined it as a distinct organ in his book “Historia Animalium” (in Greek “Περί τα ζώα ιστορίαι”), with Rufus of Ephesus and Galen adding more information in the following centuries (Tsuchiya, Kuroki et al. 2015). The word pancreas is a combination of the Greek words “pan” and “kreas”, which mean “all” and “meat”, respectively (Tsuchiya and Fujisawa 1997). These first studies regarding the structure of the pancreas were initially performed on the bodies of birds sacrificed for divination purposes, which was very common in ancient times, and later on apes (Tsuchiya, Kuroki et al. 2015). It was not until 1682 and onwards that physicians actually described the duct and revealed the multiple functions of this complex organ (thoroughly reviewed in (Busnardo, DiDio et al. 1983)).

1.2 The pancreas – physiology and structure

The pancreas consists of three different cell types, each of which corresponds to a different functional unit: the exocrine part, the endocrine part, and the supportive tissue. The exocrine pancreas contains acinar cells and ducts, with the former ones producing a number of digestive enzymes such as amylase, lipase, chymotrypsin and trypsin, and the latter ones transporting these enzymes further into the duodenum and comprising the component of the pancreas from which the vast majority of neoplasms arise (Figure 1). The endocrine pancreas consists of cells named “islets of Langerhans” crucial for hormones production including glucagon, insulin and somatostatin, and although endocrine malignancies are not uncommon, they still occur less frequently than the exocrine cancers. The supportive tissue is a mixture of immune cells, fibroblasts and vessels, and it is rarely implicated in neoplastic conditions (Basturk, Coban et al. 2010).

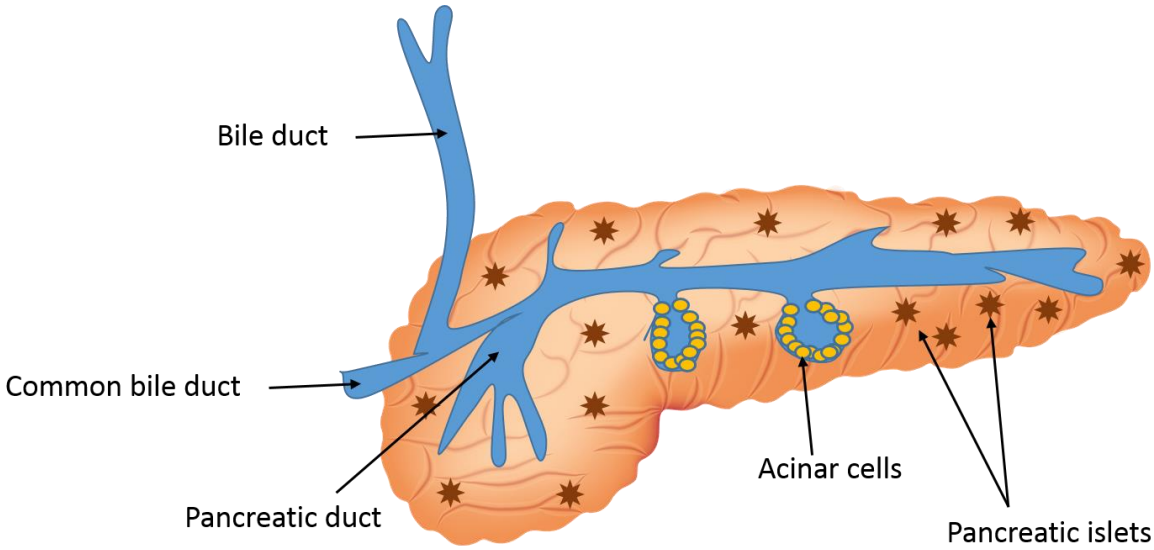


Figure 1 Structure of the pancreas. The pancreatic, common bile and bile duct, as well as acinar cells and pancreatic islets are displayed.

1.3 Pancreatic ductal adenocarcinoma – pancreatic intraepithelial neoplasia and genetics

Pancreatic ductal adenocarcinoma (PDAC) accounts for more than 85% of pancreatic malignancies and for this reason the term pancreatic cancer is often used to refer to this specific type. PDAC has a very rapid progression and eventually metastasizes to other organs such as liver and lymph nodes. Most patients develop a non-resectable tumour by the time they reach a diagnosis, and this greatly contributes to the lethality of the disease; the survival rate 5 years post diagnosis is 8-11% (Lambert, Schwarz et al. 2019). It is now known that PDAC does not arise *de novo*, but from pre-invasive lesions that gradually progress histologically and genetically into forming the invasive tumour (Koorstra, Feldmann et al. 2008). These lesions were initially acknowledged as pancreatic intraepithelial neoplasias (PanINs) during a National Cancer Institute meeting in 1999 in Utah (Hruban, Adsay et al. 2001). In brief, low-grade PanINs (PanIN-1A, PanIN-1B) comprise of columnar cells with unaffected nuclear polarity and can exhibit flat or papillary epithelium, respectively. In intermediate grades (PanIN-2), the nuclei lose polarity, get crowded and enlarged, and exhibit pseudo-stratification, while mitoses are infrequent. Lastly, high-grade PanINs (PanIN-3), alternatively called “carcinoma-in-situ”, exhibit extended loss of polarity, nuclear abnormalities and mitoses occurrence (Hruban, Adsay et al. 2001, Hruban, Takaori et al. 2004). Although PanINs are present in the pancreatic ducts that are less than 5mm (microscopic lesions), there are other forms of non-invasive neoplastic lesions that can be clinically detectable due to the mass formation (macroscopic lesions), such as

intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs). In MCNs, the epithelium comprises of columnar cells containing mucin and its supporting stroma expresses receptors for hormones such as progesterone and estrogen. IPMNs appear in pancreatic ducts of bigger size than PanINs and can be further categorized into “branch duct type” or “main duct type”, depending on the part of the duct involved (reviewed in (Koorstra, Feldmann et al. 2008)).

The genetic evolution from PanINs to PDAC includes mutations of oncogenes, genome-maintenance and tumour-suppressor genes, deregulation of the telomeres, aberrant epigenetic regulation, and abnormal signalling. The vast majority of PDAC cases (>90%) involve gain-of-function point mutations-harbouring *KRAS*, with the PanIN grade being correlated to the overall mutation numbers. (Eser, Schnieke et al. 2014). This underlines the importance of *KRAS* oncogene as one of the driving factors for PDAC initiation and progression. Opposite to oncogenes, tumour-suppressor genes impede abnormal cell proliferation, and their inactivation can promote cancer progression. *DPC4/SMAD4/MADH4*, *TP53* and *p16INK4A/CDKN2A* are the main tumour suppressor genes found to be inactivated in PanINs, due to either promoter hypermethylation, homozygous deletion or mutation of one copy, accompanied by loss of the other copy. Among the genome-maintenance genes compromised in pancreatic cancer are *hMLH1* and *hMLH2*, alteration of which leads to microsatellite instability, and gene members of the Fanconi anaemia family such as *FANCG*, *FANCC* and *BRCA2*. Inactivation of both copies of *BRCA2* (as well as *TP53*) is an event that happens in later stages of PDAC progression. As for the telomere length, it has been observed that it can be unusually short in early stages of the disease progression and in >90% of low-grade cases, and this can result in chromosomal fusion and therefore instability, adding even more to the abnormal genetic burden of PDAC. Finally, methylations of CpG islands in gene promoters occur as early events, whereas changes in mucins, cyclin D1 and cyclooxygenase-2 protein expression are detected in different stages, as are components of the Notch and Hedgehog signalling pathways (reviewed in (Koorstra, Feldmann et al. 2008)). Overall, it is widely accepted that PDAC progression is multifactorial and there is a high genetic complexity underlying the disease (Laghi, Orbetegli et al. 2002).

Although pancreatic cancer is a disease the progression of which follows a specific sequence of genetic mutations, clones that do not follow the expected tumour lineage often arise. As a result its genome presents as a mosaic with high instability, the provenance of which is yet to be elucidated. High genome instability often points towards events such as polyploidization and chromothripsis. A recent informatic

analysis of more than 100 PDAC genomes revealed the existence of polyploid tumours enriched for mutations in the *TP53* gene. In an attempt to put a time stamp on the polyploidization occurrence during the tumour evolution timeline, further analysis revealed that this phenomenon succeeded mutational events correlated with age and double-strand break repair, and on the contrary preceded copy numbers changes. Strikingly, it was shown that more than half of the examined PDAC cases had undergone at least one chromothripsis event which in many cases affected the genetic loci of either *KRAS* or *SMAD4*, and in addition to that it was passed on from the primary to the metastatic tumour. During the same study, single cell sequencing experiments directly proved that PDAC progression does not necessarily follow the established model of consecutive genetic mutations (Notta, Chan-Seng-Yue et al. 2016). The effect of chromothripsis in the context of cancer progression has also been previously studied by other groups, which concluded that this phenomenon can result from exogenous and endogenous factors such as ionizing radiation or during the cellular division, respectively. In regards to the latter, the cell's repair machinery detects and tries to restore the fragmented DNA which occurs during anaphase, resulting in major rearrangements which confer a detrimental phenotype to the cell and put it in a fast forward mode down the path to cancer development (Stephens, Greenman et al. 2011).

As aforementioned, almost 90% of PDAC cases carry *KRAS* mutations which result in a constitutively active protein that participates in a number of feedback loops and activates multiple downstream cascades imperative for cancer initiation as well as maintenance, the most important of which are the Rapidly Accelerated Fibrosarcoma / Mitogen-Activated Protein Kinase (RAF/MAPK) and Phosphoinositide 3-Kinase / 3-phosphoinositide-dependent protein kinase 1 (PI3K/PDK1) pathways (Lemstrova, Brynychova et al. 2017). These two pathways are crucial for PDAC treatment as *KRAS* itself is an undruggable cancer target. At this point it is worth mentioning that although the majority of KRAS mutations are yet not targetable, there has been some improvement in inhibitor development regarding the rare variant $KRAS^{G12C}$. More specifically, the first *in vitro* selective $KRAS^{G12C}$ inhibitor, named ARS-853, was developed in 2016 (Patricelli, Janes et al. 2016). This category of inhibitors later entered clinical trials, with the first drug candidate being MRTX849 (Hallin, Engstrom et al. 2020). Another successful candidate is AMG150, which exhibited remarkable results when combined with anti-PD-1 immunotherapy in the case of *in vivo* colon cancer models. Increased infiltration of $CD8^+$ T cells, dendritic cells and macrophages, and increased expression of specific interferons and chemokines, as well as MHC I

class antigens, were among the observed effects. AMG150 is currently in the clinical trial stage, offered as treatment for $KRAS^{G12C}$ advanced solid tumours (Canon, Rex et al. 2019).

Although mutations are very commonly found in PI3K and RAF kinases, the same does not apply to their effector proteins, for which a number of inhibitors are undergoing clinical testing (Collisson, Trejo et al. 2012). Regarding the RAF/MAPK pathway, pharmacological inhibition of MEK1/2 kinase *in vivo* has been shown to enhance the effect of Abraxane, a chemotherapeutic drug comprising of a conjugation of paclitaxel with albumin (Awasthi, Monahan et al. 2018), while in *in vitro* studies inhibition of the pathway results in increased levels of $p21^{Kip1}$ and concomitant cell cycle arrest (Gysin, Lee et al. 2005).

On the other side, amplified PI3K isoforms have been shown to be upregulated in many cancers, with the p110γ being the predominant one in PDAC (Edling, Selvaggi et al. 2010). A study by Eser *et al*. highlighted the importance of PDK1, a downstream effector of PI3K, as a pancreatic tissue-specific KRAS effector, noting its crucial role in PDAC establishment, acinar-to-ductal metaplasia (ADM) and cell plasticity (Eser, Reiff et al. 2013). *In vivo* experiments using transgenic mouse models showed that the expression of the mutated $p110\alpha^{H1047R}$ in the pancreas initiated ADM, resulting in PanINs (from PanIN-1A to PanIN-3), and exhibiting an almost identical phenotype to $Kras^{G12D}$ – driven PDAC in terms of metastatic events and survival, as well as activation of the same components of the PI3K pathway. Aberrant expression of the PI3K pathway at the early stages of pancreatic malignancy was also validated in human tissue microarrays, cell lines and xenografts. Interestingly, *PDK1* ablation in the pancreas epithelium resulted in successful blockage of PDAC and premalignant PanINs in transgenic mice, and was not accompanied by lethality and impaired pancreatic development apart from glucose tolerance defects which did not develop further. On the contrary, deletion of PDK1 in non-small cell lung cancer (NSCLC) transgenic mice models harbouring $Kras^{G12D}$ mutation had no effect in disease progression, demonstrating that despite the common genetic background, there is cell-specificity in regards to PDK1-dependent cancer initiation and progression (Eser, Reiff et al. 2013). More studies have recently provided further evidence, directly or indirectly linking PDK1 to pancreatic cancer. For instance, PDK1 has been shown to be regulated by the tumour suppressors miR-375 and miR-1271, with their expression being inversely correlated in pancreatic cancer (Zhou, Song et al. 2014, Xie, Huang et al. 2018), whereas its direct pharmacological inhibition has been shown to impair pancreatic cancer cells survival (Falasca, Chiozzotto et al. 2010) and its deletion has

been recently linked to re-sensitization of PDAC cells to gemcitabine (Li, Mullinax et al. 2018). An extensive analysis of PDK1 structure, function, involvement in chemoresistance and its pharmacological targeting in pancreatic cancer can be found in chapter 4 in the form of two peer-reviewed article reviews and one peer-reviewed research article.

1.4 Epidemiology

Pancreatic cancer occurrence is relatively low compared to other malignancies, however mortality levels are high in global ranking even in cases of early diagnosis. The rates of pancreatic cancer incidence greatly vary between countries, implying that the manifestation of the disease is attributed not only to genetic but also to epigenetic factors (Klein and Hepatology 2019). There is a number of factors involved in pancreatic cancer epidemiology, divided into three main categories: established, putative and dietary risk factors. In 2018, Hungary was the country with the highest incidence of pancreatic cancer globally (AICR 2018) and men are 1,5 times more likely to get affected than women. Lack of a current screening method makes prevention crucial and therefore identification of risk factors can assist in the explanation for differences related to gender and nationality, and serve as a powerful tool (Ilic and Ilic 2016).

1.4.1 Established factors

Heredity, age, smoking, obesity and diabetes mellitus are factors with great impact on the disease occurrence. Pancreatic cancer risk dramatically increases after 50 years of age, with the average diagnosis age being 72 years (Jiao and Li 2010). Inheritable traits have been found to play a role in 5-10% of all cases (Greer, Whitcomb et al. 2007). The disease can be linked to cystic fibrosis, hereditary pancreatitis or accompany as part of an existing malignancy syndrome (Lynch, Lanspa et al. 1989). Such syndromes include the familial atypical multiple-mole melanoma, Peutz-Jeghers syndrome, hereditary breast-ovarian cancer syndrome, familial adenomatous polyposis, Li-Fraumeni syndrome, hereditary pancreatitis and hereditary nonpolyposis colon cancer (Jiao and Li 2010). Cigarette smoking is responsible for 25% of cases. Studies have shown that long term exposure of animal models to tobacco-specific *N*-nitrosamines led to a development of pancreatic malignancies, whereas DNA adducts and compounds specific for tobacco could be detected in human pancreatic tissue and juice of affected individuals (reviewed in (Lochan, Reeves et al. 2011)). Additionally, smoking has been linked specifically to pancreatic

cancer K-ras mutations (Blackford, Parmigiani et al. 2009). However, there are no strong data supporting a contributory role of passive smoking to this malignancy (Jiao and Li 2010). Obesity, the second modifiable factor for pancreatic cancer, can be defined by the body mass index (BMI). BMI between 25.00 and 29.99 indicates a pre-obese state (overweight), whereas individuals with BMI > 30.00 are classified as obese (DoH 2014). A number of studies and meta-analyses have positively correlated obesity to pancreatic cancer onset (de Gonzalez, Sweetland et al. 2003, Larsson, Orsini et al. 2007, Renehan, Tyson et al. 2008). Of special interest is the fact that individuals exhibiting main and upper body weight gain (waist, chest and shoulders) have a higher relative risk of pancreatic cancer compared to those exhibiting peripheral weight gain (thighs, hips or equal overall distribution), independently of their BMI (Patel, Rodriguez et al. 2005). However, it is known that abdominal adiposity can lead to glucose intolerance; therefore it is possible that elevated insulin levels or insulin-like growth factors (IGFs) may promote malignant transformation of the pancreatic duct cells (Jiao and Li 2010). Diabetes mellitus (or Type 2 diabetes) is both a predisposing factor and an early indicator for pancreatic cancer onset (Gullo, Pezzilli et al. 1994, Fisher 2001) and almost 1% of sufferers over 50 years old will be diagnosed with the malignancy (Chari, Leibson et al. 2005).

1.4.2 Putative risk factors

Alcohol and pancreatitis are the two main suspect risk factors for pancreatic cancer. Although moderate alcohol consumption does not appear to have a significant role, heavy alcohol consumption (especially of liquor) could create a pro-inflammatory environment that contributes to the onset of the disease when combined with other predisposing factors (Jiao, Silverman et al. 2009, Rohrmann, Linseisen et al. 2009), and is therefore identified as a pancreatitis trigger. Chronic pancreatitis can at the same time result in pancreatic cancer. Although pancreatitis usually precedes pancreatic cancer it can also be its consequence, since pancreatic cancer interferes with the flow of the pancreatic enzymes. Nonetheless, pancreatitis–related inflammation can create a pro-oncogenic environment and further studies are needed to clarify the association between pancreatic inflammation and oncogenesis (Jiao and Li 2010). Acute pancreatitis has recently been linked to PDAC as well, nevertheless research outcomes are mostly conflicting at this stage (Kirkegård, Cronin-Fenton et al. 2018).

1.4.3 Dietary factors

Another factor affecting pancreatic cancer risk is diet, more specifically low fruit intake and increased consumption of red meat. A cohort study showed that high consumption of processed meat was positively correlated with an increased risk for disease manifestation. Additionally, the significant driving factor was associated with meat-derived saturated fat (Nöthlings, Wilkens et al. 2005). Regarding fruit and vegetable consumption, results significantly vary (Gold and Goldin 1998), however flavonols and especially kaempferol were shown to associate with risk reduction (Nöthlings, Murphy et al. 2007). In general, no clear conclusions can be drawn regarding the effects of putative and dietary factors on pancreatic cancer risk, and this is due to the great heterogeneity of studies, populations and assessment tools. Further research and extensive studies need to be carried out in order to understand the possible interactions between genetic predisposition and exogenous factors, and to be able to identify individuals of high risk (Jiao and Li 2010).

1.5 Biomarkers

Despite the fact that the epidemiology and genetic background of pancreatic cancer have been thoroughly investigated and comprehended, it still remains an aggressive disease with an 8-11% survival rate within a 5 years post-diagnosis period. The manifestation of the disease comes along with unspecific symptoms such as weight loss, jaundice and back pain, therefore complicating timely diagnosis (Jenkinson, Earl et al. 2015). The facts that the majority of patients are inoperable and diagnosis is made via fine-needle aspiration, dramatically limit the amount of specimens available for research purposes regarding biomarker discovery (Jenkinson, Earl et al. 2015), and the genetic heterogeneity and instability of PDAC hamper the establishment of a biomarker suitable for all patient groups (Campbell, Yachida et al. 2010, Yachida, Jones et al. 2010). In addition to this, pre-existing pathological conditions such as Type 2 diabetes may affect biomarker performance (Pannala, Leirness et al. 2008), as can obstructive jaundice, which is associated with tumours detected at the head of the pancreas (Sener, Fremgen et al. 1999). It has been recently shown that the presence or absence of obstructive jaundice can negatively affect the specificity and sensitivity of candidate biomarkers, therefore reducing their capacity to discriminate between PDAC and benign conditions (Tonack, Jenkinson et al. 2013). Another potential challenge for diagnostic biomarker discovery is the fact that the vast majority of PDAC cases are diagnosed at an advanced stage and therefore the candidate biomarkers resulting from those samples may be reflective of this late stage solely, and be unsuitable for stages prior to symptoms manifestation. To address this, more

prospective cohort studies need to be carried out, in order to detect candidate molecules for each of the stages of the disease progression (Jenkinson, Earl et al. 2015). To achieve successful biomarker discovery it is important that cohort studies include a wide range of samples sources deriving from healthy and high-risk individuals, patients with benign conditions of the pancreas, diagnosed PDAC patients, as well as a variety of sample types such as pancreatic juice, urine, blood, tissue and saliva, whenever this is feasible (Jenkinson, Earl et al. 2015).

1.5.1 Lewis antigens and CA19.9

Lewis antigens are part of the histo-blood group antigens (HBGAs), which are oligosaccharides with similar structure divided in four groups (type 1 to type 4). Lewis b (Le^b) (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-) is derived from the H type 1 antigen (Fucα1-2Galβ1-3GlcNAcβ1-) which is formed after the addition of an α2-fucosyl residue (α2Fuc) to the type 1 chain (Galβ1-3GlcNAcβ1-) by the secretor enzyme fucosyltransferase 2 (FUT2). Fucosyltransferase 3 (FUT3) is responsible for the addition of an α4Fuc to the type 1 and the H type 1 chain, resulting in Lewis a (Le^a) (Galβ1-3(Fucα1-4)GlcNAcβ1-) or Lewis b. For the production of Le^a only FUT3 activity is essential- and therefore it is a monofucosylated carbohydrate, whereas for Le^b both FUT2 and FUT3 need to be active- and therefore it is a difucosylated carbohydrate (Kubota, Kumagai et al. 2012). As those enzymes are primarily expressed in the gastrointestinal tract, Lewis antigens are predominantly found in the respective cell type (Nishihara, Hiraga et al. 1999). The secretor enzyme FUT2 is active in approximately 80% of the Caucasian population. Individuals with a non-secretor phenotype express the Le^a carbohydrate, providing that FUT3 is active. It is important to underline that FUT2 and FUT3 both compete for the type 1 chain and therefore the carbohydrate expression depends on the enzyme activity (Mattos 2016).

The carbohydrate antigen19.9 (CA19.9), otherwise known as carbohydrate antigen sialyl-Lewis α, was characterized three decades ago (Magnani, Steplewski et al. 1983) and is currently the golden standard for pancreatic cancer diagnosis and monitoring (Galli, Basso et al. 2013). Nevertheless, based on the aforementioned, CA19.9 expression is higher in individuals with Le^{a+b-} and variable in Le^{a-b+} ones. Although CA19.9 expression should be null in Le^{a-b-} individuals and 10% of Caucasian population, there have been cases where advanced pancreatic cancer patients of these genetic backgrounds express CA19.9 up to some extent (>100 U/ml) (Orntoft, Holmes et al. 1991, Hamada, Taniguchi et al. 2012). Kim et al., have determined the

optimal cut-off to be >37 U.ml in order for CA19.9 to discriminate between benign and malignant disease of the pancreas with 87% specificity and 77% sensitivity, after the evaluation of more than 20,000 individuals bearing malignant or benign pancreatic condition, or being asymptomatic (Kim, Kim et al. 1999). However, apart from the Lewis antigen status, there are more implications involved in the evaluation of CA19.9 as a biomarker for pancreatic cancer diagnosis, one of them being tumour size. When the tumour is smaller than 3cm, the sensitivity of CA19.9 drops at 55%, thus allowing the disease to be undetectable at the time of screening. In addition to this, CA19.9 is elevated in benign conditions of the gastrointestinal tract such as pancreatitis, biliary obstruction and cirrhosis, resulting in high incidence of false- positive results (Steinberg 1990). Other markers for pancreatic cancer have been explored and are summarized in Table 1, nevertheless they do not exhibit higher diagnostic value or they have the same or more limitations with CA19.9, which results in this carbohydrate antigen being the current standard to which all potential biomarkers are compared.

Table 1: Current biomarkers in pancreatic cancer

CA proteins and Overexpressed proteins			
Marker	***Sensitivity***	***Specificity***	***References***
CA242	41-75%	85-95%	(Haglund, Lundin et al. 1994, Ventrucci, Ubalducci et al. 1998, Ozkan, Kaya et al. 2003)
CAM 17.1	67-78%	76-91%	(Parker, Makin et al. 1992, Gansauge, Gansauge et al. 1996)
MUC-1	77%	95%	(Gold, Modrak et al. 2006)
CEACAM1	85%	98%	(Simeone, Ji et al. 2007)
Osteopontin	80%	97%	(Koopmann, Fedarko et al. 2004)
DNA markers			
Marker		***References***	
K-ras		(Dressman, Yan et al. 2003, Shi, Eshleman et al. 2004, Diehl, Li et al. 2005)	
p53		(Sturm, Hruban et al. 1998, Wikman, Lu et al. 2000, Dahl, Stenberg et al. 2007)	
Hypermethylated genes		(Jansen, Fukushima et al. 2002, Sato, Ueki et al. 2002, Sato, Fukushima et al. 2003)	
Mitochondrial DNA markers		(Kassauei, Habbe et al. 2006)	
MicroRNA		(Szafranska, Davison et al. 2007)	
Telomerase		(Iwao, Hiyama et al. 1997, Tsutsumi, Tsujiuchi et al. 1997, Pearson, Chiao et al. 2000, Mishra, Zhao et al. 2006)	

1.6 Exosomes

1.6.1 Biology of Exosomes

A significant part of intercellular communication is achieved through the shedding of extracellular vesicles (EVs), a conserved process adopted by almost all organisms. EVs are implicated in both physiological and pathological conditions and their diameter range is 30-1000nm. The two predominant EV classes are microvesicles and exosomes, which differ in size, biogenesis and composition (Cicero, Stahl et al. 2015). Exosomes are mostly found in a range of 30-150nm (Vestad, Llorente et al. 2017) and they are derived from the late endosomes (Stoorvogel, Kleijmeer et al. 2002). More specifically, early endosomes are the main recipients of endocytosed cargo, which get gradually enriched in intraluminal vesicles (ILVs) and turn into late endosomes or multivesicular bodies (MVBs). MVBs can either stay in a neutral state- acting as a kind of "storage" for the accumulated vesicles, they can fuse with lysosomes and thus their cargo gets degraded, or last and most importantly, they can fuse with the plasma membrane of the cell and release the vesicles- which from that point are termed exosomes, in the intercellular space [reviewed in (Stoorvogel, Kleijmeer et al. 2002)]. The existence of exosomes was first described in 1983 when during *in vitro* maturation of sheep reticulocytes, simultaneous physical ablation of the transferrin receptor was observed. Experimental work showed that during maturation, reticulocytes released vesicles enriched in the transferrin receptor protein, with very distinct profile from that of the donor cells' plasma membrane. Those vesicles could be pelleted following 100,000 x g centrifugation and visualized under the electron microscope (Pan and Johnstone 1983). Depending on their source of origin exosomes have distinct protein cargo, however common proteins include tetraspanins (CD81, CD9, CD63, CD82), chaperones and Tsg101 (Stoorvogel, Kleijmeer et al. 2002). Hydrophobic tails are proposed to be crucial for the MVBs lipid sorting (Mukherjee, Soe et al. 1999) as in the case of lysobisphosphatidic acid (LBPA) (Kobayashi, Beuchat et al. 1999), while B cell-derived exosomes exhibit abundance of sphingomyelin and cholesterol, which are implicated in lipid raft formation (Stoorvogel, Kleijmeer et al. 2002). Sorting of proteins and lipids at the MVBs level is what determines the exosomes' composition, and takes place in two steps. The first one occurs at the limiting membrane with protein segregation and selection, and the second one involves the inclusion of the selected cargo in newly formed vesicles

exhibiting inward budding behaviour (Hirsch, Fedorko et al. 1968, Holm, Kayser et al. 1993). MVBs biogenesis is linked to lipid metabolism, with phosphoinositide 3-kinase (PI3K) being crucial for vesicle formation (Futter, Collinson et al. 2001), and proteins such as the Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) (Komada and Soriano 1999) and the endosomal sorting complexes required for transport (ESCRT) (Bishop and Woodman 2001) significantly impact this process.

The mechanisms responsible for ILVs formation get initiated by the targeting of specific molecules-cargo to the MVBs, therefore the first checkpoint for the formation of exosomes is the expression of proteins which will become exosomal cargo (Palmulli and van Niel 2018), such as in the case of syndecan in HeLa cells (Baietti, Zhang et al. 2012). A crucial step following the expression of the proteins destined to be exosomal cargo is their ability to have access to the endosomes in order to be sorted. Mutated proteins can be retained in specific subcellular compartments and therefore never reach the endosomal compartment (Trajkovic, Hsu et al. 2008). Exosomal cargo proteins are known to reach their destination via endocytosis, which can either be dependent or independent of clathrin or dynamin, and with ubiquitination being an important part of the process (Mayor and Pagano 2007, Walseng, Bakke et al. 2008, Piper, Dikic et al. 2014). A second checkpoint is the fine balance between targeting proteins for ILVs sorting or recycling, which is regulated by syntenin. In the presence of phosphatidylinositol 4,5-bisphosphate $PI(4,5)P_2$ and Arf6, syntenin can recycle syndecans to the plasma membrane; however, when acting in combination with ALIX, it directs syndecans to the ILVs. When in a complex, these three proteins promote exosomal release and also mediate Arf6-regulated ILVs budding (Palmulli and van Niel 2018). An important event taking place in the early steps of the process, is the early endosomes' maturation which depends on the RAB5/RAB7 interchange, and the ESCRT-dependent inward folding and fission of their membranes, required for ILV formation (Scott, Vacca et al. 2014, Hurley 2015). During ILV formation the sorting machinery is recruited by the cargo protein which is surrounded by a clathrin coat through the action of ESCRT-0 and -I. Removal of the coat allows membrane scission and deubiquitinating enzymes recruitment via ESCRT-II and –III, so that subsequent exosomal cargo proteins can be ubiquitin–free, prior to the ILVs release into the multivesicular endosomes (MVEs) lumen. ILVs formation can also take place in an ESCRT-independent manner, which results in CD63-enriched vesicles. In general, exosome biogenesis is regulated up to some extent by both ESCRT-dependent and non-dependent mechanisms, however increasing evidence has been pointing to the fact that ILVs degradation is mostly linked to ESCRT-dependency whereas release

as extracellular vesicles is linked to ESCRT-independency (Palmulli and van Niel 2018). The destiny of MVEs is to fuse with the lysosomes and release of ILVs as exosomes means that this step has to be avoided, nevertheless there is still very little comprehension of the mechanisms that promote exosomal release and are unfavourable towards fusion with the lysosomes (Eitan, Suire et al. 2016). Up to date, proteins known to be involved in the secretion of exosomes are members of the Rab family including RAB11, RAB35, RAB7 and RAB27A/B (Palmulli and van Niel 2018), and although it is a constitutive process, extracellular stimuli can affect secretion rate or exosomal cargo, for instance in the case of chemotherapy where increased secretion serves as a way for the cancer cell to reduce intracellular drug levels and confer resistance to neighbouring naive cells through altered levels of exosomal active molecules (Safaei, Larson et al. 2005, Chen, Cai et al. 2014).

Apart from exosome biogenesis and secretion, cellular recognition once the vesicles are released in the intercellular space or the body fluids is a mostly unexplored area of research. The main mechanisms through which this recognition can take place are free floating, adhesion and antigen recognition (McKelvey, Powell et al. 2015). According to the first mechanism, exosomes can be detected in body fluids for a very short period of time before being recruited to organs such as the spleen or liver by immune cells (Saunderson, Dunn et al. 2014). This recruitment can be facilitated by opsonisation, as suggested by the presence of opsonins such as lactoadherin on the exosomal surface (Blanc, Barres et al. 2007), or by exosomal expression of chemokines which act as chemoattractants (Chen, Guo et al. 2011). In the case of adhesion, molecules from both parties are required to be involved. In T cell – exosomes interaction, binding and strong adhesion depends both on ICAM-1, CD44, CD49d and CD11a expression by the cell and CD81 and CD9 expression on the exosome surface (Morelli, Larregina et al. 2004, Zech, Rana et al. 2012). Antigen recognition might be the simplest of the aforementioned mechanisms as it is solely driven by the exosomal major histocompatibility complex (MHC) molecules, which are strongly conserved between the parental cell and the corresponding exosomes (Wieckowski, Visus et al. 2009). MHC class I exosomal expression exerts immunosuppressive activity such as inhibition of natural killer (NK) and $CD8^+$ cells activation (Kshirsagar, Alam et al. 2012), whereas MHC class II expression acts as an immune stimulant. It is not a coincidence that MHC class II molecules are present in exosomes derived from antigen – presenting cells such as dendritic cells (DCs) (Vincent-Schneider, Stumptner-Cuvelette et al. 2002).

Once the exosome has been successfully recognized by the recipient cell it is time for it to be internalized in order to release its cargo and elicit a response, an event that can happen via soluble and juxtacrine signalling, fusion, phagocytosis, macropinocytosis, and endocytosis mediated by rafts and receptors (McKelvey, Powell et al. 2015). Soluble and juxtacrine signalling is a mechanism adopted by cancer – derived exosomes which carry death ligands on their surface, and requires alternative splicing or cleavage of the ligand and juxtaposition of exosomal and cellular ligand and receptor, respectively (Taylor, Gerçel-Taylor et al. 2003). Examples of exosomal and plasmatic membrane fusion have been reported in the case of microvesicles targeted to activated platelets in order to transfer P-selectin glycoprotein ligand-1 (PSGL-1) and tissue factor (Del Conde, Shrimpton et al. 2005), and in the case of melanoma exosomes (Parolini, Federici et al. 2009). PI3K together with Na^+ are the main regulators of macropinocytosis, a method of uptake in which microparticles are surrounded by protrusions of the cell plasma membrane that form an invagination (Tian, Zhu et al. 2014). Endocytosis mediated by receptors and rafts is also called clathrin – mediated endocytosis in the case of the former, and the latter includes clathrin – independent endocytosis as well as caveolae dependent and independent endocytosis (Mayor and Pagano 2007). Whether the exosome will elicit a cellular response or it will deliver its cargo once it reaches its target recipient cell, is likely dependent on the method of uptake. For instance, endocytosis is inevitably linked to the endosomal pathway and the subsequent degradation of the exosomal cargo whereas fusion and soluble and juxtacrine signalling elicit cellular responses (McKelvey, Powell et al. 2015).

1.6.2 Current methods on EV and exosome research

1.6.2.1 Exosome isolation methods

There has recently been a rapidly growing interest of the scientific community towards the study of EVs and specifically exosomes, due to their important role in cell to cell communication in physiological and pathological states (Yáñez-Mó, Siljander et al. 2015), as well as their potential as source of biomarkers and as means of drug delivery (György, Hung et al. 2015, Kourembanas 2015). As a result, methods of isolation of these EVs is a field of ongoing development and standardization is crucial for reproducibility reasons. Currently, the most popular methods of isolation consist of ultracentrifugation and size exclusion by filtration or chromatography, either as stand-alone techniques or in combination (Thery, Amigorena et al. 2006). The vast majority of EV researchers to date, use the ultracentrifugation as their main isolation method

from cell culture supernatant or biological fluids, with the procedure involving initial low-speed centrifugations for the depletion of debris such as apoptotic bodies or platelets, or alternatively exclusion of bigger particles by filtration, and then high speed centrifugation at 100,000 g for the pelleting of the desired EVs (Thery, Amigorena et al. 2006). Sedimentation is dependent on particle density as well as distance to the bottom of the tube, meaning that higher speeds are more efficient for pelleting larger particles (Ismail, Wang et al. 2013). It is very common for EVs to aggregate at this point, and pellet resuspension in PBS can alleviate this issue. Alternatively, a density gradient can be performed, where EVs will travel through different sucrose densities, get dissociated from possible contaminants or aggregates and finally float at a density of approximately 1.1-1.10 g/ml (Thery, Amigorena et al. 2006). In regards to size exclusion by filtering, the particle is required to physically pass through a certain pore size, so one can select between large EV exclusion and small EV retention, whereas in chromatography a column can allow for the retention and subsequent elution of the desired particle population (Thery, Amigorena et al. 2006). It should be noted here that the forces applied on a particle in order to pass through a physical barrier might negatively affect its structure, therefore caution is needed so that they are always kept at a minimum (György, Módos et al. 2011).

Apart from the aforementioned, other methods for EVs and exosomes isolation have developed in parallel. A workflow which delivers higher specificity but lower yield recovery is that of immunoaffinity isolation, where EVs are captured based on specific proteins located on their surface. For instance, in a case of immuno-depletion, HIV-1 virions and exosome populations in Jurkat cells supernatant have been successfully separated with the use of CD45-coated beads. Caution is needed in this case as well, since a marker is not always represented on the surface of all the vesicles of a specific class (Coren, Shatzer et al. 2008). A method commonly used in commercial exosome isolation kits is polymeric precipitation which involves less experimental steps than other more classic isolation methods, it has however raised queries regarding co-precipitation of contaminants and has generally been reported as a less desirable isolation method in comparison to density gradient ultracentrifugation (Alvarez, Khosroheidari et al. 2012, Yamada, Inoshima et al. 2012). In the recent years microfluidics is a constantly growing field for exosome isolation. The devices used are small in size and a small amount of starting material is required – therefore the same applies for reagents, while sensitivity is increased; as a result it would make an attractive method for medical diagnostic laboratories. In order to achieve bigger contact surface and higher chance of capturing the vesicles of interest, herringbone

grooves are the pattern of preference for the channels fabricated. The channel surface is coated with antibodies targeting specific markers on the surface of the vesicles and the starting material (low-speed centrifuged serum or ultracentrifuged cell culture supernatant) passes through it at specific flow rates (Chen, Skog et al. 2010, Hisey, Dorayappan et al. 2018). Earlier studies have analysed glioblastoma multiforme serum and cell supernatant samples using this method, and direct image analysis of the captured exosomes fixed on the channel confirmed their unscathed morphology. The superiority of microfluidics over other immunoaffinity methods was proposed, such as that of magnetic beads where a series of steps including washings and centrifugations may result in loss of significant portion of the initial sample (Chen, Skog et al. 2010). While microfluidics have demonstrated increased capacity to selectively capture specific exosomes, the release of those exosomes in a state that allows their use in sensitive downstream applications still remained an issue until recently. In 2018, a research group managed to capture and release intact exosomes in terms of surface and morphology by taking into account the nature of bonds developed in antigen-antibody interactions and using buffers for pH modifications in order to break them. The innovation of this study lied in the use of glycine-HCl buffer of pH 2.2 in order to recover captured exosomes which retain functionality (Hisey, Dorayappan et al. 2018).

1.6.2.2 Exosome characterization methods

The morphology and the size of EVs and specifically exosomes are important for their biological functions as well as their exploitation as drug delivery systems. A number of characterization methods have been developed so far, the most widely used probably being electron microscopy (EM). The principle behind this technique is the collection of two electron beams, a primary that passes through the samples and a secondary that is generated immediately after, and their concomitant magnification. Incorporation of immunogold labelling in the procedure enables for collection of additional information regarding the presence or not of specific surface markers. For biological samples, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are the common choice for exosome characterization. These methods achieve high resolution, however as in all methods, drawbacks are always present. For instance, dehydration and fixation of the samples can sometimes alter the native morphology, while damage can also be caused by the electron beam. These problems can be addressed with the use of cryo-TEM, with which the samples are kept in amorphous ice at approximately -200^{0}C (Szatanek, Baj-Krzyworzeka et al.

2017). Despite the fact that exosomes exhibit a central depression when studied under TEM, it is interesting to note that vesicles diameters do not vary significantly between different methods of characterization such as SEM and TEM (Wu, Deng et al. 2015). In the case of atomic force microscopy (AFM), a three-dimensional image is created based on the analysis of the movement of a tip attached at the free end of a cantilever, which scans a flat – usually mica- surface on which the vesicles of interest are attached. This is a contact-free scan and movement detection is achieved via combination of a photodiode and a laser. As in EM, antibodies can be used for the collection of biochemical information (Szatanek, Baj-Krzyworzeka et al. 2017)**.**

A common method for the estimation of EV size is that of dynamic light scattering (DLS), in which particle detection is achieved through monitoring of their Brownian motion. This method can be applied for a range of sizes (1nm-6μm), however it is not suitable for polydisperse samples (Szatanek, Baj-Krzyworzeka et al. 2017). Under the last decade, a technique that has been adapted by the majority of laboratories is the Nanoparticle Tracking Analysis (NTA), which also detects single particles via their Brownian motion. NTA does not only calculate the average sizes of particles in suspension but is also an absolute quantitative method. The analysed particles get illuminated by a laser beam and a CCD camera records the scattered light. Particle size is determined based on the two-dimensional Stokes-Einstein equation. NTA analysis can be highly affected by the presence of contaminants found within the sample or the reagents used during sample preparation, therefore appropriate precautions need to be taken prior to analysis (Dragovic, Gardiner et al. 2011, Sokolova, Ludwig et al. 2011). An alternative to NTA could be the resistive pulse sensing (RPS) analysis, which has been commercialized by IZON and applied with the qNano instrument which has its base at the Coulter principle (Garza-Licudine, Deo et al. 2010). The analysis system is comprised of two chambers each of which contains an electrode, connected via a <1μm pore – sized polyurethane membrane. Upon electric field application the EVs move towards the positively or negatively charged electrode and a change in the electrical resistance is detected, which is correlated to particle volume; therefore the size of EVs can be calculated. As a drawback, the time required for the analysis of each sample can be significantly affected by pore clogging (Momen-Heravi, Balaj et al. 2012).

Flow cytometry is another common technique for microparticle analysis, and it reveals information about surface morphology (granulation) and size of the studied sample thanks to the scattered light initiating from a laser beam targeted at the suspended

microparticles of interest. Currently available flow cytometry instruments can analyse 500nm-200nm sized vesicles, therefore posing an obstacle for exosome characterization (Chandler, Yeung et al. 2011, van der Pol, Coumans et al. 2014). Between forward-scattered light (FSC) and side-scattered light (SSC) as a trigger channel in exosomes analysis by flow cytometry, SSC tends to lead to higher detection sensitivity, as FSC is affected by multiple parameters and is extremely variable among different flow instruments (Nebe-von-Caron 2009). Another drawback of flow cytometry in EV detection is the "swarming" effect presented in high concentration EV samples. This term is used to describe a group of EVs which are falsely detected as a single event by the instrument, due to the fact that in order for a signal that overcomes the threshold to be detected, a number of vesicles need to scatter light simultaneously (van der Pol, van Gemert et al. 2012). Apart from the issues regarding detection, size characterization is also strenuous. Instruments get calibrated with beads of known diameter which are often added to the sample, and based on that measurements the size of the studied vesicles can be determined. Nevertheless, refractive index, shape and other characteristics affect the light scatter, and with the EVs and beads being highly different in native properties, their concomitant scattered light cannot allow for correlation and subsequent size determination (Curl, Bellair et al. 2005). Tailor-made approaches have lately been developed for improved exosomes characterization by flow cytometry, and these mostly include commercially available antibody-coated latex beads which can selectively bind to the vesicles of interest and artificially increase their size, therefore making them visible to the instrument (Caby, Lankar et al. 2005).

1.6.2.3 Guidelines for EVs studies

In order to promote uniformity and reproducibility in EV research, the International Society of Extracellular Vesicles (ISEV) has released the Minimal Information for Studies of Extracellular Vesicles (MISEV) which was last updated in 2018 (MISEV2018). Those guidelines are aimed to provide researchers with tools to critically design experiments and interpret the outcomes, and to highlight possible issues that need to be taken into consideration before attributing certain characteristics to EVs. Points of consideration exist regarding the collection of EVs from cell culture conditioned media, such as method of culture and conditions of harvest, method of preparing the EV-depleted media (if no commercial EV-depleted FBS/FCS is being used), and state of cells at the time of harvest (cell death etc.), as well as from biological fluids. For instance, parameters of consideration regarding

blood as a source of EVs would include age, gender, volume of starting material, type of anticoagulant and collection tube, and the absence / presence (and to which extent) of haemolysis, among others.

Regarding EV preparations, it needs to be highlighted that absolute separation of EVs from other biological molecules is unrealistic and not achievable, and that the degree of purity that needs to be achieved is highly determined by the downstream applications and the ultimate experimental question that is being explored. An ISEV survey in 2015 revealed that the differential ultracentrifugation was the scientific community's choice of preference regarding methods for EVs isolation, and up to 20% of participants combined it with secondary methods, examples of which are size exclusion chromatography (SEC) and density gradients. An inverse correlation exists between the recovery of the EVs and their specificity, both of which are dependent of the separation method as depicted by the graph in Figure 2.

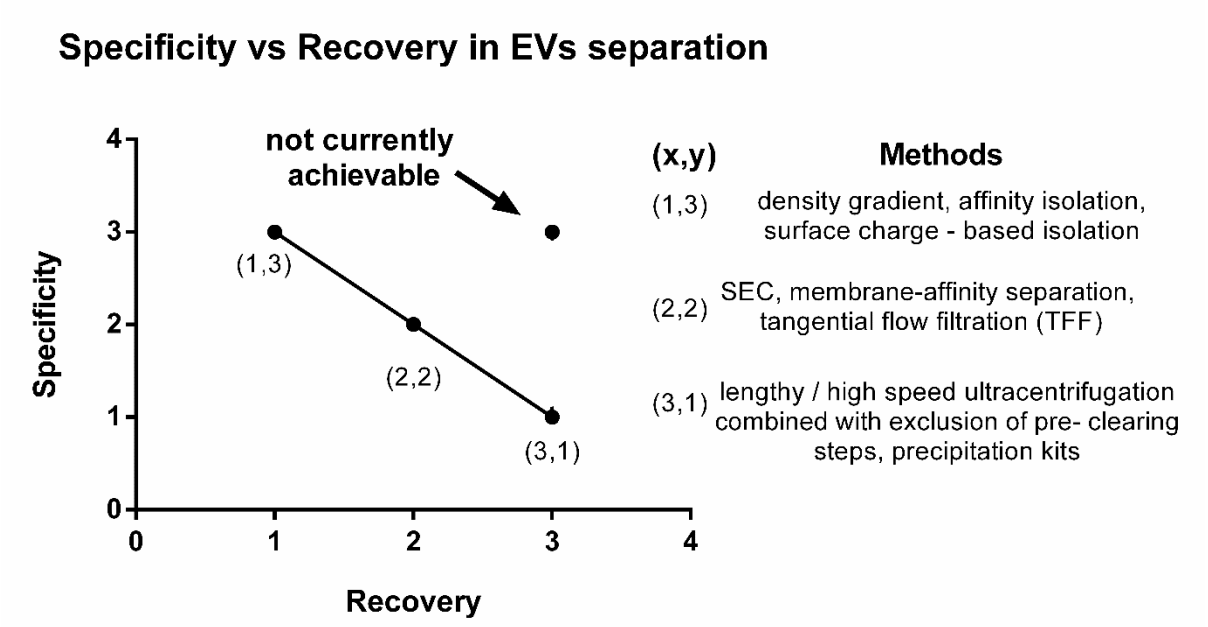


Figure 2 Correlation between specificity and recovery in EVs separation and corresponding methods. 1 = low, 2 = intermediate, 3 = high

The MISEV guidelines also suggest valuable EV characterization criteria, starting from quantification and stating that a single absolute method does not exist, nevertheless the two most widely accepted and applied are total number of particles and total amount of protein. In regards to protein content, positive as well as negative

controls need to be included. The first should include cytosolic as well as transmembrane proteins, and the latter can refer to proteins that derive from compartments of the cell different than the endosomes or the plasma membrane. As for single vesicle characterization, ideally two separate but complementary methods should be performed; for instance electron microscopy and single particle analysis.

In summary, the position statement of the MISEV2018 concludes that every rule has its exceptions and that the purpose of those guidelines which will be constantly evolving, is to improve the EV research field and not impede it. Researchers are recommended to meet the criteria but if this is not the case, they are also encouraged to explain their unique circumstances (Théry, Witwer et al. 2018).

1.6.3 Physiological roles of exosomes

Exosomes are important mediators of cellular homeostasis maintenance, as they relieve the cell from undesirable and potentially toxic cargo such as misfolded proteins (Guo, Bellingham et al. 2015). Rapid alteration of gene expression can as well be achieved via exosomal RNA release as for instance in the case of lymphocytes which upon activation promote exosomal release of miR-150, a known repressor of their functions including differentiation (de Candia, Torri et al. 2013), while this mechanism is also used for the removal of RNA degradation residues and small non-coding RNAs (van Balkom, Eisele et al. 2015). Exosomes are important effectors of intercellular and inter-organ communication, and in this section their role in a number of physiological conditions is going to be discussed.

Pregnancy represents a complicated form of immunomodulation in which the mother has to develop tolerance towards the foetus so it is not rejected as foreign tissue, and also maintain her ability to fight exogenous pathogens without risking the foetus's healthy development. In this finely balanced scenario, EVs play a central role (Nair and Salomon 2018). In order for the foetus to avoid being targeted by the immune system of the mother, the placenta releases exosomes containing the Fas and TNF-related apoptosis–inducing ligands (FasL and TRAIL ligands) which induce maternal T cells, lymphocytes and activated peripheral blood mononuclear cells (PBMCs) apoptosis and therefore grant immunotolerance to the developing embryo (Tannetta, Dragovic et al. 2013, Elfeky, Longo et al. 2017). At the same time, a subpopulation of cells named synctiotrophoblasts (STBs) shed vesicles enriched in UL16-binding proteins (ULBPs) and MHC class I-related antigen A and B, which bind to NKG2D receptors on immune cells and result in immunosuppression via cytotoxic T cells and

NK cells apoptosis (Hedlund, Stenqvist et al. 2009). Other immune–suppressant proteins found in placenta and STBs–derived exosomes are PD–L1, syncitin-1 and HLA-G (Nair and Salomon 2018). However, apart from their immunosuppressive capacity, circulating exosomes in pregnancy are reported to also have immunostimulatory effects such as maternal monocytes recruitment at the embryonic / maternal interaction areas and increased expression of pro – inflammatory cytokines (Atay, Gercel-Taylor et al. 2011). Interestingly, trophoblast cells are capable of reacting to pathogens by uptaking the maternal macrophage – derived exosomes and use them for pro – inflammatory cytokines expression (Holder, Jones et al. 2016). In summary, circulating exosomes in pregnancy act as the orchestrators of a complex mother / foetus crosstalk, which has a distinct immunomodulatory profile in each trimester in order to facilitate a successful pregnancy (Nair and Salomon 2018).

Exosomes and microvesicles are also known to facilitate intercellular central nervous system (CNS) signalling. Following CNS injury the microglia secrete exosomes capable of triggering inflammatory responses. This was shown in studies using murine models in which treatment with ATP derived from astrocytes activated sphingomyelinase downstream of P2X7R, which in turn instigated IL-1β bearing exosomes release from the microglia (Iraci, Leonardi et al. 2016). Under normal conditions axonal integrity and survival is dependent on a crosstalk between the axons and the surrounding mature oligodendrocytes (ODCs) which form myelin sheaths. This crosstalk is mediated via ODCs–secreted exosomes enriched in axon–supporting myelin proteins such as proteolipid protein, an ODC–specific glycoprotein and basic protein (Bakhti, Winter et al. 2011, Frühbeis, Fröhlich et al. 2013). Interestingly, neurons are able to regulate exosome secretion from ODCs through their activity. Electric signals initiating from the axons lead to glutamate release which in turn triggers NMDA and AMPA-mediated calcium signalling from the ODCs, resulting in exosome shedding from the latter. Notably these exosomes have been shown to be able to regulate myelin sheaths biogenesis and protect the neurons from the damaging effects of oxidative stress (Krämer-Albers, Bretz et al. 2007, Bakhti, Winter et al. 2011). Neurons have also been shown to increase their synaptic activity through the of uptake microglia-derived EVs, which positively affect their sphingolipid metabolism (Antonucci, Turola et al. 2012).

Cardiovascular diseases (CVDs) are currently the leading cause of death, being responsible for one third of deaths on a global level (WHO 2017). In case of myocardial infarction incidence reperfusion of the vessel is imperative, nevertheless

it is always accompanied by some level of injury named “reperfusion injury”, the extent of which is correlated to morbidity and mortality (Neri, Riezzo et al. 2017). Ischaemic preconditioning (IPC) is an effective procedure for the protection of the heart in *in vivo* models but also a rather impractical one when it comes to humans, therefore increased interest is focused on remote IPC (RIPC), in which short ischaemic episodes applied in a distant organ can confer reperfusion injury resistance to the heart (Heusch, Bøtker et al. 2015). The functional part of the heart, the cardiomyocytes, are cells that have reached their terminal differentiation state and this makes it almost impossible to recover or improve the function of a compromised myocardium (Madonna, Van Laake et al. 2016). Attempts using stem cells injections have so far failed (Madonna, Van Laake et al. 2016), nevertheless there have been indications that remaining stem cell-derived soluble factors may mediate promising effects (Davidson, Takov et al. 2017). Mesenchymal stem cells (MSCs)–derived exosomes have been shown to successfully reduce the infarct size of mice experiencing myocardial ischemia within 24 hours (Lai, Arslan et al. 2010), and further studies revealed that the exosomal cardio protective activity was independent of the MSCs source (Lai, Arslan et al. 2010). Moreover, MSCs–exosomes were able to enhance the expression of the sarco(endo)plasmic reticulum calcium-ATPase 2 (SERCA2) enzyme and the L-type voltage-dependent Ca^{2+} channel in artificial cardiac tissue, therefore suggesting a potential impact on the contractility of the muscle (Mayourian, Cashman et al. 2017). Exosomes derived from foetal cardiac progenitor cells (CPCs) increased the migration potential of epithelial cells *in vitro* (Vrijsen, Sluijter et al. 2010), whereas exosomes derived from CPCs obtained by patients undergoing heart surgery were able to minimize scarring and cell apoptosis and improve blood vessel thickness when injected in the compromised infarct area (Barile, Lionetti et al. 2014). Human plasma exosomes have also been shown to have cardio protective effects upon administration to rodents or primary cells, an event that has been attributed to the HSP70 exosomal protein, which activates the reperfusion injury salvage kinase (RISK) pathway via TLR4 stimulation (Vicencio, Yellon et al. 2015). This underscores the possibility of the existence of a continuous exosome-driven cardio protective pathway.

Exosomes also play a critical role in the regulation and fine balance of the immune system. They serve as native antigens carriers and are implicated in antigen presentation to the T cells, they transfer RNA and they are able to instigate or suppress immune responses (Robbins and Morelli 2014). Antigen presenting cells (APCs) and their derived exosomes maintain the same topology, and therefore the

latter express MHC class molecules able to stimulate T helper and cytotoxic T cells. However due to their size and Brownian motion–driven dispersion, free vesicles have significantly decreased stimulatory activity compared to their donor APCs (Blanchard, Lankar et al. 2002). This activity can be increased via dendritic cells–vesicles interaction mediated by adhesion molecules (Vincent-Schneider, Stumptner-Cuvelette et al. 2002, Segura, Guérin et al. 2007). Comparison of the stimulatory activity of exosomes derived from mature and immature DCs revealed that the former expressed higher ICAM1 which promoted binding to and activation of T cells, which in turn dictate changes in the conformation of their surface integrins, resulting in higher affinity for ICAMM1 presenting exosomes (Segura, Nicco et al. 2005, Nolte, Buschow et al. 2009). Other molecules mediating exomes binding to target cells include MFG-E8 and phosphatidylserine (PS) (Véron, Segura et al. 2005, Miyanishi, Tada et al. 2007). Interestingly, the majority of extracellular vesicles remain on the surface of mature DCs whereas they become internalized by immature ones (Montecalvo, Shufesky et al. 2008). Exosomes also participate in a process called “cross-dressing”, which is the transfer between APCs of pre-processed peptide–MHC complexes which do not require further antigen processing (Smyth, Afzali et al. 2007). In addition, native antigens such as tumour and B-cell receptor antigens are exposed to APCs with the help of extracellular vesicles. DCs uptake tumour-bearing cancer exosomes and following antigen processing they cross-present them to cytotoxic T cells (Wolfers, Lozier et al. 2001). DCs residing in B-cell follicles (FDCs) present complement–activating immune complexes on their surface, which are recognized by B–cells. However FDCs lack the ability of MHC class-II synthesis and therefore acquire those molecules by captivation of MHC-II bearing vesicles, facilitated by MFG-E8 and ICAM1 from the side of the FCD and integrin LFA1, PS and C3 fragments from the side of the vesicle (El Shikh and Pitzalis 2012). Surface proteins that hamper the membrane attack complex assembly and control the deposition of C3b offer complement lysis protection to the exosomes involved in this procedure (Clayton, Harris et al. 2003). Last but not least, DCs, NK and $CD8^+$ cells are in a position to produce FasL–enriched exosomes and release them against target cells, resulting in their elimination (Peters, Borst et al. 1991). In conclusion, it is clear that extracellular vesicles can have a positive or negative impact on immune responses, and deciphering the exact *in vivo* role of those vesicles depending on their source of origin is the first step towards their exploitation as therapeutic tools.

1.6.4 Exosomes in cancer and pancreatic cancer

Local and systemic signalling is essential for cancer progression and dissemination, and EVs and specifically exosomes are known to play important roles in supporting these processes (Wortzel, Dror et al. 2019). Extensive proteomic characterization of EVs derived from sixty cancer cell lines representing nine distinct origins, including prostate, kidney, leukaemia, colon, breast, brain, lung, ovary and melanoma, revealed that among the 6,071 unique proteins identified, 213 of them were common to all 60 sample types and were comprised of proteins involved in EV biogenesis and secretion as well as protein trafficking. The proteins which exhibited differential expression were associated with their tissue of origin and could therefore serve as a tool in biomarker discovery. Notably, during clustering analysis, EVs from metastatic cell lines tended to cluster together and separately from other non-metastatic ones from the same tissue of origin (Hurwitz, Rider et al. 2016). EVs have the advantage of reflecting the whole tumour cells population and therefore the complete mutational landscape, on the contrary to tissue biopsies which are a snapshot of the local tumour environment (Kalluri 2016). As a result, EVs are a valuable source of circulating biomarkers which could complement the already existing cancer biomarkers such as CA19.9 (pancreatic cancer) and CA-125 (ovarian cancer), which unfortunately do not exhibit absolute specificity and / or sensitivity (Fritsche and Bast 1998, Wilbur 2008).

Exosomes, the most well-studied and characterized EV subclass, contain active proteins which facilitate cancer proliferation and dissemination. For instance, the epidermal growth factor receptor (EGFR) was present in gastric cancer exosomes (Zhang, Deng et al. 2017), and its ligand Amphiregulin was detected in both breast and colon cancer exosomes, increasing the metastatic potential of neighbouring cancer cells (Higginbotham, Beckler et al. 2011). Interestingly, colorectal cancer patients express both the receptor and the ligand in their circulating blood exosomes (Higginbotham, Zhang et al. 2016). Exosomes are also known to contain RNA which can be referred to as “exosomal shuttle RNA” and includes among others functional messenger and microRNA (mRNA and miRNA), a proportion of which are absent from the donor cells’ cytoplasm (Valadi, Ekström et al. 2007). Exosomes derived from glioblastoma tumours primary cells were enriched in the mRNA of a specific splice variant of EGFR, the EGFRvIII, and the same splice variant was also detected in serum–derived exosomes of glioblastoma patients. The importance of EGFRvIII is that not only it is commonly found in this type of tumours but it also affects the responsiveness of patients to treatment with EGFR-inhibitors, and therefore the ability to test for the EGFRvIII status via a non-invasive method is of great value (Skog,

Würdinger et al. 2008). Prostate cancer exosomes next generation sequencing (NGS) screening revealed the presence of almost 2000 miRNAs, the most abundant of which were miR-21-5p and miR-100-5p. The first was shown to activate fibroblasts and promote their migration while causing upregulation of matrix metalloproteinases (MMPs) such as MMP-13, -9 and -2, whilst the latter has been previously correlated with the disease (Sánchez, Andahur et al. 2016). Recent findings reveal that miRNA exosomal loading assists in balancing the cellular miRNAs levels. More specifically, cancer cells appeared to sort tumour suppressor miRNAs in exosomes in a fashion that was positively correlated to the disease grade (the higher the grade the higher the exosomal miRNA levels), while they preferentially retained the oncogenic ones. This indicates an effective pathway through which cancer cells ensure their survival and the concomitant disease progression (Teng, Ren et al. 2017). Studies have also confirmed the existence of amplified oncogenes and retrotransposons, as well as nuclear and mitochondrial DNA in exosomes (Guescini, Genedani et al. 2010, Balaj, Lessard et al. 2011). A subsequent study on different cancer types demonstrated that the greater part of the total exosomal DNA was in fact double stranded and comprised a precise reflection of the mutational landscape of the parental cells, therefore attracting attention regarding its translational significance as a potential biomarker. Moreover, cancer cells tended to secrete exosomes with higher DNA levels (Thakur, Zhang et al. 2014). Mitochondrial DNA (mtDNA) present in cancer-associated fibroblasts (CAFs)–derived exosomes has been recently proven to play a major role in breast cancer therapy resistance, and more specifically in the estrogen receptor-positive (ER^+) type. Although this cancer is initially sensitive to hormonal therapy and enters a dormant state, relapse can occur after long periods of time and in this case the disease is resistant to treatment (Sansone, Savini et al. 2017). Based on the knowledge that mtDNA loss leads to reduction of cancer cells metastatic potential and that nanotubes and intercellular organelles serve as means of intracellular mitochondria and mtDNA transfer (Tan, Baty et al. 2015), it was demonstrated that during the transition from dormant to hormone therapy – resistant ER^+ breast cancer, stroma cells and more specifically CAFs secrete exosomes containing mtDNA, which once uptaken by dormant cells allows the latter to regain their oxidative phosphorylation capacity and promote the manifestation of the metastatic disease (Sansone, Savini et al. 2017). More recently, CAFs were shown to secrete exosomes containing miR-92a-3p, which through targeting and negatively regulating downstream targets, attenuates loss of the mitochondrial function and triggers the Wnt/β-catenin signalling cascade in colorectal cancer (Hu, Wang et al. 2019). Nevertheless, multiple evidence shows towards the bidirectional relationship existing

between cancer cells and the tumour microenvironment, especially CAFs which are the prevalent cell type in the tumour stroma, and the crucial role of exosomes (Naito, Yoshioka et al. 2017). For instance, cancer exosomes were shown to carry biologically active (transforming growth factor beta) TGF-β, the levels of which were positively correlated to those of betaglycan expressed on the exosomes surface. The recipient fibroblasts exhibited elevated levels of SMAD-driven signalling cascade and also increased expression of α-smooth muscle actin (α-SMA) (Webber, Steadman et al. 2010). Conversely, exosomes derived from naïve CAFs or from CAFs exposed to chemotherapeutic drugs were capable of mediating cancer stem cells drug resistance and boost their ability to form tumour spheres, respectively (Hu, Yan et al. 2015).

In the context of pancreatic cancer, PSCs-derived exosomes were shown to be effectively uptaken by PDAC cells and promote their tumorigenic characteristics such as proliferation, migration and CXCL2/CXCL1 cytokine expression, with the latter being correlated with disease progression (Takikawa, Masamune et al. 2017). The combined detection of both exosomes and circulating tumour cells (CTCs) has also been shown to be a valuable tool in the PDAC diagnostic field. The currently available commercial CTCs kits do not exhibit very high detection rates and their performance is significantly affected by the collection method (for instance portal versus peripheral blood). Exosomes have also been proven to be a valuable source of biomarkers; nevertheless the sensitivity and specificity notably improve when combined with specific protein-signatures. In a recent study, a method that combines CTCs and GPC-1^{+} exosomes detection was able to successfully identify all PDAC patients with 80% specificity; the deviation being due to the presence of autoimmune diseases (Buscail, Alix-Panabières et al. 2019).

PDAC-derived exosomes are also able to sustain a highly immunosuppressive environment by hampering the functions of a number of immune cells. It has been demonstrated that by transferring the miR-212-3p to DCs, they indirectly lower MHC II levels by inhibiting the expression of its transcription factor, the regulatory factor X-associated protein (RFXAP). This ultimately results in immune tolerance (Ding, Zhou et al. 2015). In addition to this, exosomal transfer of miRNA-203 in DCs downregulates the expression of the toll-like receptor 4 (TLR4), leading to decreased release of antitumorigenic cytokines such as TNF-α and IL-12 (Zhou, Chen et al. 2014). The cytotoxic activity of NK cells has also been shown to be severely restrained upon exposure to murine PDAC-derived exosomes (Katsiougiannis, Chia et al. 2016).

1.7 Metabolic reprogramming – Lipid metabolism

1.7.1 Lipid metabolism in cancer

In 2000, the outstanding diversity of human malignancies was rationalized and broken down into six categories, which are known as the six hallmarks of cancer and include replicative immortality, cell death resistance, continuous proliferative signalling, invasion and metastasis, angiogenesis induction and last but not least, growth suppressors evasion (Hanahan and Weinberg 2000). Following a decade of intensive research in the field, another characteristic of cancer cells was added to that list: the metabolic reprogramming. This adjustment is necessary for the rapidly dividing cancer cells to maintain their high proliferative rate (Hanahan and Weinberg 2011). In the presence of oxygen (aerobic conditions), glucose is initially converted to pyruvate in the cytosol via a process called glycolysis, which will later enter the mitochondria. Oxygen insufficiency limits the pyruvate levels entering the mitochondria and favours glycolysis. However, cancer cells have established a state called “aerobic glycolysis”, in which regardless of the oxygen availability, their metabolism greatly relies on the glycolytic pathway and mitochondria no longer play a role in the metabolism of glucose. This is also known as the Warburg effect (Warburg 1930, House, Warburg et al. 1956, Warburg 1956). Based on a theory initially expressed in the 1960’s, the demanding metabolic processes that give rise to the components which will be incorporated into the newly synthesized cancer cells are fuelled by the intermediate products of the intensified glycolysis (Potter 1958, Vander Heiden, Cantley et al. 2009). The genetic mosaicism of cancer and the development of new tumour-related blood vessels result in oscillating spatiotemporal levels of oxygenation of the tumour mass. As a result, some tumour types exhibit two symbiotic subpopulations of cells: the hypoxic one behaves according to the Warburg effect and converts glucose into lactate, whereas the more oxygenated one takes advantage of the first population and metabolizes its waste (lactate) in order to fuel its own (Semenza 2008, Feron 2009, Kennedy and Dewhirst 2010). Cancer cells with high proliferative rate also show a zeal towards lipids, which they obtain by either increasing lipogenesis or scavenging exogenous lipids, while any excess lipid content is stored in lipid droplets (LDs) inside the cell for later use (Accioly, Pacheco et al. 2008, Qiu, Ackerman et al. 2015). Despite their increased lipogenic activity, cancer cells highly depend on fatty acids (FA) import and their further incorporation in the β-oxidation pathway, as this is the predominant

metabolic process in cancers with low glycolytic rates such as prostate cancer (Liu, Zuckier et al. 2010). Depending on the number of carbons (chain length), backbone type and the double bond location, different types of FA occur. Apart from their role in the maintenance of the cell membrane rigidity and protein anchoring via lipid raft formation, lipid composition and ratio of species can directly or indirectly play a major role in cancer progression. For instance, polyunsaturated FAs are susceptible to peroxidation and therefore to oxidative stress, nevertheless lipid saturation is specifically increased in tumours with lipogenic profile, rendering cancer cells immune to this type of cell death. Moreover, as saturated FAs have straighter tails than the non-saturated ones, they tend to be more densely packed and reduce the membrane fluidity and the subsequent uptake of chemotherapeutic drugs (Rysman, Brusselmans et al. 2010). Phospholipid (PL) signatures can also be indicative of the type of malignancy and its stage. Quantitatively-wise, phosphatidylinositol (PI), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were found to be greatly enriched in malignant breast tissues in comparison to neighbouring healthy tissue, whereas the latter was also found to exhibit a great degree of saturated FAs linked to more dismal overall prognosis (Hilvo, Denkert et al. 2011, Guenther, Muirhead et al. 2015). On the other side, these three PLs were lower in lymphoma than in normal tissue, with the indicative increased lipid being phosphatidylglycerol (PG) (Eberlin, Gabay et al. 2014).

Important for cell physiology and cell survival are also lipid rafts, which comprise of sphingolipids and cholesterol and serve as hosts for receptor and proteins with pro-oncogenic properties (Mollinedo and Gajate 2015). The PI3K pathway is highly dependent on lipid rafts as one of its main proteins, Akt, anchors at that site and its constant activation is linked to lipid raft enrichment in cancer cells (Zhuang, Kim et al. 2005, Adam, Mukhopadhyay et al. 2007). Cancer spread is also dependent on lipid rafts as they have direct impact on cytoskeleton modulation and focal adhesion complexes formation (Jeon, Kim et al. 2010, Wang, Bi et al. 2013). Yet, lipid rafts do not always promote cell survival. Formations known as “clusters of apoptotic signalling molecule-enriched rafts” (CASMERs) recruit death receptors and apoptotic proteins- thus initiating cell death (Mollinedo and Gajate 2015). CASMER formation reduces the apoptotic threshold of colon cancer cells, while pharmacological depletion of cholesterol reverses this effect (Delmas, Rebe et al. 2004). Aberrations in the expression of lipid metabolism – related proteins can cause endoplasmic reticulum stress (ERS) which in turn instigates cancer cell death via the unfolded protein response (UPR) pathway. Pharmacological manipulation of the cells’ lipid content,

such as increase of free cholesterol or ceramide, has been also shown to lead to the same result, therefore indicating new therapeutic opportunities (Salazar, Carracedo et al. 2009, Ríos-Marco, Martín-Fernández et al. 2013, Kato and Nishitoh 2015).

1.7.2 Lipid metabolism in pancreatic cancer

Altered lipid metabolism plays a major role in pancreatic cancer, since not only pancreatic cancer cells proliferation exhibits lipid dependency, but also *in vivo* work has directly linked fibrosis and overall enhanced *KRAS* activity and pancreatic cancer progression with high fat diet (Clerc, Bensaadi et al. 1991, Philip, Roland et al. 2013). The consumption of total fats, and especially their level of saturation greatly affects the manifestation and progress of the disease. While polyunsaturated FAs (PUFAs) exhibit a preventive function, those with one or no single bonds are disease-promoting factors. The location of the double bond in PUFAs is of importance, as n-3 molecules have been shown to exert anti-tumour activity whereas n-6 ones can instigate the proliferation of certain pancreatic cancer cells subpopulations (Sunami, Rebelo et al. 2017). PDAC has great demand for cholesterol, displayed by the overexpression of the low-density lipoproteins receptor (LDLR) and the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in respective mouse models (Guillaumond, Bidaut et al. 2015). Patients suffering from pancreatic inflammation and benign or malignant tumours such as IPMNs and PDAC respectively, exhibit higher levels of serum fatty acid synthase (FASN) compared to healthy individuals, and major EGFR-related signalling pathways known for their contribution to pancreatic cancer initiation and progression such as the MEK/ERK or PI3K/Akt cascades, increase the expression of FASN via the upregulation of another protein, the sterol regulatory element-binding protein 1 (SREBP1) (Walter, Hong et al. 2009, Ardito, Grüner et al. 2012, Navas, Hernández-Porras et al. 2012). Both those proteins are inversely correlated to survival and response to chemotherapy (Sun, He et al. 2015, Tadros, Shukla et al. 2017). In order to replenish the FAs fuelling their growth, pancreatic cancer cells alter the acetate and glutamine catabolism pathways. Normally, glutamine gets metabolised in two steps in the mitochondria and enters the TCA cycle in the form of α-ketoglutarate (α-KG). Pancreatic cancer cells can initiate a non-canonical pathway for glutaminolysis which takes place in the cytoplasm and produces pyruvate, which enters the mitochondria to eventually lead to acetyl-coA production, a molecule essential for both cholesterol and FA synthesis. Pancreatic cancer cells that undergo metabolic stress, have been also shown to elevate their

levels of acyl-CoA synthetases in order to metabolise acetate as an alternative source of acetyl-CoA. Acetate can be acquired through diet, such as ethanol or processed food, or can be synthesized *de novo* intracellularly (Sunami, Rebelo et al. 2017). HIF-1α, a protein often overexpressed in PDAC (Hao 2015), is a known mediator of lipid droplets accumulation. Lipid droplets serve as a cellular neutral lipid storage and comprise mostly of cholesteryl ester (CE) and triacylglycerol (TG), and related proteins such as acyl-CoA diacylglycerol and acyl-CoA cholesterol acyltransferase (DGAT and ACAT, respectively) enzymes. Pharmacological inhibition of those enzymes, especially of ACAT-1, has been shown to hamper PDAC progression in murine models (Sunami, Rebelo et al. 2017).

Tumour microenvironment plays a crucial role in cancer progression and the investigation of the effects of different cell types on PDAC cells and vice versa, is of interest. Pancreatic stellate cells (PSCs) are the most abundant cell type in the tumour stroma and *in vitro* experiments have shown that PSCs-conditioned medium can significantly and specifically increase PDAC cells' mitochondrial activity through secretion of alanine, a non-essential amino acid. Transamination of alanine leads to pyruvate conversion, which can then enter the TCA cycle. PDAC cells exhibit strong preference for the carbon which is provided from alanine rather than glucose, therefore the latter can be used in abundancy in biosynthesis of other molecules including serine. Interestingly, secretion of alanine is dependent on PSCs autophagy, which in turn is a result of PDAC cells – PSCs crosstalk, a finding which was validated also in *in vivo* studies and highlights the clinical significance of this bidirectional relationship (Sousa, Biancur et al. 2016). Evidence underscoring the importance of this crosstalk towards the establishment and progression of the disease, came to light earlier this year. Up to now, the source of lysophosphatidic acid (LPA) found inside the tumour had not been elucidated, and considering the poor vasculature characterizing the disease, it was highly unlikely that it was supplied by the circulation. It has now been established that PDAC cells express higher levels of autotaxin and increase the number of LPA receptors on their surface, and at the same time activate the PSCs within their vicinity. The latter undergo a "lipidome remodelling" and secrete LPC, which is then hydrolysed to LPA by autotaxin. This is a perfect example of how PDAC cells take advantage of alternative routes in order to sustain their metabolic needs, and suggests of a potential therapeutic target within the tumour microenvironment which allows the interruption of the harmful tumour-stroma crosstalk without the ablation of the latter (Auciello, Bulusu et al. 2019).

1.8 Study Aims

The first aim was to characterize the proteomic cargo of PDAC- and pancreatic epithelial cells-derived exosomes together with their respective cells, in order to identify key proteins enriched in cancer exosomes, which are known to be implicated in cancer hallmarks and could potentially be used as biomarkers.

The second aim was to characterize the lipidome of PDAC- and pancreatic epithelial cells-derived exosomes together with their respective cells, and compare it to that of ovarian and prostate cancer cells and exosomes. In case of detection of a specific PDAC-exosomes signature, appropriate downstream assays would be performed to examine potential biological effects.

The third aim was to explore the possibility of targeting the PDK1 protein in pancreatic cancer. PDK1 is a major component of the PI3K pathway, known to be constitutively active in PDAC and generating lipids which serve as second messengers. This would be done by pharmacological inhibition and siRNA downregulation of PDK1, and identification of downstream effector proteins though which its function is exerted.

CHAPTER 2

Chapter 2: Characterization of the proteome of pancreatic cancer-derived exosomes as potential biomarkers for PDAC

This chapter is displayed in the form of a peer-reviewed research paper

Publication 1

Emmanouilidi, A., Paladin, D., Greening, D. W., & Falasca, M. (2019). Oncogenic and Non-Malignant Pancreatic Exosome Cargo Reveal Distinct Expression of Oncogenic and Prognostic Factors Involved in Tumor Invasion and Metastasis. *Proteomics*, *19*(8), 1800158.

2.1 Article synopsis

2.1.1 Background

Pancreatic cancer is a disease with dismal prognosis and a less than 10% chance of survival within 5 years from diagnosis. The most common type of the malignancy arises from the head of the pancreas and is called pancreatic ductal adenocarcinoma (PDAC). Due to the lack of symptoms and sensitive and specific biomarkers, early detection is rare. By the time of diagnosis, the cancer has in the vast majority of cases spread, rendering the surgical resection of the tumour prohibitive. In order to obtain pancreatic tissue for further biopsy in order to make a diagnosis, fine-needle aspiration (FNA) can be performed; it is nevertheless a procedure that involves risks and causes discomfort to the patient, highlighting the urge for the development of another type of biopsy which will involve more accessible samples and more accurate biomarkers.

Exosomes are a subgroup of extracellular vesicles secreted from cells in both physiological and pathological states, and carry selective cargo. Exosomes contain molecules such as nucleic acids, proteins and lipids, and they are present in all types of body fluids including blood, urine, saliva and breast milk; characteristics which place them in the centre of attention of the scientific community in regards to biomarkers discovery. In this study, in order to evaluate the protein cargo of pancreatic cancer-derived exosomes, human pancreatic cancer and non-malignant epithelial cell

lines were used, and their cargo was compared to that of their corresponding exosomes.

2.1.2 Experimental design in brief

Human non-malignant epithelial pancreatic duct cells (HPDE and hTERT-HPNE) and pancreatic cancer cells (AsPC-1, BxPC3 and MiaPaCa2) were grown in 245 x 245 x 25mm culture dishes until they reached approximately 80% confluency, and the supernatant was ultracentrifuged at 100,000 x g. Cells and exosomes samples were lysed in SDS and subjected to GeLC-MS/MS, with raw data being processed using the UniProt database, and identified proteins undergoing pathway analysis using the KEGG and DAVID databases. Exosomes were additionally characterized with western blotting, scanning electron microscopy (SEM), and Nanoparticle Tracking Analysis (NTA), complying to MISEV criteria.

2.1.3 Results in brief

A total of 1294 and 2748 proteins were identified in the exosomes and cells sample groups. A number of established pancreatic cancer biomarkers were significantly upregulated in the cancer cells-derived exosomes compared to their non-malignant counterparts, while more than half of the top 50 most upregulated cancer-exosomes proteins were identified by the Human Protein Atlas as being correlated with poor prognosis. Non-malignant exosomes upregulated proteins were involved in insulin secretion and cell adhesion, while in the malignant ones, the upregulated proteins were involved in the pre-metastatic niche formation and overall disease development.

2.1.4 Concluding remarks in brief

This study is among the few which compare the cancer derived- exosomes proteome not only to their donor cells but also to non-malignant cells and exosomes from the same tissue origin. Proteins uniquely identified in the malignant exosomes such as claudin-1 and EGFR highlight their potential key role in disease establishment and/or progression, while the differential expression of splicing factors across different sample groups depicts the significance of alternative splicing in the disease's landscape. Malignant exosomes contain upregulated proteins which are implicated in cancer hallmarks such as immune system surveillance evasion, metastasis, proliferation and invasion, as well as proteins involved in the stroma regulation, adding to the role of exosomes as important players in the cancer cells-tumour stroma crosstalk. The great biological significance of the pancreatic cancer-exosomes and

their abundance in bodily fluids, renders them good candidates for biomarker discovery and liquid biopsies development.

Publication 1

RESEARCH ARTICLE

Exosome Proteomics

Proteomics

www.proteomics-journal.com

Oncogenic and Non-Malignant Pancreatic Exosome Cargo Reveal Distinct Expression of Oncogenic and Prognostic Factors Involved in Tumor Invasion and Metastasis

Aikaterini Emmanouilidi, Dino Paladin, David W. Greening, and Marco Falasca**

Exosomes are small extracellular membrane vesicles important in intercellular communication, with their oncogenic cargo attributed to tumor progression and pre-metastatic niche formation. To gain an insight into key differences in oncogenic composition of exosomes, human non-malignant epithelial and pancreatic cancer cell models and purified and characterized resultant exosome populations are utilized. Proteomic analysis reveals the selective enrichment of known exosome markers and signaling proteins in comparison to parental cells. Importantly, valuable insights into oncogenic exosomes (362 unique proteins in comparison to non-malignant exosomes) of key metastatic regulatory factors and signaling molecules fundamental to pancreatic cancer progression (KRAS, CD44, EGFR) are provided. It is reported that oncogenic exosomes contain factors known to regulate the pre-metastatic niche (S100A4, F3, ITGβ5, ANXA1), clinically-relevant proteins which correlate with poor prognosis (CLDN1, MUC1) as well as protein networks involved in various cancer hallmarks including proliferation (CLU, CAV1), invasion (PODXL, ITGA3), metastasis (LAMP1, ST14) and immune surveillance escape (B2M). The presence of these factors in oncogenic exosomes offers an understanding of select differences in exosome composition during tumorigenesis, potential components as prognostic and diagnostic biomarkers in pancreatic cancer, and highlights the role of exosomes in mediating crosstalk between tumor and stromal cells.

1. Introduction

Pancreatic cancer is very difficult to detect and diagnose and is largely considered incurable, with a 5-year survival rate limited to ≈7%.[1] Pancreatic cancer can be of endocrine or exocrine nature; however, pancreatic ductal adenocarcinoma (PDAC) arising from exocrine cells and most commonly found in the head of the pancreas, is the most common type of pancreatic cancer (more than 90% of cases).[1] Unfortunately, less than one-fourth of diagnosed patients present with a resectable tumor, and few of them survive 5 years post-operatively.[7] These facts demonstrate the urgent need to understand the molecular mechanisms of pancreatic cancer and to identify novel and more effective diagnostic and prognostic biomarkers.[3]

Toward this, many studies have focused on body fluid-derived biomarkers such as cell-free nucleic acids, circulating tumor cells and proteins, and extracellular vesicles (EVs), namely exosomes.[4] Exosomes are endosomal-derived small EVs that facilitate intercellular communication in physiological and malignant conditions.[5] Exosomes carry molecules such as oncoproteins and oncopeptides, RNA species, lipids, and DNA fragments from parental to recipient cells, initiating phenotypic changes.[5] Emerging evidence suggests that exosomes have crucial roles in cancer development, including pre-metastatic niche formation and metastasis.[5–7] While the complexities surrounding the therapeutic potential of exosomes continue to be unraveled,[8] the use of exosomes as diagnostic tools is of significant focus for the field. In pancreatic cancer, efforts have been focused toward the use of exosomes in understanding cell communication and disease biology[4] and in diagnostic and therapeutic procedures.[9]

Previous studies have identified glypican-1 (GPC1),[10] different types of mucins, the multidrug resistance protein 1 (MDR1) and the cystic fibrosis transmembrane conductance regulator (CFTR) in these vesicles, highlighting the potential of exosomes as circulating markers of pancreatic cancer.[11] Further, pancreatic cancer exosomes have been shown to target and modulate pre-metastatic niche formation in the liver through macrophage migration inhibitory factor (MIF), initiating profound phenotypic changes in the liver to coordinate metastasis.[12] In addition, exosomal integrin expression has been shown to be of prognostic value, in regard to determining the organotropic metastasis dissemination to the liver and lung.[13]

Interestingly, although a number of studies have performed proteomic analysis of pancreatic cancer-derived exosomes, the

A. Emmanouilidi, Dr. D. Paladin, Prof. M. Falasca
Metabolic Signaling
School of Pharmacy and Biomedical Sciences
Curtin Health Innovation Research Institute
Curtin University
6102, Perth, Western Australia, Australia
E-mail: marco.falasca@curtin.edu.au
Dr. D. W. Greening
Baker Heart and Diabetes Institute
Department of Biochemistry and Genetics
La Trobe Institute for Molecular Science
La Trobe University
3086, Melbourne, Victoria, Australia
E-mail: d.greening@latrobe.edu.au

DOI: 10.1002/pmic.201800158

field still requires further detail regarding comparisons to their non-malignant counterpart. In an effort to exploit the property of the exosomes to serve as a source of circulating disease markers, and at the same time gain insights into the molecular events of pancreatic malignancy, we performed biophysical characterization and proteomic analysis of human pancreatic cancer-derived exosomes as well as non-malignant pancreatic epithelial models from pancreas duct cells, in comparison to parental cells. The potential clinical value of exosomes was supported by the fact that our approach identified several known and established biomarkers of pancreatic cancer in oncogenic exosomes. We further provide a correlation between the mutational landscape of cancer models in this study, and their composition within exosomes, indicating that even within cancer exosomes there is selective composition based on the oncogenic cells producing these EVs. We reveal key differences in exosomes derived from pancreatic cancer cells, which will from now on be referred to as "oncogenic" or "malignant," including metastatic and epithelial-to-mesenchymal promoting factors (S100A4, SDCBP), and signal transduction components (HMGB1, LGALS3, MET, ITGA3, ARF6). It is becoming increasingly clear that in addition to the soluble-secreted cytokines and chemokines that mediate cell communication at primary and secondary tumor sites,[14] extracellular membranous vesicles, including exosomes, are important regulators of the tumor microenvironment and metastatic dissemination.

Significance Statement

In this study, we comprehensively investigated the proteome of exosomes from both human pancreatic cancer cell lines and non-malignant human pancreatic epithelial cell lines. Applying proteomic analysis, we identified 362 proteins uniquely expressed in oncogenic exosomes, most of them related to pancreatic cancer progression such as key metastatic regulators and signaling molecules. We believe that our study will open new lines of investigation to further explore the pathological role of cancer cell-derived exosomes as well as the physiological role of non-malignant epithelial exosomes—important in the understanding of exosome biology and intercellular signaling.

2. Experimental Section

2.1. Cell Culture and Maintenance

Pancreatic cancer cell lines AsPC-1, BxPC3 and MiaPaCa2, as well as immortalized pancreas duct cells hTERT-HPNE were obtained from ATCC, and human pancreatic duct epithelial (HPDE) cells were kindly provided from Prof Hemant Kocher (Queen Mary University of London). Pancreatic cancer cell lines used had genomic mutations in the following genes (Sanger COSMIC database): AsPC1 (*CDKN2A, FBXW7, KRAS, MAP2K4, TP53*); BxPC3 (*CDKN2A, MAPK2K4, SMAD4, TP53*); MiaPaCa2 (*KRAS, TP53*). AsPC-1 and BxPC3 cells were routinely cultured in RPMI-1640 medium (Sigma) supplemented with 10% FBS (Bovogen Biologicals), MiaPaCa2 and hTERT-HPNE in Dulbecco's modified Eagle's medium (DMEM) (Sigma) supplemented with 10% and 5% FBS respectively, and HPDE cells were maintained in Keratinocyte-SFM (Life Technologies) supplemented with 0.05 mL bovine pituitary extract (BPE) and 0.005 µg mL^{-1} human recombinant EGF (Life Technologies). All media were supplemented with 100 units mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. Cells were maintained in humidified incubators at 37 °C with 5% CO_2.

2.1.1. Cell Line Characterization

Cells were seeded in a 96-well plate (3000 cells per well) in media containing either FBS or commercial exosome-depleted FBS (Thermo Fisher Scientific) and were incubated together with Cytotox Red (Essen Bioscience) dye for 72 h, allowing for quantification of dead cells. Cells were subsequently stained with Vybrant Green Stain (Thermo Fisher Scientific), allowing for the quantification of total cells. Visualization and quantification of the stained nuclei were carried out using Incucyte Zoom Live-Cell Analysis system (Essen Bioscience) and GraphPad Prism v6.01 used for statistical analysis (two-way ANOVA, Sidak's multiple comparisons test). Data are shown as the average of three independent experiments performed in triplicates ±SEM.

2.2. Exosome Isolation

Cells were seeded in 245 × 245 × 25 mm Corning culture dishes (500 cm^2 each—two per biological replicate, approximately 4 × 10^6 cells per dish) in their respective medium, with FBS replaced with exosome-depleted FBS (Thermo Fisher Scientific) to avoid cross-species exosome contamination. The average seeding density was 4 × 10^6 cells per dish, and cells were grown until ≈80% confluency. After 72 h following incubation, conditioned medium (CM) was collected and pooled (50 mL total), and centrifuged at 450 × *g* for 5 min followed by 2000 × g for 10 min at 4 °C, to remove cell debris and apoptotic vesicles. CM was then passed through a 0.22 µm filter to remove microvesicles, and the flow-through subjected to ultracentrifugation at 100 000 × *g* for 2 h at 4 °C. The remaining pellet was resuspended in PBS and washed (100 000 × *g* for 2 h at 4 °C), and the pellet reconstituted in PBS, aliquoted, and stored at −20 °C.[15] Freeze-thaw cycles were limited to a single thaw phase.

2.3. Whole Cell lysate

Cells grown in 245 × 245 × 25 mm Corning culture dishes (500 cm^2 each) were washed twice with ice-cold PBS, and cells exposed to 300 µL of 4× Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% LDS, 0.005% bromophenol Blue) (BioRad). Samples were left on ice for 10 min, scraped, before being stored at −80 °C. Prior to total protein quantitation, cell lysates were centrifuged at 14 000 × *g* for 5 min.

2.4. Protein Quantification

Sample protein quantification was determined using either 1D SDS-PAGE/SYPRO Ruby (Thermo Fisher Scientific) protein

staining densitometry as described[15] or microBCA assay (Thermo Fisher Scientific).

2.5. Western Blotting

For exosomal lysis prior to western blotting, samples were resuspended in RIPA buffer (NaCl 150 mM, Nonidet P-40 1%, Sodium deoxycolate 0.5%, EDTA 2.5 mM, SDS 0.1%, Tris-HCl 25 mM). Prior to electrophoresis, lysed samples (cells and exosomes, 15 µg) were heat treated at 95 °C for 5 min. Samples were electrophoresed on a 10% polyacrylamide gel at 120 V and transferred to a 0.45 µm nitrocellulose membrane, using the Trans-Blot SD cell (BioRad, #170-3940). Membranes were blocked for 1 h at room temperature (RT) with 3% BSA-TBST 0.05%, and probed with the indicated primary antibodies overnight at 4 °C. All antibodies were purchased from Cell Signaling Technology and were used at a dilution of 1:1000, apart from TSG101 (Novus Biologicals, 1:500) and Glypican-1 (GeneTex Inc., 1:1000). Membranes were washed in TBST (3 × 10 min) at RT, followed by incubation with secondary anti-mouse or anti-rabbit antibodies (1:20 000) for 1 h at RT and visualized with ChemiDoc MP Imaging System (BioRad).

2.6. Scanning Electron Microscopy

Exosomes (reconstituted in PBS, from 50 mL of CM), were mixed with equal volume of 5% glutaraldehyde (Sigma) on a covered glass coverslip and left overnight at RT. Samples underwent serial dehydration with 10, 20, 40, and 90% ethanol solution, and coverslips mounted on aluminium stubs using carbon conductive tape. Imaging was performed by the Microscopy and Microanalysis Facility, John de Laeter Centre, Faculty of Science and Engineering, Curtin University, Bentley, WA.

2.7. Mass Spectrometry–Based Proteome Profiling

Proteomic sample preparation was performed as described,[16] in biological triplicate (with technical duplicate).[17] Briefly, protein samples (10 µg) were lysed in SDS sample buffer and proteins denatured at 95 °C (5 min). Proteins were separated by SDS-PAGE (150 V, 15 min) and visualized by Imperial Protein Stain (Thermo Fisher Scientific). Samples were excised into equal fractions ($n = 2$), reduced with 2 mM tri(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma-Aldrich, C4706) at 22 °C for 4 h on gentle rotation, followed by alkylation with 25 mM iodoacetamide for 30 min at 25 °C in the dark, and digested with trypsin (Promega, V5111) at a 1:50 enzyme-to-substrate ratio for 16 h at 30 °C[18] . The peptide mixture was acidified to a final concentration of 2% formic acid, 0.1% trifluoroacetic acid (TFA) and centrifuged at 16 000 × g for 15 min. Subsequently, peptides were purified and extracted using reverse-phase C18 StageTips (Sep-Park cartridges, Waters, MA) in 85% v/v acetonitrile (ACN) in 0.5% v/v formic acid (FA). Peptides were lyophilized and acidified with buffer containing 0.1% FA, 2% ACN.

Proteomic experiments were performed in biological triplicate, with technical replicates ($n = 2$), with MIAPE-compliance.[19,20] A nanoflow UPLC instrument (Ultimate 3000 RSLCnano, Thermo Fisher Scientific) was coupled online to a Q-Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific) with a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were loaded (Acclaim PepMap100, 5 mm × 300 µm i.d., µ-Precolumn packed with 5 µm C18 beads, Thermo Fisher Scientific) and separated (BioSphere C18 1.9 µm 120 Å, 360/75 µm × 400 mm, NanoSeparations) with a 120-min gradient from 2–100% v/v phase B (0.1% v/v FA in 80% v/v ACN) (2–100% 0.1% FA in acetonitrile (2–40% from 0–100 min, 40–80% from 100–110 min at a flow rate of 250 nL min^{-1} operated at 55 °C.

The mass spectrometer was operated in data-dependent mode where the top 10 most abundant precursor ions in the survey scan (350–1500 Th) were selected for MS/MS fragmentation. Survey scans were acquired at a resolution of 60 000, with an MS/MS resolution of 15 000. Unassigned precursor ion charge states and singly charged species were rejected, and peptide match disabled. The isolation window was set to 1.4 Th and selected precursors fragmented by HCD with normalized collision energies of 25 with a maximum ion injection time of 110 ms. Ion target values were set to 3e6 and 1e5 for survey and MS/MS scans, respectively. Dynamic exclusion was activated for 30 s. Data were acquired using Xcalibur software v4.0 (Thermo Fisher Scientific). Raw mass spectrometry data are deposited in PeptideAtlas (#PASS01331).

2.8. Database Searching and Protein Identification

Raw data were pre-processed as described[21] and processed using MaxQuant[22] (v1.6.0.1) with Andromeda (v1.5.6), using a Human-only (UniProt #71 785 entries) sequence database (January 2018). Data were searched as described[19] with a precursor ion tolerance of 10 ppm, fragment tolerance of 0.5 Da and minimum peptide length 6, with false discovery rate <1% at the peptide and protein levels, tryptic digestion with up to two missed cleavages, cysteine carbamidomethylation as fixed modification, and methionine oxidation and protein N-terminal acetylation as variable modifications, and data analyzed with label-free quantitation (LFQ).[23] LFQ intensities for all unique and razor peptides were included, with zero intensity values replaced with a constant value of 1 (imputation, constant value) to calculate fold change ratios. As a result, LFQ intensity values were averaged, LFQ intensity values were normalized for protein length, and fold change ratios calculated. Contaminants, and reverse identification were excluded from further data analysis. Protein identifications were accepted, if they reached greater than 99% probability (protein FDR < 1%) and contained at least two identified unique peptides (q-values < 1%). Resulting p-values were adjusted by the Benjamini-Hochberg multi-test adjustment method for a high number of comparisons[24] and statistics performed as previously described.[25] For pathway analyses, Kyoto Encyclopedia of Genes and Genomes (KEGG) and NIH Database for Annotation, Visualization and Integrated Discovery Bioinformatics Resources 6.7 (DAVID) resources were utilized using recommended analytical parameters.[26] For gene ontology enrichment and network analyses, UniProt (www.uniprot.org) database resource (biological process, molecular function) was

utilized. Interaction mapping and protein association pathways were performed using STRING (https://string-db.org/). For such pathway and enrichment analyses, *Homo Sapiens* background genome was utilized. Differentially expressed proteins were identified using the criteria: Fold change ratios $>\pm 2.0$ and $p < 0.05$, with identifications in at least two biological sample replicates. Clustering of samples was performed by principal component analysis (PCA) and visualized using ggplot2[27] and ggfortify (https://cran.r-project.org/web/packages/ggfortify/index.html). The heat map of proteins was performed using gplots (https://cran.r-project.org/web/packages/gplots/index.html).

3. Results and Discussion

This study aimed to analyse the composition of pancreatic cancer cell-derived exosomes in comparison to their non-malignant counterparts, toward understanding how malignancy alters oncogenic cargo exosome for the potential to determine molecular drivers of pancreatic cancer and as disease-specific biomarkers.

3.1. Exosome Isolation & Characterization from Cancer and Non-Cancer Models

A complete workflow for the isolation and characterization of exosomes from human non-malignant pancreatic duct models (HPDE, hTERT-HPNE) and cancer cell models (AsPC1, BxPC3, and MiaPaCa2) is provided (**Figure 1**A and Figure S1, Supporting Information). Cells maintained in exosome-depleted FBS were shown to have limited effect on cell viability over 72 hours – therefore we utilized 72 h incubation for CM collection (Figure S2A–C, Supporting Information). For exosome isolation, CM was differentially centrifuged and filtered (0.22 µm), prior to ultracentrifugation. To confirm enrichment of exosome markers, western immunoblotting was performed (relative to cell lysate counterpart) using TSG101 and the absence of endoplasmic reticulum marker calnexin, confirming that exosomes based on their protein marker enrichment were identified in all models (Figure 1B). Further, exosomes were morphologically characterized using SEM (Figure 1C), confirming their spherical shape and size within the reported range of 30–150 nm.[28]

3.2. Proteomic Analysis of Exosomes Reveal Distinct Protein Composition to Parental Cells

3.2.1. Exosomes Exhibit Unique and Distinct Cargo in Comparison to Their Corresponding Cell Line

We next compared the protein profiles of purified exosomes and parental cells using GeLC-MS-MS. MS-based proteomics analysis revealed 2748 cellular and 1294 exosomal proteins across all sample groups.

In comparison to parental cells, 1023 proteins were expressed in both exosomes and their corresponding cell line, while 271 proteins were specific to exosomes, indicating the select enrichment in exosomes of these lowly-expressed proteins in cells (**Figure 2**A). Of these, various tetraspanin components including TSPAN4/5/6/7/8/9/14/15, and CD63, in addition to CD9, CD59, CD81, CD82, and exosomal marker proteins TSG101 and Alix (PDCD6IP), were identified, several components of which have been extensively reported associated with exosome formation.[29] This indicates that we have specifically enriched for exosomes in this study.

Based on global heatmap protein expression correlation revealed key differences between exosome and cell lysate profiles, and a high degree of similarity in biological replicates for each sample group (Figure 2B). KEGG pathway and GO analysis based on DAVID database revealed that exosomes are significantly enriched in proteins involved in the complement and coagulation cascades (hsa04610, 4.20E-08), focal adhesion (hsa04510, 1.40E-02), and cell adhesion molecules (hsa04514, 1.20E-03), and proteins associated with extracellular matrix and receptor interaction (hsa04512, 4.00E-06), and endocytosis (hsa04144, 8.10E-03) (Figure 2C).

3.2.2. Distinct Cell Profiles between Non-Malignant Epithelial and Pancreatic Cancer

We report significant differences in cellular protein composition between non-malignant epithelial and pancreatic cancer models. A total of 1534 proteins were commonly identified between both cell groups, while 389 proteins were specific to non-malignant cells, and 263 proteins were identified in malignant cells only. For differential protein expression (LFQ ratio ≥ 2 or ≤ -2, $p < 0.05$), 590 (27% total) proteins were significantly upregulated in malignant cells, while 435 (20% total) proteins were downregulated in expression relative to non-malignant cells (Figure S3, Supporting Information).

Non-malignant epithelial cells uniquely express proteins that act as tumor suppressors and sustain the physiological cellular processes such as ARFIP1,[30] NES[31] HNRNPH1,[32] and ACY1.[33] Other proteins uniquely identified in non-malignant epithelial cells include—but are not restricted to—TNFAIP2, P4HA2, FLNC, CSH1, CDH13, ITGA1, FGF2, COL1A2, and COL5A1.

Furthermore, other proteins of interest enriched in cancer cells include Histone H2A type 1-C, aldehyde dehydrogenase (dimeric NADP-preferring), 28S ribosomal protein S29 (mitochondrial), Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-5, ribosomal RNA small subunit methyltransferase NEP1 and HIG1 domain family member 2A (mitochondrial). It is clear that, in these pancreatic cancer cells, select proteins are identified as enriched in comparison to their non-malignant counterparts, suggestive to play important functional roles in the development and progression of tumorigenesis.

3.2.3. Proteomic Analysis Depicts Differences in Malignant and Non-Malignant Exosomes Cargo, and Reveals Novel Oncogenic Exosome Cargo

We next questioned whether the composition of exosomes differed between pancreatic cancer and non-malignant states. For exosomes, GeLC-MS/MS profiling[34] identified a total of 1294

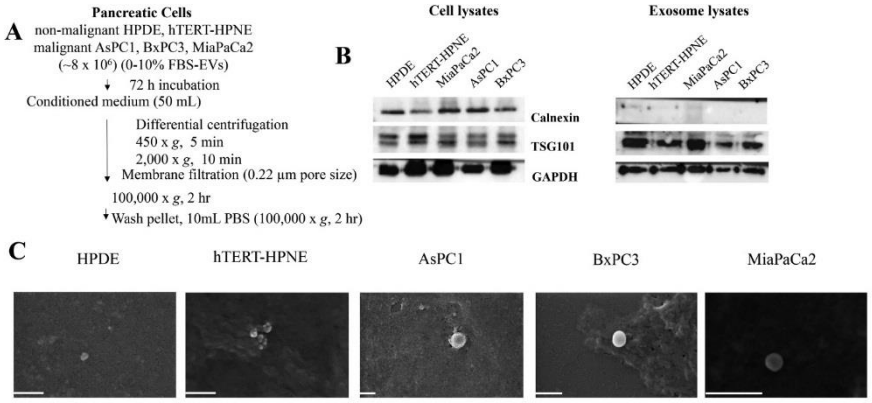


Figure 1. Isolation and characterization of non-malignant pancreatic epithelial and pancreatic cancer cell exosomes. A) Experimental workflow for exosome isolation from non-malignant (HPDE, hTERT-HPNE) and cancer (BxPC3, MiaPaCa2, AsPC1) cell conditioned media. B) For Western blotting, cell lysate and exosome preparations (10 µg) were separated by 1D-SDS-PAGE, electro transferred, and probed with GAPDH, exosome marker TSG101 and the endoplasmic reticulum marker, calnexin. C) Exosomes were viewed with scanning electron microscopy (SEM). Scale bar 200 nm.

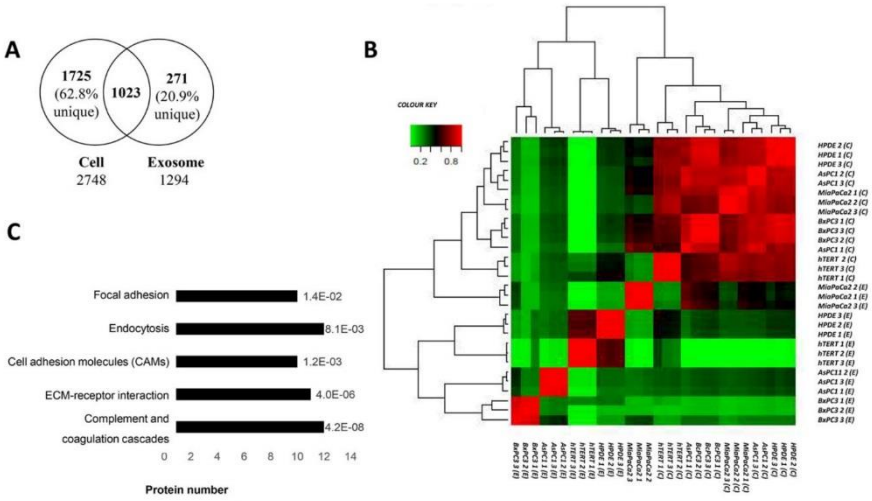


Figure 2. Proteome profiling of cancer and non-malignant pancreatic exosomes. A) Proteins from cancer and non-malignant exosomes and their corresponding cell lysates (hTERT-HPNE, HPDE, AsPC1, BxPC3, MiaPaCa2) were separated by 1D-SDS-PAGE, gel sections subjected to in-gel tryptic digestion, with mass spectrometry-based proteomic profiling performed. Venn diagram of cell and exosome proteome (all models combined) reveal 1023 proteins commonly identified, while 1725 (62.8% unique identifications) and 271 (20.9% unique identifications) proteins were specific for each sample group, respectively. B) Correlation expression heatmap of exosome (E) and cell lysates (CL) for each biological replicate ($n = 3$). C) KEGG Pathway analysis based on DAVID Bioinformatics Resource knowledgebase of proteins specifically identified in exosomes (relative to cells), representing significant enrichment ($p < 0.05$) of pathways associated with complement and coagulation cascades, ECM-receptor interaction, cell adhesion molecules, endocytosis, and focal adhesion.

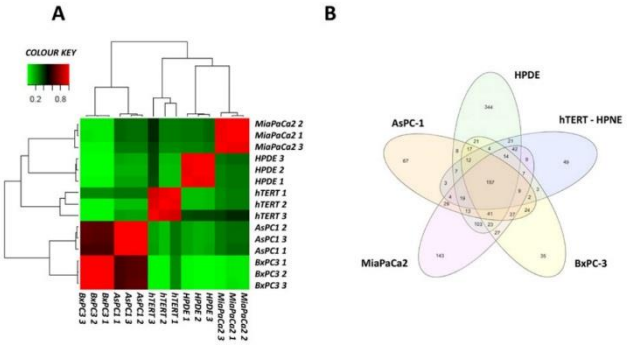


Figure 3. Proteomic profiling reveals global characterization of exosomes. A) Correlation expression heatmap of exosome samples for each biological replicate ($n = 3$). B) A five-way Venn diagram of proteins identified in exosomes, with 157 proteins commonly identified in all five exosome models, while uniquely identified exosomal proteins for each model include 143 proteins (MiaPaCa2), 344 proteins (HPDE), 49 proteins (hTERT-HPNE), 67 proteins (AsPC1), and 35 proteins (BxPC3), respectively.

proteins, comprising 880 and 932 in cancer and non-malignant exosomes, respectively, and 518 proteins common to both exosome types, as shown in the Venn diagram in **Figure 3**B. A global analysis of all five cellular and exosomal models showed that not only the exosomal protein cargo is distinct from the cellular one, but also that the abundance of the components highly differs, as depicted in Figure 3A. In regard to the proteins common between malignant and non-malignant exosome models, many are involved in exosome biogenesis (e.g., proteins involved in the endosomal sorting complex required for transport (ESCRT) machinery such as PDCD6, PDC6IP/Alix), coordination of intracellular vesicle trafficking (e.g., tetraspanins such as CD9 and CD82,[35] SDCBP, small Rab GTPases such as RAB1B, RAB5C, RAB7A, RAB10, RAB14, RAB11B, and RAB35[36]), and annexins such as ANXA1, ANXA2, ANXA4, ANXA5, and ANXA11.[37] Overall, 66 of the 1294 exosomal proteins identified in this study (5.1%) have not been reported in ExoCarta (Table S1, Supporting Information), including mucin-2 and claudin-11.

3.3. Exosomes Contain Distinct Protein Biomarkers in Pancreatic Cancer

Importantly, we report several known pancreatic cancer biomarkers in exosomes from pancreatic cancer models, including significant upregulated expression of CD151, LGALS3BP, and histone H2BE, . as reported by Castillo et al..,[38] We have also identified members of the claudins (CLDN) family in cancer-derived exosomes, such as claudin-1 and -3 (CLDN1, CLDN3). CLDN1 promotes breast cancer though interaction with EPCAM, Ephrin B1 (EFNB1), and CD9,[39] with both EFNB1 and CD9 being significantly upregulated in our cancer-derived exosomes. Claudins have been reported to co-express with mucins, proteins known for their contribution to pancreatic cancer progression,[40] and we are the first to report the significant upregulated expression and detection of MUC5B uniquely in pancreatic cancer-derived exosomes, along with the identification of other members of the mucin family including MUC1, 4, 2, 5AC, 17, and 13.

In our study, GPC1 was identified in both malignant and non-malignant epithelial exosomes, in accordance to Castillo et al.[38]Another protein of interest is the Macrophage migration inhibitory factor (MIF), exclusively identified in exosomes derived from a metastatic model of pancreatic cancer (MiaPaCa2 cells). Importantly, in agreement with previous studies,[41] we have also detected tetraspanin-8 (Tspan8), hepatocyte growth factor receptor (MET), and significant elevated expression of CD44 antigen in malignant exosomes. A complete table of known PDAC biomarkers identified in our exosomes, along with relevant references, can be found in **Table 1**.

To provide further insights into the composition of malignant exosomes, we analyzed the 50 highly upregulated proteins in our cancer-derived exosomes (including all upregulated proteins irrespective of the significance of this change in expression) to reveal >60% have been reported to correlate with poor prognosis in pancreatic cancer according to the Human Protein Atlas[42] (Table S2, Supporting Information). Interestingly, Serine protease 23 (PRSS23), significantly upregulated in expression in malignant exosomes, has been demonstrated to correlate with poor prognosis. Serine proteases have been previously proposed to be candidate biomarkers in pancreatic cancer screening.[43] Another key component identified in malignant exosomes was the G protein–coupled receptor, class C, group 5, member A (GPRC5A), which promotes pancreatic cancer cell proliferation and migration.[44] Further, ADP-ribosylation factor 6 (ARF6), a mutant *KRAS* downstream target which acts through c-Myc to sustain the Warburg effect (pro-metabolic function) in pancreatic cancer cells,[45] and has been previously detected in exosomes secreted from different tumors,[46,47] but not pancreatic cancer.

Our study underlines the importance of oncogenic exosome cargo, the potential to further understand the molecular function of these cargo, and the potential for use in liquid biopsies as carriers of prognostic and diagnostic PDAC biomarkers (in the case of PRSS23). Further, such insights provide leads to

Table 1. Known exosomal pancreatic cancer biomarkers, verified in our study.

Protein accession	Protein description	Gene name	Non-malignant pancreatic exosome LFQ (mean LFQ intensity, models: hTERT, HPNE)	Oncogenic pancreatic exosome LFQ (mean LFQ intensity, models: AsPC1, BxPC3, MiaPaCa2)	LFQ ratio (oncogenic and non-malignant pancreatic exosome cargo)	*p*-Value (Benjamini-Hochberg multi-test adjustment method)	Reference
P48509	CD151 antigen	CD151	279629.8	603368.5	2.2	4.19E-04	[36]
Q08380	Galectin-3-binding protein	LGALS3BP	5344900.3	9471908.8	1.8	1.41E-04	
Q16778	Histone H2B type 2-E	HIST2H2BE	1215003.9	43260935.6	35.6	4.06E-76	
U3KQK0	Histone H2B	HIST2H2BF	231755.0	11744578.3	50.7	1.53E-83	
P35052	Glypican-1	GPC1	86599.2	123776.9	1.4	NS	[9]
P16070	CD44 antigen	CD44	162537.1	399524.3	2.5	2.70E-05	[40]
P08581	Hepatocyte growth factor receptor	MET	ND	23344.0	23344.0	NS	
P19075	Tetraspanin-8	Tspan8	1108931.1	ND	9.018E-07	NS	
P14174	Macrophage migration inhibitory factor	MIF	ND	532402.9	532402.9	NS	[12]

ND, not detected; NS, non-significant.

further understand oncogenic transformation of pancreatic cancer (as distinct from non-malignant counterparts), and the capacity of exosomes to potentially mediate changes in their local environment, to facilitate changes in cell metabolism (ARF6) as well as resistance to chemotherapy (GPRC5A, MUC-1, PCDH1).

3.4. Understanding the Composition of Non-Malignant Exosomes

To gain insight into the composition of non-malignant exosomes, we analyzed the distinct proteome profile of exosomes from non-malignant models (relative to composition of exosomes from pancreatic models) (**Figure** 4A). Among the significantly enriched components in the non-malignant exosomes included Rho GDP-dissociation inhibitor 1 (ARHGDIA), involved in Rho GTPases activation/ inactivation cycle and associated with several cancers including breast, non-small cell lung, and glioma.[48] Other insulin secretion-related proteins identified in non-malignant exosomes include the significantly enriched expression of hypoxia upregulated protein 1 (HYOU1)[49] and identification of activin A (INHBA).

Our data reveal that non-malignant pancreatic exosomes are also significantly enriched in protein cargo implicated in controlled cell adhesion (GTP binding/GTPase activity, extracellular matrix and receptor interaction) (RAN, TGM2, RAB1B;RAB1C, FSCN1, LAMB3) and metabolic signaling pathways including glycolysis, gluconeogenesis, the TCA cycle and amino acid biosynthesis (PKM, TPI1, PFKL, G6PD, PGM1, MDH1/2, PGAM1) (Figure 4B), as well as proteins crucial for spatiotemporal regulation of insulin secretion (PARK7, DYNLL1).

3.5. Cancer Cell Mutational Landscape Reflects Differences in Oncogenic Exosome Protein Cargo

We further correlated the mutational background of different pancreatic cell models, and how this affected the composition of exosomes. Previously, in colorectal cancer-derived exosomes, mutant KRAS status dramatically affected the composition of the exosome proteome.[50] It is interesting to note that regarding *KRAS* wild-type exosomes (HPDE, hTERT-HPNE, BxPC3) CTNNB1 was uniquely detected and CAPZA1, RSU1 and TLDC1 were uniquely and significantly expressed. Interestingly, although the Ras suppressor protein 1 (RSU1) has been previously identified in exosomes derived from ovarian[47] and squamous carcinoma,[51] this is the first study identifying this specific protein in non-malignant epithelial pancreatic cell-derived exosomes, but not in the respective cancer-derived ones.

3.6. Oncogenic Exosomes Contain Components Known to Modulate Metastatic Niche and Tumor Progression

Tumor-derived exosomes have been reported to have the ability to facilitate tumor growth and metastasis.[12,52,53] Importantly, pancreatic cancer exosome proteome was shown to contain several key metastatic factors known to regulate the pre-metastatic niche and metastasis.[7,53,54] Several mediators of tissue invasion, intravasation and metastasis were upregulated in the malignant exosomes, including Hepatocyte growth factor receptor (MET), Disintegrin and metalloproteinase domain-containing protein 9 (ADAM9) and significant differential expression of galectin-3 (LGALS3) (**Table 2**). Importantly, in pancreatic cancer exosomes, we report the identification of CLDN1, YES1, EGFR, and GPC1 with the differential expression of these proteins in malignant exosomes validated by antibody-based western blotting (Figure 4C). Protein enrichment analysis revealed that angiogenesis, innate immune response and cell adhesion were among the pathways that exhibited significant enrichment ($p < 0.05$) in malignant exosomes compared to their non-malignant counterpart (Figure 4D). Interestingly, CLDN1 and EGFR were identified based on mass spectrometry and western immunoblotting in all three malignant exosomal groups and were not detected in

Table 2. Proteins implicated in cancer hallmarks, upregulated in our malignant exosomes.

Protein accession	Protein description	Gene name	Non-malignant pancreatic exosome LFQ (mean LFQ intensity, models: hTERT, HPNE)	Oncogenic pancreatic exosome LFQ (mean LFQ intensity, models: AsPC1, BxPC3, MiaPaCa2)	LFQ ratio (oncogenic and non-malignant pancreatic exosome cargo)	*p*-Value (Benjamini-Hochberg multi-test adjustment method)	Molecular function/process
P11279	Lysosome-associated membrane glycoprotein 1	LAMP1	ND	251181.9	251181.9	NS	*Metastasis*
Q9Y5Y6	Suppressor of tumorigenicity 14 protein	ST14	ND	34206.6	34206.6	2.20E-43	
O00592	Podocalyxin	PODXL	ND	119833.9	119833.9	NS	*Invasion*
Q9UGM3	Deleted in malignant brain tumors 1 protein	DMBT1	ND	45255.1	45255.1	1.07E-20	
Q7L576	Cytoplasmic FMR1-interacting protein 1	CYFIP1	10558.5	38907.7	3.7	1.12E-13	
P26006	Integrin alpha-3	ITGA3	249308.6	892615.4	3.6	6.01E-11	
P39060	Collagen alpha-1(XVIII) chain	COL18A1	ND	427081.6	427081.6	NS	*Angiogenesis*
O60687	Sushi repeat-containing protein SRPX2	SRPX2	ND	276854.5	276854.5	NS	
Q99828	Calcium and integrin-binding protein 1	CIB1	ND	60516.7	60516.7	2.20E-41	
Q13443	Disintegrin and metalloproteinase domain-containing protein 9	ADAM9	ND	50123.2	50123.2	NS	
P08581	Hepatocyte growth factor receptor	MET	ND	23344.0	23344.0	NS	
O75340	Programmed cell death protein 6	PDCD6	280329.0	1103649.2	3.9	8.92E-16	
P09429	High mobility group protein B1	HMGB1	ND	1323834.6	1323834.6	NS	*Migration*
P17931	Galectin-3	LGALS3	ND	306782.3	306782.3	1.02E-47	
Q9UIW2	Plexin-A1	PLXNA1	ND	35694.4	35694.4	2.69E-59	
P98160	Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	230718.6	1558232.8	6.8	2.24E-37	
P04083	Annexin A1	ANXA1	1344711.0	7732107.9	5.8	2.82E-13	
O00560	Syntenin-1	SDCBP	2439015.7	8844407.2	3.6	7.64E-09	
O15230	Laminin subunit alpha-5	LAMA5	113471.4	377880.0	3.3	2.35E-43	
P06748	Nucleophosmin	NPM1	ND	970785.0	970785.0	NS	*Proliferation*
P10909	Clusterin	CLU	ND	720947.0	720947.0	NS	
P02786	Transferrin receptor protein 1	TFRC	18853.5	365814.0	19.4	3.46E-39	
Q03135	Caveolin-1	CAV1	406482.6	1785445.2	4.4	1.75E-12	
Q8WUM4	Programmed cell death 6-interacting protein	PDCD6IP	643310.3	2046532.3	3.2	1.58E-10	
Q9Y265	RuvB-like 1	RUVBL1	98968.4	259527.6	2.6	4.76E-03	

ND, not detected; NS, non-significant.

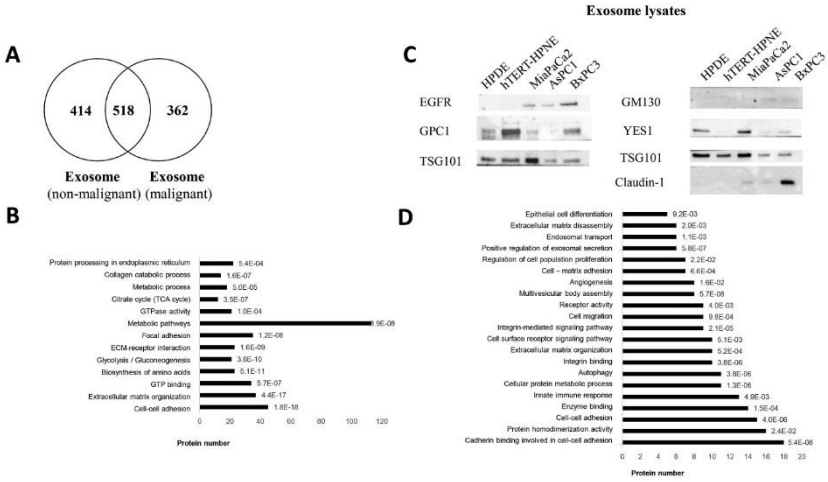


Figure 4. Exosome proteome reveals key insights into oncogenic and non-malignant cargo. A) A two-way Venn diagram of exosome proteome indicating combined non-malignant (hTERT-HPNE, HPDE) and malignant (AsPC1, BxPC3, MiaPaCa2) models, respectively. B) KEGG Pathway and GO analysis based on DAVID Bioinformatics Resource knowledgebase of proteins uniquely identified in non-malignant exosomes, representing significant enrichment ($p < 0.05$) of pathways associated with cell adhesion, GTP binding/GTPase activity, metabolic processes, ECM organization, and endoplasmic reticulum protein processing. C) For Western blot validation exosomes lysates were probed with anti-claudin1, anti-YES1, anti-EGFR, and anti-GPC1 primary antibodies. TSG101 was used as a loading control and GM130 as a negative exosomal marker. D) Gene Ontology (GO) analysis based on DAVID Bioinformatics Resource knowledgebase of proteins uniquely identified in malignant pancreatic exosomes, representing significant enrichment ($p < 0.05$) of pathways associated with cell adhesion, innate immune response, integrin binding and signaling, and angiogenesis.

non-malignant exosomes, raising the potential of these markers as key factors in pancreatic cancer exosome biology.

Exosomes have been found to enhance the migrating potential of cancer cells via Wnt signaling upregulation.[55] In our analysis, we have detected multiple Wnt-related proteins which are upregulated in the cancer-derived exosomes, the proteins known to modulate and promote tumor progression. These include, the significant upregulation of syndecan-1 (SDC1), caveolin-1 (CAV1), and nicastrin (NCSTN). Other migration-promoting proteins identified include the collagen alpha-1(XVIII) chain (COL18A1), galectin-3 (LGALS3) ($p < 0.05$), sushi repeat-containing protein (SRPX2), podocalyxin (PODXL)—which is correlated with poor prognosis in PDAC,[56] calcium and integrin-binding protein 1 (CIB1) ($p < 0.05$), and plexin-A1 (PLXNA1) ($p < 0.05$).

We further report various components in cancer exosomes previously attributed to pre-metastatic niche formation[5–7,12,13] (**Table 3**). A number of factors have a specific contribution in the formation of the pre-metastatic microenvironment, including primary tumor hypoxia and bone marrow-derived cell (BMDC) recruitment which promotes immunosuppression, among others.[57] The potential for tumor-derived exosomes to control the establishment of organ-specific pre-metastatic niches has been demonstrated by select integrins.[13] In this study, we report unique identification of ITGB5 and significant differential expression of ITGB4 (attributed as key selectors for liver and lung tropism, respectively[58]) in cancer-derived exosomes. Immune system-related proteins upregulated in the PDAC exosomes include the high mobility group protein B1/2 (HMGB1/2), membrane cofactor protein (CD46), Complement C4-A (C4A;C4B), and DNA-dependent protein kinase catalytic subunit (PRKDC). Another distinct exosomal protein which is implicated in immunosuppression, is the prostaglandin E2 synthase (PTGES3). Other proteins upregulated in our cancer exosomes and related with pre-metastatic niche formation are Annexin A1 (ANXA1),[59] (MET)[60] and protein S100-A4 (S100A4).[61]

3.7. Selective Enrichment or Downregulation of Alternative Splicing Regulators in Cancer Cells and Exosomes

Alternative splicing (AS) is the main reason behind the immense protein diversity occurring in nature and defects in the AS process have been shown to reiterate hallmarks of cancer. Two major protein groups with competing functions in AS are the serine and arginine-rich (SR) proteins and the heterogeneous nuclear ribonucleoproteins (hnRNPs). An analysis of proteomic composition regarding SRs identified SRSF7, 10, 11, 2, 9, 6, 1, and 3 in both malignant and non-malignant cell models, with only SRSF1 and SRSF3 significantly upregulated, while SRSF1, 7, 2, 3, and 6 were uniquely expressed in the cancer-derived exosomes.

Table 3. Proteins detected in the malignant exosomes, known to contribute in the pre- metastatic niche formation.

Protein accession	Protein description	Gene name	Non-malignant pancreatic exosome LFQ (mean LFQ intensity, models: hTERT, HPNE)	Oncogenic pancreatic exosome LFQ (mean LFQ intensity, models: AsPC1, BxPC3, MiaPaCa2)	LFQ ratio (oncogenic and non-malignant pancreatic exosome cargo)	*p*-Value (Benjamini-Hochberg multi-test adjustment method)	Reference
P08581	Hepatocyte growth factor receptor	MET	ND	23344.0	23344.0	NS	[60]
P18084	Integrin beta-5	ITGB5	ND	62772.8	62772.8	NS	[12]
P16144	Integrin beta-4	ITGB4	64385.3	475123.7	7.4	4.78E-63	
P26447	Protein S100-A4	S100A4	ND	917297.0	917297.0	NS	[61]
P04083	Annexin A1	ANXA1	1344711.0	7732107.9	5.8	2.82E-13	[59]
P16070	CD44 antigen	CD44	162537.1	399524.3	2.5	2.70E-05	[73]

ND, not detected; NS, non-significant.

A number of hnRNPs show significant upregulation in cancer cells, including hnRNP-H2/UL2/AS/A2B1/U/C/F/A0/M, whereas only hnRNPC is significantly upregulated in cancer exosomes. The differential expression of SR (splicing enhancers) and hnRNP (splicing suppressors) proteins in malignant and non-malignant cells and corresponding exosomes depicts the importance of AS in cancer and its effect on a number of cancer hallmarks. Among the proteins analyzed in the current study, proteins known for their altered AS pattern and their severe impact in PDAC progression were identified in both malignant cells and exosomes, including CD44 and EGFR[62] and the identification of specific isoforms and SR proteins phosphorylation levels in cell and exosome samples would be of prognostic and diagnostic significance.

4. Concluding Remarks

The opportunity to exploit exosomes as biomarkers is an area of intense investigation in biomedical and clinical research. In an effort to evaluate the significance of exosomes in pancreatic cancer, not only as sources of biomarkers but also as oncogenic signaling mediators contributing to disease progression, we performed in-depth proteomic analysis of exosomes from human non-malignant epithelial and pancreatic cancer cell models, in addition to their donor cells. We demonstrated the significant upregulation of key known malignant factors including various cell invasive and metastatic components (CLDN1, EGFR), in addition to proteins known to correlate with poor prognosis (GPRC5A, PRSS23, and ITGA3). Interestingly, proteins uniquely identified in the malignant pancreatic exosomes could be categorized into four clusters including proteins related to extracellular matrix (for instance disassembly), cancer progression (angiogenesis, proliferation, autophagy), signaling (integrins, receptor activity), and stroma regulation. We further identified known modulators of pre-metastatic niche formation including NCSTN, ANXA1, and S100A4. A notable remark is the selective enrichment of the malignant exosomes in AS regulating factors such as SR proteins, compared to hnRNPs. Importantly, GPC1 was detected in exosomes from malignant and non-malignant models (Figure 4C). The non-malignant epithelial cells used in this study are pancreatic duct epithelial cells immortalized via hTERT insertion (hTERT-HPNE) or transformed with HPV oncoproteins (HPDE). hTERT insertion is shown to induce genomic instability and cells acquire epigenetic traits, therefore exhibiting different phenotypes comparing to their donor counterparts,[63] whereas HPV-induced immortalization is accompanied by p53 degradation and c-Myc overexpression, among others.[64] Human Protein Atlas[42] depicts the differential expression of GPC1 among various cancer, immortalized, and transformed cell lines. In all cases, due to their ability to undergo limitless divisions, their inevitable genomic instability (aneuploidy, deletions, etc.) and their altered epigenetic status, the use of immortalized cells as normal controls should take into account these limitations.[65] Castillo et al.[38] did not detect GPC1 in pancreatic cancer-derived exosomes despite the multiple antibodies used (Abcam (ab137604), Thermo Fisher (PA5-28055 and PA5-24972) and Sigma-Aldrich (SAB2700282)); however, they identified the protein selectively in the non-neoplastic exosomes.[38] Yang et al.,[66] reported that an EV signature including EGFR, EpCAM, GPC1, WNT2, and MUC1 was able to distinguish between PDAC and other pancreatic conditions with 86% sensitivity and 81% specificity; nevertheless, GPC1 could not deliver such results as a single marker.[66] In our study, we analyzed exosomes from two non-malignant and three malignant pancreatic cell lines and mass spectrometry analysis detected GPC1 in both malignant and non-malignant exosome types; however, it did not reveal a significant differential expression in either of the two groups. Indeed, western blotting analysis confirmed the presence of GPC1 in both exosome groups. The antibody used in our study was GTX104557 (GeneTex, Inc), different from what the two aforementioned studies, or Melo et al. used, which was discontinued. Discrepancies between different studies can be attributed to different antibodies, different genetic alterations occurring during cell passaging and the inevitable gap between in vitro and in vivo settings. The results of our study in regard to GPC1 support the notion of the establishment of a combination of markers rather than individual proteins, in order to successfully distinguish PDAC from other pathologic conditions of the pancreas, taking into account the great genetic heterogeneity of the disease and the need to screen large populations.

Further studies investigating how different cells following tumorigenesis alter their exosome composition, and insights into the functional capacity of these exosomes in transferring,

initiating, and regulating tumorigenesis in recipient cells remain in question.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was supported by Avner Pancreatic Cancer Foundation (grants to M.F.) and AB Analitica. A.E. is supported by the Curtin International Postgraduate Research Scholarship (CIPRS)/AB Analitica Scholarship. D.W.G. is supported by National Health and Medical Research Council of Australia project Grants #1141946 and #1139489, La Trobe Institute for Molecular Science Fellowship, La Trobe University Leadership Research Focus grant, and La Trobe University Start-up Fund. A.E. and M.F. also acknowledge the infrastructure and staff support provided by CHIRI, School of Pharmacy & Biomedical Sciences and Faculty of Health Sciences, Curtin University. Part of this research was undertaken using the Zeiss Neon 40EsB Cross-beam FESEM instrumentation (ARC LE0775553) at the John de Laeter Centre, Curtin University.

Conflict of Interest

D.P. is a consultant at AB Analitica (Padova, Italy). The remaining authors declare no conflict of interest.

Keywords

exosomes, metastasis, pancreatic cancer, pre-metastatic niche, proteomics

Received: August 3, 2018
Revised: February 28, 2019
Published online: April 9, 2019

[1] M. Falasca, M. Kim, I. Casari, *Biochim. Biophys. Acta, Rev. Cancer* **2016**, *1865*, 123.
[2] M. De La Cruz, A. P. Young, M. T. Ruffin, *Am. Fam. Physician* **2014**, *89*, 626.
[3] R. Mayeux, *NeuroRx* **2004**, *1*, 182.
[4] E. A. Armstrong, E. W. Beal, J. Chakedis, A. Z. Paredes, D. Moris, T. M. Pawlik, C. R. Schmidt, M. E. Dillhoff, *J. Gastrointest. Surg.* **2018**, *1*, 737.
[5] R. Xu, A. Rai, M. Chen, W. Suwakulsiri, D. W. Greening, R. J. Simpson, *Nat. Rev. Clin. Oncol.* **2018**, *15*, 617.
[6] a) D. W. Greening, S. K. Gopal, R. A. Mathias, L. Liu, J. Sheng, H. J. Zhu, R. J. Simpson, *Semin. Cell Dev. Biol.* **2015**, *40*, 60; b) Z. Zeng, Y. Li, Y. Pan, X. Lan, F. Song, J. Sun, K. Zhou, X. Liu, X. Ren, F. Wang, J. Hu, X. Zhu, W. Yang, W. Liao, G. Li, Y. Ding, L. Liang, *Nat. Commun.* **2018**, *9*, 5395; c) M. P. Plebanek, N. L. Angeloni, E. Vinokour, J. Li, A. Henkin, D. Martinez-Marin, S. Filleur, R. Bhowmick, J. Henkin, S. D. Miller, I. Ifergan, Y. Lee, I. Osman, C. S. Thaxton, O. V. Volpert, *Nat. Commun.* **2017**, *8*, 1319; d) Y. Liu, Y. Gu, Y. Han, Q. Zhang, Z. Jiang, X. Zhang, B. Huang, X. Xu, J. Zheng, X. Cao, *Cancer Cell* **2016**, *30*, 243; e) B. Costa-Silva, N. M. Aiello, A. J. Ocean, S. Singh, H. Zhang, B. K. Thakur, A. Becker, A. Hoshino, M. T. Mark, H. Molina, J. Xiang, T. Zhang, T. M. Theilen, G. Garcia-Santos, C. Williams, Y. Ararso, Y. Huang, G. Rodrigues, T. L. Shen, K. J. Labori, I. M. Lothe, E. H. Kure, J. Hernandez, A. Doussot, S. H. Ebbesen, P. M. Grandgenett, M. A. Hollingsworth, M. Jain, K. Mallya, S. K. Batra, W. R. Jarnagin, R. E. Schwartz, I. Matei, H. Peinado, B. Z. Stanger, J. Bromberg, D. Lyden, *Nat. Cell Biol.* **2015**, *17*, 816; f) A. Hoshino, B. Costa-Silva, T. L. Shen, G. Rodrigues, A. Hashimoto, M. Tesic Mark, H. Molina, S. Kohsaka, A. Di Giannatale, S. Ceder, S. Singh, C. Williams, N. Soplop, K. Uryu, L. Pharmer, T. King, L. Bojmar, A. E. Davies, Y. Ararso, T. Zhang, H. Zhang, J. Hernandez, J. M. Weiss, V. D. Dumont-Cole, K. Kramer, L. H. Wexler, A. Narendran, G. K. Schwartz, J. H. Healey, P. Sandstrom, K. J. Labori, E. H. Kure, P. M. Grandgenett, M. A. Hollingsworth, M. de Sousa, S. Kaur, M. Jain, K. Mallya, S. K. Batra, W. R. Jarnagin, M. S. Brady, O. Fodstad, V. Muller, K. Pantel, A. J. Minn, M. J. Bissell, B. A. Garcia, Y. Kang, V. K. Rajasekhar, C. M. Ghajar, I. Matei, H. Peinado, J. Bromberg, D. Lyden, *Nature* **2015**, *527*, 329.
[7] T. Jung, D. Castellana, P. Klingbeil, I. Cuesta Hernandez, M. Vitacolonna, D. J. Orlicky, S. R. Roffler, P. Brodt, M. Zoller, *Neoplasia* **2009**, *11*, 1093.
[8] R. S. Conlan, S. Pisano, M. I. Oliveira, M. Ferrari, I. Mendes Pinto, *Trends Mol. Med.* **2017**, *23*, 636.
[9] a) D. K. Yadav, X. Bai, R. K. Yadav, A. Singh, G. Li, T. Ma, W. Chen, T. Liang, *Oncotarget* **2018**, *9*, 26900; b) X. Zhang, S. Shi, B. Zhang, Q. Ni, X. Yu, J. Xu, *Am. J. Cancer Res.* **2018**, *8*, 332.
[10] S. A. Melo, L. B. Luecke, C. Kahlert, A. F. Fernandez, S. T. Gammon, J. Kaye, V. S. LeBleu, E. A. Mittendorf, J. Weitz, N. Rahbari, *Nature* **2015**, *523*, 177.
[11] X. Osteikoetxea, M. Benke, M. Rodriguez, K. Pálóczi, B. W. Sódar, Z. Szvicsek, K. Szabó-Taylor, K. V. Vukman, Á. Kittel, Z. Wiener, *Biochem. Biophys. Res. Commun.* **2018**, *499*, 37.
[12] B. Costa-Silva, N. M. Aiello, A. J. Ocean, S. Singh, H. Zhang, B. K. Thakur, A. Becker, A. Hoshino, M. T. Mark, H. Molina, *Nat. Cell Biol.* **2015**, *17*, 816.
[13] A. Hoshino, B. Costa-Silva, T.-L. Shen, G. Rodrigues, A. Hashimoto, M. T. Mark, H. Molina, S. Kohsaka, A. Di Giannatale, S. Ceder, *Nature* **2015**, *527*, 329.
[14] B. Psaila, D. Lyden, *Nature Rev.* **2009**, *9*, 285.
[15] B. J. Tauro, D. W. Greening, R. A. Mathias, S. Mathivanan, H. Ji, R. J. Simpson, *Mol. Cell. Proteomics* **2013**, *12*, 587.
[16] a) A. Rai, D. W. Greening, M. Chen, R. Xu, H. Ji, R. J. Simpson, *Proteomics* **2018**, e1800148; b) W. Suwakulsiri, A. Rai, R. Xu, M. Chen, D. W. Greening, R. J. Simpson, *Biochim. Biophys. Acta Proteins Proteom.* **2018** S1570-9639(18)30206-1.
[17] S. K. Gopal, D. W. Greening, R. A. Mathias, H. Ji, A. Rai, M. Chen, H. J. Zhu, R. J. Simpson, *Oncotarget* **2015**, *6*, 13718.
[18] a) H. M. Duivenvoorden, J. Rautela, L. E. Edgington-Mitchell, A. Spurling, D. W. Greening, C. J. Nowell, T. J. Molloy, E. Robbins, N. K. Brockwell, C. S. Lee, M. Chen, A. Holliday, C. I. Selinger, M. Hu, K. L. Britt, D. A. Stroud, M. Bogyo, A. Moller, K. Polyak, B. F. Sloane, S. A. O'Toole, B. S. Parker, *J. Pathol.* **2017**, *243*, 496; b) D. W. Greening, H. Ji, M. Chen, B. W. Robinson, I. M. Dick, J. Creaney, R. J. Simpson, *Sci. Rep.* **2016**, *6*, 32643.
[19] S. K. Gopal, D. W. Greening, R. A. Mathias, H. Ji, A. Rai, M. Chen, H. J. Zhu, R. J. Simpson, *Oncotarget* **2015**, *6*, 13718.
[20] D. W. Greening, H. P. Nguyen, K. Elgass, R. J. Simpson, L. A. Salamonsen, *Biol. Reprod.* **2016**, *94*, 38.
[21] V. Gorshkov, T. Verano-Braga, F. Kjeldsen, *Anal. Chem.* **2015**, *87*, 6319.
[22] J. Cox, M. Mann, *Nat. Biotechnol.* **2008**, *26*, 1367.
[23] C. A. Luber, J. Cox, H. Lauterbach, B. Fancke, M. Selbach, J. Tschopp, S. Akira, M. Wiegand, H. Hochrein, M. O'Keeffe, M. Mann, *Immunity* **2010**, *32*, 279.
[24] Y. Benjamini, Y. Hochberg, *J. R. Stat. Soc. Ser. B Stat. Methodol.* **1995**, *57*, 289.
[25] B. J. Tauro, R. A. Mathias, D. W. Greening, S. K. Gopal, H. Ji, E. A. Kapp, B. M. Coleman, A. F. Hill, U. Kusebauch, J. L. Hallows, D.

Shteynberg, R. L. Moritz, H. J. Zhu, R. J. Simpson, *Mol. Cell. Proteomics* **2013**, *12*, 2148.
[26] W. Huang da, B. T. Sherman, R. A. Lempicki, *Nat. Protoc.* **2009**, *4*, 44.
[27] H. Wickham, *ggplot2: Elegant Graphics for Data Analysis*, Springer Science & Business Media, New York **2009**.
[28] G. Raposo, W. Stoorvogel, *J. Cell Biol.* **2013**, *200*, 373.
[29] a) R. Xu, D. W. Greening, H. J. Zhu, N. Takahashi, R. J. Simpson, *J. Clin. Invest.* **2016**, *126*, 1152; b) D. W. Greening, R. J. Simpson, *Expert Rev. Proteomics* **2018**, *15*, 887.
[30] H. Gehart, A. Goginashvili, R. Beck, J. Morvan, E. Erbs, I. Formentini, M. A. De Matteis, Y. Schwab, F. T. Wieland, R. Ricci, *Dev. Cell* **2012**, *23*, 756.
[31] Y. Matsuda, Z. Naito, K. Kawahara, N. Nakazawa, M. Korc, T. Ishiwata, *Cancer Biol. Therapy* **2011**, *11*, 512.
[32] H. Bent, H. R. Hanne, V. Henrik, D. Kurt, L. Xiangdang, G. Pavel, M. Peder, G. Borbala, T. Niels, E. C. Julio, *J. Biol. Chem.* **1995**, *270*, 28780.
[33] X. Wei, J. Li, H. Xie, Q. Ling, J. Wang, D. Lu, L. Zhou, X. Xu, S. Zheng, *Cancer Lett.* **2014**, *351*, 117.
[34] R. J. Simpson, L. M. Connolly, J. S. Eddes, J. J. Pereira, R. L. Moritz, G. E. Reid, *Electrophoresis* **2000**, *21*, 1707.
[35] a) I. Nazarenko, S. Rana, A. Baumann, J. McAlear, A. Hellwig, M. Trendelenburg, G. Lochnit, K. T. Preissner, M. Zoller, *Cancer Res.* **2010**, *70*, 1668; b) S. Rana, M. Zoller, *Biochem. Soc. Trans.* **2011**, *39*, 559.
[36] a) M. Fukuda, *Cell. Mol. Life Sci.* **2008**, *65*, 2801; b) M. T. Lee, A. Mishra, D. G. Lambright, *Traffic* **2009**, *10*, 1377; c) H. Stenmark, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 513.
[37] C. Thery, M. Ostrowski, E. Segura, *Nat. Rev. Immunol.* **2009**, *9*, 581.
[38] J. Castillo, V. Bernard, F. San Lucas, K. Allenson, M. Capello, D. Kim, P. Gascoyne, F. Mulu, B. Stephens, J. Huang, *Ann. Oncol.* **2017**, *29*, 223.
[39] B. Zhou, A. Moodie, A. A. Blanchard, E. Leygue, Y. Myal, *J. Clin. Med.* **2015**, *4*, 1960.
[40] a) M. Tanaka, J. Shibahara, N. Fukushima, A. Shinozaki, M. Umeda, S. Ishikawa, N. Kokudo, M. Fukayama, *J. Histochem. Cytochem.* **2011**, *59*, 942; b) N. Jonckheere, N. Skrypek, I. Van Seuningen, *Cancers* **2010**, *2*, 1794.
[41] H. Wang, S. Rana, N. Giese, M. W. Büchler, M. Zöller, *Int. J. Cancer* **2013**, *133*, 416.
[42] M. Uhlen, C. Zhang, S. Lee, E. Sjostedt, L. Fagerberg, G. Bidkhori, R. Benfeitas, M. Arif, Z. Liu, F. Edfors, K. Sanli, K. von Feilitzen, P. Oksvold, E. Lundberg, S. Hober, P. Nilsson, J. Mattsson, J. M. Schwenk, H. Brunnstrom, B. Glimelius, T. Sjoblom, P. H. Edqvist, D. Djureinovic, P. Micke, C. Lindskog, A. Mardinoglu, F. Ponten, *Science* **2017**, 357.
[43] J. Gao, F. Zhu, S. Lv, Z. Li, Z. Ling, Y. Gong, C. Jie, L. Ma, *Oncol. Rep.* **2010**, *23*, 1683.
[44] E. Jahny, H. Yang, B. Liu, B. Jahnke, F. Lademann, T. Knosel, P. Rummele, R. Grutzmann, D. E. Aust, C. Pilarsky, A. Denz, *PLoS One* **2017**, *12*, e0170390.
[45] C. Liang, Y. Qin, B. Zhang, S. Ji, S. Shi, W. Xu, J. Liu, J. Xiang, D. Liang, Q. Hu, Q. Ni, X. Yu, J. Xu, *Cancer Lett.* **2017**, *388*, 303.
[46] a) S. Mathivanan, J. W. Lim, B. J. Tauro, H. Ji, R. L. Moritz, R. J. Simpson, *Mol. Cell. Proteomics* **2010**, *9*, 197; b) I. Lazar, E. Clement, M. Ducoux-Petit, L. Denat, V. Soldan, S. Dauvillier, S. Balor, O. Burlet-Schiltz, L. Larue, C. Muller, L. Nieto, *Pigm. Cell Melanoma Res.* **2015**, *28*, 464.
[47] B. Liang, P. Peng, S. Chen, L. Li, M. Zhang, D. Cao, J. Yang, H. Li, T. Gui, X. Li, K. Shen, *J. Proteomics* **2013**, *80*, 171.
[48] B. Zhang, *Drug Resist. Updates* **2006**, *9*, 134.
[49] W. D'Hertog, M. Maris, G. B. Ferreira, E. Verdrengh, K. Lage, D. A. Hansen, A. K. Cardozo, C. T. Workman, Y. Moreau, D. L. Eizirik, *J. Proteome Res.* **2010**, *9*, 5142.
[50] M. Demory Beckler, J. N. Higginbotham, J. L. Franklin, A. J. Ham, P. J. Halvey, I. E. Imasuen, C. Whitwell, M. Li, D. C. Liebler, R. J. Coffey, *Mol. Cell. Proteomics* **2013**, *12*, 343.
[51] J. E. Park, H. S. Tan, A. Datta, R. C. Lai, H. Zhang, W. Meng, S. K. Lim, S. K. Sze, *Mol. Cell. Proteomics* **2010**, *9*, 1085.
[52] H. Peinado, S. Lavotshkin, D. Lyden, *Semin. Cancer Biol.* **2011**, *21*, 139.
[53] a) J. P. Sleeman, *Cancer Metastasis Rev.* **2012**, *31*, 429; b) D. Castellana, F. Zobairi, M. C. Martinez, M. A. Panaro, V. Mitolo, J. M. Freyssinet, C. Kunzelmann, *Cancer Res.* **2009**, *69*, 785.
[54] a) D. X. Nguyen, P. D. Bos, J. Massague, *Nat. Rev. Cancer* **2009**, *9*, 274; b) A. J. Minn, G. P. Gupta, P. M. Siegel, P. D. Bos, W. Shu, D. D. Giri, A. Viale, A. B. Olshen, W. L. Gerald, J. Massague, *Nature* **2005**, *436*, 518; c) B. Psaila, D. Lyden, *Nat. Rev. Cancer* **2009**, *9*, 285; d) J. A. Joyce, J. W. Pollard, *Nat. Rev. Cancer* **2009**, *9*, 239; e) C. Grange, M. Tapparo, F. Collino, L. Vitillo, C. Damasco, M. C. Deregibus, C. Tetta, B. Bussolati, G. Camussi, *Cancer Res.* **2011**, *71*, 5346.
[55] V. Luga, J. L. Wrana, *Cancer Res.* **2013**, *73*, 6843.
[56] K. Saukkonen, J. Hagström, H. Mustonen, A. Juuti, S. Nordling, C. Fermér, O. Nilsson, H. Seppänen, C. Haglund, *PLoS One* **2015**, *10*, e0129012.
[57] J. Sceneay, M. J. Smyth, A. Moller, *Cancer Metastasis Rev.* **2013**, *32*, 449.
[58] A. Hoshino, B. Costa-Silva, T. L. Shen, G. Rodrigues, A. Hashimoto, M. Tesic Mark, H. Molina, S. Kohsaka, A. Di Giannatale, S. Ceder, S. Singh, C. Williams, N. Soplop, K. Uryu, L. Pharmer, T. King, L. Bojmar, A. E. Davies, Y. Ararso, T. Zhang, H. Zhang, J. Hernandez, J. M. Weiss, V. D. Dumont-Cole, K. Kramer, L. H. Wexler, A. Narendran, G. K. Schwartz, J. H. Healey, P. Sandstrom, K. Jorgen Labori, E. H. Kure, P. M. Grandgenett, M. A. Hollingsworth, M. de Sousa, S. Kaur, M. Jain, K. Mallya, S. K. Batra, W. R. Jarnagin, M. S. Brady, O. Fodstad, V. Muller, K. Pantel, A. J. Minn, M. J. Bissell, B. A. Garcia, Y. Kang, V. K. Rajasekhar, C. M. Ghajar, I. Matei, H. Peinado, J. Bromberg, D. Lyden, *Nature* **2015**, *527*, 329.
[59] A. Walther, K. Riehemann, V. Gerke, *Mol. Cell* **2000**, *5*, 831.
[60] D. Gao, N. Joshi, H. Choi, S. Ryu, M. Hahn, R. Catena, H. Sadik, P. Argani, P. Wagner, L. T. Vahdat, J. L. Port, B. Stiles, S. Sukumar, N. K. Altorki, S. Rafii, V. Mittal, *Cancer Res.* **2012**, *72*, 1384.
[61] Z.-H. Li, N. G. Dulyaninova, R. P. House, S. C. Almo, A. R. Bresnick, *Mol. Biol. Cell* **2010**, *21*, 2598.
[62] a) T. J. Loh, H. Moon, H. N. Jang, Y. Liu, N. Choi, S. Shen, D. R. Williams, D.-W. Jung, X. Zheng, H. Shen, *BMB Rep.* **2016**, *49*, 612; b) X.-P. Li, X.-W. Zhang, L.-Z. Zheng, W.-J. Guo, *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 6724; c) K. Tsubouchi, K. Minami, N. Hayashi, Y. Yokoyama, S. Mori, H. Yamamoto, M. Koizumi, *J. Radiat. Res.* **2017**, *58*, 816; d) D. Galiana-Arnoux, F. Lejeune, M.-C. Gesnel, J. Stevenin, R. Breathnach, F. Del Gatto-Konczak, *J. Biol. Chem.* **2003**, *278*, 32943; e) K. A. Adamczyk, S. Klein-Scory, M. M. Tehrani, U. Warnken, W. Schmiegel, M. Schnolzer, I. Schwarte-Waldhoff, *Life Sci.* **2011**, *89*, 304; f) M. Signaevsky, J. Hobbs, J. Doll, N. Liu, G. A. Soff, *Semin. Thromb. Hemostasis* **2008**, *34*, 161; g) B. Ünlü, V. Bogdanov, H. Versteeg, *Thromb. Res.* **2017**, *156*, 1; h) G. T. Gerotziafas, V. Galea, E. Mbemba, A. Khaterchi, M. Sassi, H. Baccouche, C. Prengel, P. van Dreden, M. Hatmi, J. F. Bernaudin, *Thromb. Res.* **2012**, *129*, 779.
[63] a) H. Vaziri, J. A. Squire, T. K. Pandita, G. Bradley, R. M. Kuba, H. Zhang, S. Gulyas, R. P. Hill, G. P. Nolan, S. Benchimol, *Mol. Cell. Biol.* **1999**, *19*, 2373; b) J. Wang, G. J. Hannon, D. H. Beach, *Nature* **2000**, *405*, 755.
[64] X. F. Pei, *Carcinogenesis* **1996**, *17*, 1395.
[65] B. W. Futscher, *Adv. Exp. Med. Biol.* **2013**, *754*, 179.
[66] K. S. Yang, H. Im, S. Hong, I. Pergolini, A. F. Del Castillo, R. Wang, S. Clardy, C. H. Huang, C. Pille, S. Ferrone, R. Yang, C. M. Castro, H. Lee, C. F. Del Castillo, R. Weissleder, *Sci. Transl. Med.* **2017**, 9.

CHAPTER 3

Chapter 3: Lipidomic analysis of PDAC exosomes and corresponding cells

3.1 Introduction

As mentioned in Chapter 1, the high heterogeneity of pancreatic cancer and the lack of sensitive and specific biomarkers hamper diagnosis as well as the prediction of chemotherapy outcome, therefore -omics approaches, such as lipidomics in this case, can offer a greater view of the molecular fingerprint of the disease by thoroughly quantifying a number of lipid classes and species, and allowing us to make a connection between their expression, and that of related proteins and genes.

Lipids facilitate crucial cell functions including- but not limited to- storage of energy, maintenance of the cell membrane, intercellular signalling and interactions, and of course proliferation and survival (Wenk 2005). They are categorized into eight major classes, each of which comprises of subgroups. Six of the categories refer to the lipids occurring from ketoacyl subunits condensation and include fatty acids (FAs), sphingolipids (SLs), glycerophospholipids (GLPs), glycerolipids (GLs), polyketides (PKs) and saccharolipids (SCLs), whereas the remaining two categories refer to lipids derived from isoprene units condensation and include prenol and sterol lipids (PR and ST, respectively). Lipids can also be classified into simple and complex ones, based on the numbers of molecules they yield when hydrolysed. Simple lipids result in a combination of maximum two molecular components, whereas complex ones yield three or more (reviewed in (Fahy, Cotter et al. 2011). Cell membranes are enriched in phosphatidylethanolamine (PE) and phosphatidylcholine (PC), which together with phosphatidyl -inositol, -serine, -glycerol and phosphatidic acid (PI, PS, PG and PA, respectively) comprise the greater GLPs class (Perrotti, Rosa et al. 2016). A very popular method for analysing those lipid classes is electrospray ionization mass spectrometry (ESI-MS), which allows the detection of a number of lipid species within only one experiment, whereas incorporation of liquid chromatography prior to the ESI-MS (LC ESI-MS) boosts the analytical power of the technique and adds to the accuracy and sensitivity; features highly appreciated in the biomarker discovery field (Fernandis and Wenk 2009). As lipids play major roles in cellular homeostasis, it is inevitable that they would be under scrutiny in regards to their potential as novel biomarkers in pathologies, including cancer. Indeed, there have been many cases where specific lipid species or classes have shown increased sensitivity in distinguishing malignant from non-pathologic states. Lipidomic analysis of prostate cancer tissues showed cholesteryl ester (CE) accumulation and correlation with disease staging. CE accumulation was attributed to overexpression of the scavenger

receptor class B type I (SR-BI), while cholesteryl oleate (CE18:1) effectively discriminated between malignancy, benign prostatic hyperplasia and healthy states, therefore raising the question of whether it would be a good candidate to be used alongside the prostate-specific antigen (PSA) (Li, Ren et al. 2016). In other cases, sphingolipid species were differentially expressed in metastatic and non-metastatic cases of pancreatic cancer and pancreatitis. The C16:0 ceramide, which is an insulin resistance indicator (Hla and Kolesnick 2014), and the nervonic ceramide (C24:1) were significantly enriched in pancreatic cancer samples compared to pancreatitis ones, and higher levels were positively correlated with nodal metastasis (Jiang, DiVittore et al. 2013). Despite the fact that ceramides are categorised as "pro-apoptotic" molecules, there is increasing evidence of there are found in unexpectedly high levels in malignant states (Koybasi, Senkal et al. 2004, Karahatay, Thomas et al. 2007). Phosphorylated sphingolipids were also significantly enriched in the metastatic cases, successfully separating them from non-metastatic and non-malignant ones (Jiang, DiVittore et al. 2013).

Although lipids are actively released from cells, exosomes also comprise a factor that needs to be considered, as they are also contributors of a number of lipids that are found extracellularly. A number of lipid and phospholipase-related proteins have been reported to be present in exosomes, including fatty acid binding protein (FABP), phospholipid scramblase and phosphoinositide-specific phospholipase C ε (PLCε), cyclooxygenase enzymes and secreted phospholipase A_2 ($sPLA_2$), indicating that fatty acids can either be passively carried in the exosomes but also being generated inside them (Subra, Grand et al. 2010). In addition to the abovementioned, major lipid classes found in exosomes from various cell sources include- but are not limited to PI, PS, SM, PC, COH, LPC and PE. Interestingly, certain exosomes such as those derived from B cells are shown to have a great enrichment in cholesterol compared to their donor cells, contrary to exosomes derived from reticulocytes for instance (Vidal, Sainte-Marie et al. 1989, Wubbolts, Leckie et al. 2003). In regards to cholesterol, it was in fact demonstrated that in B lymphocytes a striking 63% of the COH found in the endocytic track is contained in the MVBs, from which exosomes are ultimately released (Möbius, Van Donselaar et al. 2003).

Due to their cargo, exosomes are implicated in many metabolic diseases (Record, Poirot et al. 2014), and there are many studies linking those nanovesicles to pancreatic cancer initiation and progression. Desmoplasia is a characteristic of pancreatic cancer and it has been shown to have a dual role in the progression of the disease. A number of studies support the notion that the stroma acts as a restrain to

the tumour growth, since its depletion has shown to accelerate tumour cells proliferation and metastasis as well as correlate with higher morbidity and mortality (Rhim, Oberstein et al. 2014, Özdemir, Pentcheva-Hoang et al. 2014, Liu, Pitarresi et al. 2016). On the other hand, there are many paradigms of the stroma having a pro-tumorigenic effect and contributing to chemoresistance along with other cancer hallmarks (Hanahan and Weinberg 2011, Waghray, Yalamanchili et al. 2013, Neesse, Krug et al. 2014). It is possible that the interactions between the stroma and the pancreatic cancer epithelial cells vary depending on the tumour stage, nevertheless it is now widely accepted that exosomes are amongst the most crucial players in intracellular communication. It was recently demonstrated that tumour-associated stroma (TAS) – derived exosomes could exert anti-tumour effects to PDAC cells via specific miRNA cargo delivery (miR-145), therefore adding to the conception that stroma does restrain tumour expansion and to some extent explain why stroma depletion drives pancreatic cancer progression (Han, Gonzalo et al. 2018). miR-145 has been previously shown to induce apoptosis in PDAC cells via caspase dependent and independent pathways (Ostenfeld, Bramsen et al. 2010, Cui, Wang et al. 2014) and it comprises a target for oncogenic KRAS, which transcriptionally suppresses it (Kent, Chivukula et al. 2010). Other cell types abundantly found in the desmoplastic tumour environment are the CAFs and the pancreatic stellate cells (PSCs). Apart from being gemcitabine resistant, CAFs increase the amount of exosomes shed following exposure to the drug. In turn, those vesicles transfer Snail and microRNA-146a to neighbouring cancer cells, increasing their chemoresistance and proliferation rates (Richards, Zeleniak et al. 2017). PSCs play a central role in its maintenance not only by producing ECM components but also by communicating with other cells to nurture the tumour microenvironment. PSC-derived exosomes were shown to instigate the tumorigenic characteristics PANC-1 cancer cells, as well as expression of chemokine-related genes such as *CCL20, CXCL1* and *CXCL2*, probably as a result of transfer of microRNAs including miR-21, miR1290 and miR-1246 (Takikawa, Masamune et al. 2017). Apart from the aforementioned, exosomes have also been found to participate in immunological aspects of this malignancy. Tumour-associated antigens (TAAs) initiate humoral response, and autoimmune response associated to B-cells in malignant states manifests itself by the production of autoantibodies against those TAAs (Tsou, Katayama et al. 2016). This process is initiated years or at least months before the first symptoms of the disease appear (Tan, Low et al. 2009) and for this reason detection of these autoantibodies has a great value in liquid biopsies (Dumstrei, Chen et al. 2016). PDAC patients plasma-derived and PDAC cell culture supernatants-derived exosomes were shown to act as decoys and exhibit

immunoglobulins or TAAs respectively on their surface, in this way hampering the complement from exerting its cytotoxic effects on tumour cells (Capello, Vykoukal et al. 2019). In line with this decoy-like function of PDAC-derived exosomes, other types of tumour exosomes have been shown to sequestrate anti-Her2 and CD20 antibodies and significantly impair the efficacy of anti-cancer therapies (Battke, Ruiss et al. 2011, Ciravolo, Huber et al. 2012).

3.2 Study aims

Pancreatic cancer is a malignancy with highly deregulated lipid metabolism; it would therefore be of great value to initially analyse the lipidome of pancreatic cancer cells-derived exosomes and compare it to that of exosomes derived from other types of malignancies. The aim of Chapter 3 of this thesis is to initially perform lipidomic analysis of PDAC, ovarian and prostate cancer cells-derived exosomes and respective donor cells, in search of a distinct PDAC lipid signature, and as the next step- once a lipid of interest is detected, to perform downstream functional assays to reveal the related biological effect.

3.3 Materials and Methods

3.3.1 Cell lines

Name	Supplier and Cat#.	Type of Cells	Medium and % of FBS
hTERT-HPNE	ATCC®CRL-4023™	Pancreatic duct	75% DMEM (Sigma Cat#. D-5030 with additional 2 mM L-glutamine and 1.5 g/L sodium bicarbonate) /25%M3 Base (Incell Corp. Cat# M300F- 500) + 10 ng/ml hEGF + 5.5mM glucose + 750 ng/ml puromycin , 5% FBS
HPDE	Gift from Prof Hemant Kocher (Queen Mary University of London)	Pancreatic duct	Keratinocyte SMF + EGF + bovine pituitary extract (ThermoFisher Scientific Cat#. 17005042), no FBS
AsPC-1	ATCC®CRL-1682™	Pancreatic cancer	RPMI-1640 Medium (SIGMA Cat#. R8758), 10% FBS
BxPC-3	ATCC®CRL-1687™	Pancreatic cancer	RPMI-1640 Medium (SIGMA Cat#. R8758), 10% FBS
MiaPaCa-II	ATCC®CRL-1420™	Pancreatic cancer	DMEM (SIGMA Cat#. D6429), 10% FBS, 2.5% equine donor serum
PANC-1	ATCC®CRL-1469™	Pancreatic cancer	DMEM (SIGMA Cat#. D6429), 10% FBS
HPAF-II	ATCC®CRL-1997™	Pancreatic cancer	MEM (SIGMA Cat#. M4655), 10% FBS
RWPE-1	ATCC®CRL-11609™	Prostate epithelial	Keratinocyte SMF + EGF + bovine pituitary extract (ThermoFisher Scientific Cat#. 17005042), no FBS
PNT2	ECACC Cat#.95012613	Prostate epithelial	RPMI-1640 Medium (SIGMA Cat#. R8758), 10% FBS
PC-3	ATCC®CRL-1435™	Prostate cancer	RPMI-1640 Medium (SIGMA Cat#. R8758), 10% FBS
OSE4	Gift from Dr Yu Yu (Curtin University)	Ovarian surface epithelial	RPMI-1640 Medium (SIGMA Cat#. R8758), 10% FBS
A2780	ECACC Cat#.93112519	Ovarian cancer	RPMI-1640 Medium (SIGMA Cat#. R8758), 10% FBS

All media was supplemented with 100 units mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin (Sigma-Aldrich, Cat#. P4333). Cells were kept in humidified incubators at 37^0C with 5% CO_2 and passaged routinely.

3.3.2 Samples collection for lipidomic analysis with mass-spectrometry

For lipidomic analysis, $1.2x10^6$ cells were seeded in a T175 flask (ThermoFisher Scientific Cat#.159910) in media containing commercial exosome depleted – FBS (Thermo Fisher Scientific, Cat#.A2720801) and allowed to grow to 80% confluency. Three flasks were used in each biological replicate and a total of three independent biological replicates were performed per cell line. When cells reached 80% confluency, the supernatant from the three flasks of each biological replicate was pooled for further exosome isolation (details found in Chapter 2 – Publication 1) and the cells were trypsinized, pooled, span at 300 g for 5 minutes and snap frozen. Cells and exosomes pellets were packaged with dry ice and sent to the Baker Heart & Diabetes Institute in Melbourne, Victoria, for mass spectrometry analysis.

3.3.3 Mass spectrometry

As mentioned above, mass spectrometry analysis was performed at the Baker Heart & Diabetes Institute in Melbourne, Victoria by a designated technician. Briefly, a single phase chloroform:methanol ($CHCl_3$:MeOH) (2:1) extraction was used for lipids extraction from cells and exosomes pellets, following the methodology described in (Weir, Wong et al. 2013). Samples were randomized and each sample was extracted using 20 volumes of $CHCl_3$:MeOH. An internal standard mix (in $CHCl_3$:MeOH (1:1)) was also included during the analysis, and comprised of 10-10,000 pmol of 31 stable isotope-labelled or non-physiological lipid standards. Liquid chromatography electrospray ionisation-tandem mass spectrometry (LC ESI-MS/MS) was performed for lipidomic analysis, with Agilent Technologies equipment used for both phases of the procedure. Liquid chromatography was carried out on a Zorbax Eclipse Plus C18, 1.8 um, 100 x 2.1mm column (Agilent Technologies) using solvents A and B. Solvent A comprised of water:acetonitrile:isopropanol (50:30:20), 10mM ammonium formate and 0.05µM medronic acid, while solvent B comprised of water:acetonitrile:isopropanol (1:9:90) and 10mM ammonium formate. Temperatures for the column and the auto sampler were set at 45°C and 25°C, respectively. Lipid extracts (1ul) were injected and separated under gradient conditions with a flow rate of 400ul/min: 15% B to 50% B over 2.5 minutes, increase to 57% B over 0.1 minutes, increase to 70% B over 6.4 minutes, increase to 93% B over 0.1 minutes, increase to 96%B over 1.9 minutes, increase to 100% over 0.1 minutes and hold at 100% B for 0.9 minutes. The solvent was then decreased to 15% B over 0.2, held at 15% B for 0.7 minutes and remained at 15% B until next injection at 13.2 minutes. The first 0.8

minutes of each analytical run were diverted to waste. Electrospray ionisation-tandem mass spectrometry was performed using an Agilent 1290 HPLC coupled to an Agilent 6490 triple quadrupole mass spectrometer using settings as follows: gas temperature 150C, gas flow 17 L/min, nozzle pressure 20 psi, sheath gas temperature 200C, sheath gas flow 10L/min, capillary voltage 3500V, nozzle voltage 1000V. Dynamic multiple reaction monitoring (dMRM) was used for lipid species measurement, where data was collected for a retention time window specific to each lipid species. Mass Hunter Quant was used for the chromatographic data analysis, where relative lipid abundances were calculated by relating each area under the chromatogram for each lipid species to the corresponding internal standard. Correction factors were applied to adjust for different response factors, where these were known.

3.3.4 Lymphocytes isolation from healthy donors' whole blood

- Whole blood (50 ml) was collected from a healthy donor in 5x10ml EDTA vacutainer tubes (BD Vacutainer K2E (EDTA) REF 367525) and distributed evenly into two 50ml falcon tubes.

- PBS pH 7.4 (Gibco, REF 10010-023) was added in each tube in order to make up the volume to 35 ml.

- A total of 30 ml of Ficoll-Paque (GE Healthcare, Cat#.17-1440-03) were evenly distributed in two 50 ml falcon tubes and were overlaid with the 35ml of blood / PBS from the previous step.

- Falcons were centrifuged at 20^0C, 400 x g for 40 min, without acceleration and brake.

- After centrifugation, the buffy coat from each tube was pooled and transferred to a new 50 ml centrifuge tube. PBS was added up to 50 ml and the falcon was centrifuged at 20^0C, 300 x g, 10 minutes, with acceleration and brake.

- Supernatant was discarded, pellet was resuspend in 1.2ml FACS buffer (PBS - 5% heat-inactivated FBS) and distributed in 6 wells of a U-shaped 96-well plate (Falcon, REF 353077).

- Antibodies against CD4 (BioLegend, Cat#. #317410) and CD8 (BioLegend, Cat#. #300906) were added in each well in a 1:200 dilution. One well was left

untreated (control – no antibodies), one was treated with CD4 only and one with CD8 only; these three wells being used to set up the cell sorter. The rest of the wells were treated with both anti-CD4 and anti-CD8 antibodies.

- The plate was incubated on ice in the dark for 15 minutes, span at 300 x g for 2 minutes at RT, and cells were resuspended in FACS buffer. This step was repeated twice and after the final wash the cells were collected in special 5ml polypropylene round bottom tubes (Falcon, REF 352063) and underwent sorting in a BD FACS Jazz ™ instrument using the BD FACS™ Software Version 1.2.0142. Cells were collected in sorting buffer (PBS - 20% FBS), and were left in the incubator for 30 minutes at 37^0C to recover.

- Cells were span at 300 x g for 5 min, resuspended in RPMI without FBS in a concentration of 1 x 10^7 - 2 x 10^7 and carboxyfluorescein succinimidyl ester (CFSE) was added to a final concentration of 2.5 μM.

- Cells were left in the dark for 10 min and 20 ml of RPMI 10% FBS were added in each tube.

- A 2-3ml FBS underlay was added in each tube and cells were centrifuged at 300 x g for 5 min. This step was repeated once more and the cells were resuspended in RPMI containing 10% FBS and 0.05 mM β-mercaptoethanol (2-ME), at a final concentration of 2.5 x 10^5 cells / well.

- Cells were stimulated with CD3 (Biolegend, Cat#. 300332) and CD28 (Biolegend, Cat#. 302934) antibodies (dilutions of 1:1000 and 1:500, respectively), seeded in a U-shaped 96-well plate, 25 μg / ml of the desired exosome sample were added in the appropriate wells and the cells were left in the incubator for 7 days, at the end of which, downstream assays were performed (proliferation assay and cytokine expression assay).

3.3.5 T cell proliferation assay

- One week post treatment, lymphocytes were span at 300 x g for 5 minutes, supernatant was removed and kept in a separate plate, and the cell pellets were resuspended in FACS buffer and analysed on a BD LSRFortessa (Special Order Research Product).

- Analysis of the obtained data was performed using the FlowJo™ software Version 10.6.1.

3.3.6 Cytokine expression assay

A small portion (12.5 µl) of the supernatant kept aside from the T cell proliferation assay was used for the cytokine expression analysis, and the assay was performed using the LEGENDplex™ Multi-Analyte Flow Assay Kit (Biolegend, Cat#.740118 and when discontinued, Cat#.740809). In brief:

- Assay buffer, diluted standards, samples, pre-mixed beads and detection antibodies, all in volumes of 12.5 µl, were added in wells of a U-shaped 96-well plate and were left to incubate for 2 h, at RT, on a shaker.
- Without washing, 12.5 µl of SA-PE were added in each well and the plate was incubated for 30 min at RT on a shaker.
- The plate was span at 300 x g for 5 min and the beads were resuspended in 1X wash buffer. This step was repeated twice.
- After the last wash, beads were resuspended in 1X wash buffer (50 µl / well) and analysed on a BD LSRFortessa (Special Order Research Product).
- Analysis of the obtained data was performed using the FlowJo™ software Version 10.6.1.

3.4 Results

3.4.1 Lipidomic analysis of pancreatic, prostate and ovarian epithelial and cancer cells and their respective exosomes

Towards the search of a specific pancreatic cancer signature, lipidomic analysis was performed on the two pancreatic epithelial cell lines hTERT-HPNE and HPDE, as well as the five pancreatic cancer cell lines AsPC-1, BxPC-3, MiaPaCa-II, PANC-1 and HPAF-II, and their derived exosomes. As a comparison, prostate epithelial (RWPE-1, PNT2) and cancer (PC-3) cells and ovarian epithelial (OSE4) and cancer cells (A2780) as well as their derived exosomes were analysed at the same time. A total of 636 lipid species were identified during this analysis.

The cellular lipid bilayer consists of three predominant lipid classes, namely phospholipids, cholesterol and glycolipids. The first class is mainly comprised of SM, PC, PE and PS, while PI is detected in lower levels but is still of utmost importance

for cell signalling: PIs get cleaved by activated phospholipases and the consequent products act as second messengers which can either help activate other proteins or mediate the ERs' Ca^{2+} release. Cholesterol is also found in high levels in mammalian cell membranes and can regulate their fluidity. Interestingly, although increased cholesterol adds to the rigidity of the membrane and diminishes its permeability to small-scaled hydrophilic molecules, at the same time it prevents phase transitions. Last but not least, glycolipids expose sugars at the surface of the cells therefore mediating intercellular communications. Indicatory, the plasma membrane of liver cells was found to consist by 17% of COH, 4% of PS, 7% of PE, 24% of PC and 19% of SM (Van Meer, Voelker et al. 2008). As shown in Figure 3.1A, lipidomic analysis of the cell samples is following the aforementioned pattern, with COH, PE, SM, PS being present in intermediate and high levels consistently across the different cell lines. Other lipid classes enriched in our analysis were PE(P), PC(O), DGs and TGs, ceramides (in relatively low levels) and CE mostly in specific cell lines such as HPDE, MiaPaCa-II and A2780.

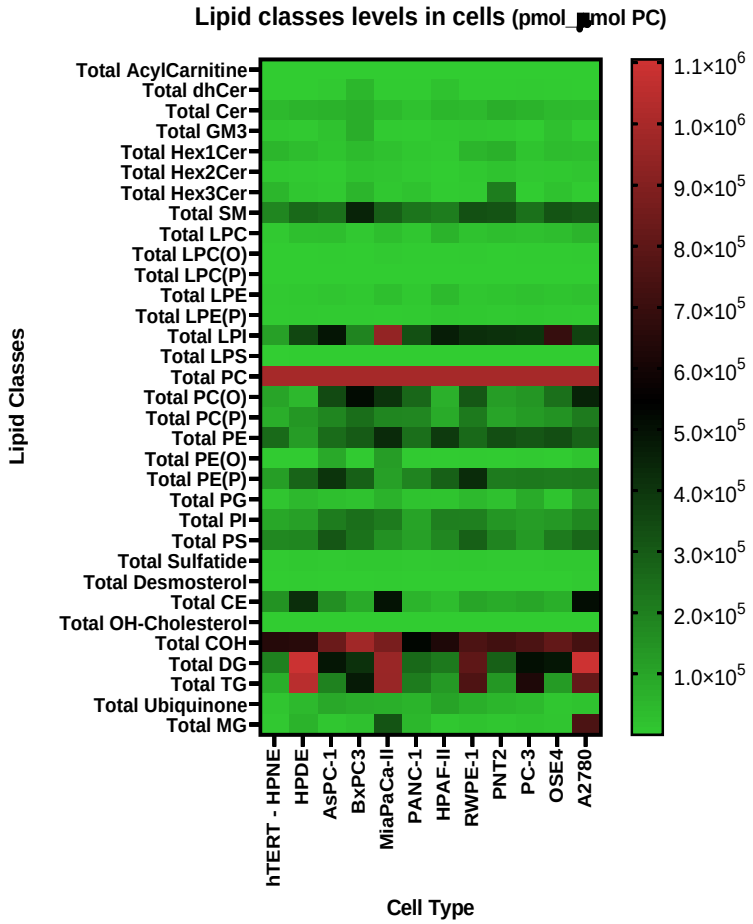
Studies performing lipidomic analysis of exosomes exhibit discrepancy in their results, resulting from parameters such as the exosomes isolation method (ultracentrifugation, ultrafiltration, SEC, etc.), the presence or absence of serum, the method selected for the lipid analysis (shotgun lipidomics, LC/MS, gas chromatography) and even the software interpreting the MS data (Simons 2018). For instance, a study on the lipidome of exosomes from different cell sources reported depletion of major lipid classes such as PI, PE and PG in exosomes and enrichment of SM in those vesicles compared to the parent cells, with CE, LPC and acyl carnitines showing no differential expression and PS, FFA and glycolipids showing enrichment (Haraszti, Didiot et al. 2016). Other studies show enrichment of COH, PS, SM and Cer in exosomes compared to their donor cells (Trajkovic, Hsu et al. 2008, Llorente, Skotland et al. 2013).

In our analysis the lipidome profile of the exosomes is distinct to that of their donor cells, as shown in Figure 3.1B. Among the most predominant lipid classes present in exosomes are SM, COH, DG and MG, with MG exhibiting higher levels in epithelial pancreatic, prostate and ovarian exosomes compared to their malignant counterparts and COH showing little fluctuation among the different exosomes types, similar to SM. LPI is highly expressed only in the AsPC-1 exosomes compared to all other types. A very interesting observation is that CE levels are consistently higher in PDAC exosomes compared to all other types, which led to further investigation of the

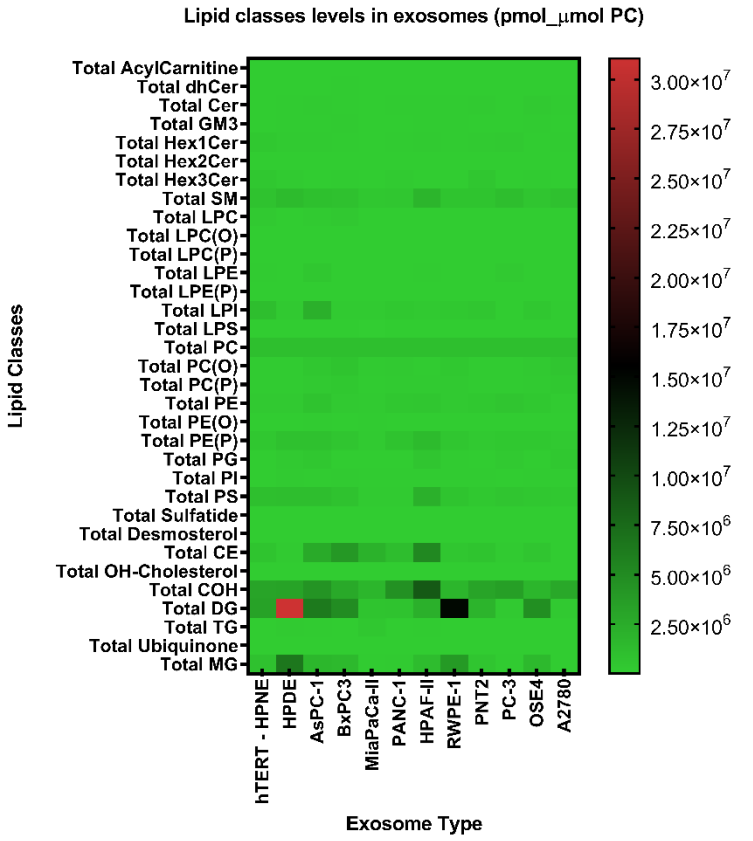
biological importance of this phenomenon, described later in this chapter. In Figure 3.1C, the relative expression of the lipid classes of exosomes compared to their donor cells is shown, where MG is the most enriched lipid class in all exosomes types apart from MiaPaCa-II and A2780. Hexosylceramides (Hex1Cer, Hex2Cer, Hex3Cer), SM, PS, PE(P) and COH are enriched in all exosomes types compared to their cell source. CE shows an interesting enrichment in PDAC exosomes compared to the other two types, which was further investigated.

To better understand the enrichment of lipid classes in exosomes compared to their source cells, the relative expression of some of the major lipid classes between the two sample groups was expressed as a ratio and is displayed in Figure 3.2. Interestingly, the ratio of CE expression in PDAC exosomes/ PDAC cells is strikingly higher compared to the ratio of the epithelial samples, while the opposite trend is observed for the prostate and ovarian samples (Figure 3.2A). COH is enriched in all exosomal samples (Figure 3.2B), as does Cer (apart from MiaPaCa-II and PC-3 where the ratio is just below 1) (Figure 3.2C), and SM (Figure 3.2D). PI, LPI and PE have higher expression in the donor cells than in their respective exosomes for the majority of the samples (Figure 3.2E, F, G, respectively) while PS is overexpressed in all samples (Figure 3.2H). It has been reported before that PS is expressed in exosomes in the outer leaflet, and especially in cases of hypoxia (Wei, Liu et al. 2016). What stands out is the high ratio of expression of PS in HPAF-II exosomes compared to cells, which is magnitudes higher than the rest of the ratios. PS is naturally expressed on the surface of cells which undergo apoptosis and it is a signal for clearance of this cell by the macrophages. At present we do not know if this is due to HPAF-II having higher basal levels of apoptosis or higher membrane levels of PS.

A


Lipid classes levels in cells (pmol_µmol PC)
Total AcylCarnitine
Total dhCer
Total Cer
Total GM3
Total Hex1Cer
Total Hex2Cer
Total Hex3Cer
Total SM
Total LPC
Total LPC(O)
Total LPC(P)
Total LPE
Total LPE(P)
Total LPI
Total LPS
Total PC
Total PC(O)
Total PC(P)
Total PE
Total PE(O)
Total PE(P)
Total PG
Total PI
Total PS
Total Sulfatide
Total Desmosterol
Total CE
Total OH-Cholesterol
Total COH
Total DG
Total TG
Total Ubiquinone
Total MG
Lipid Classes
1.1×10^6
1.0×10^6
9.0×10^5
8.0×10^5
7.0×10^5
6.0×10^5
5.0×10^5
4.0×10^5
3.0×10^5
2.0×10^5
1.0×10^5
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Cell Type

B


Lipid classes levels in exosomes (pmol_µmol PC)
Total AcylCarnitine
Total dhCer
Total Cer
Total GM3
Total Hex1Cer
Total Hex2Cer
Total Hex3Cer
Total SM
Total LPC
Total LPC(O)
Total LPC(P)
Total LPE
Total LPE(P)
Total LPI
Total LPS
Total PC
Total PC(O)
Total PC(P)
Total PE
Total PE(O)
Total PE(P)
Total PG
Total PI
Total PS
Total Sulfatide
Total Desmosterol
Total CE
Total OH-Cholesterol
Total COH
Total DG
Total TG
Total Ubiquinone
Total MG
Lipid Classes
3.00×10^7
2.75×10^7
2.50×10^7
2.25×10^7
2.00×10^7
1.75×10^7
1.50×10^7
1.25×10^7
1.00×10^7
7.50×10^6
5.00×10^6
2.50×10^6
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Exosome Type

C

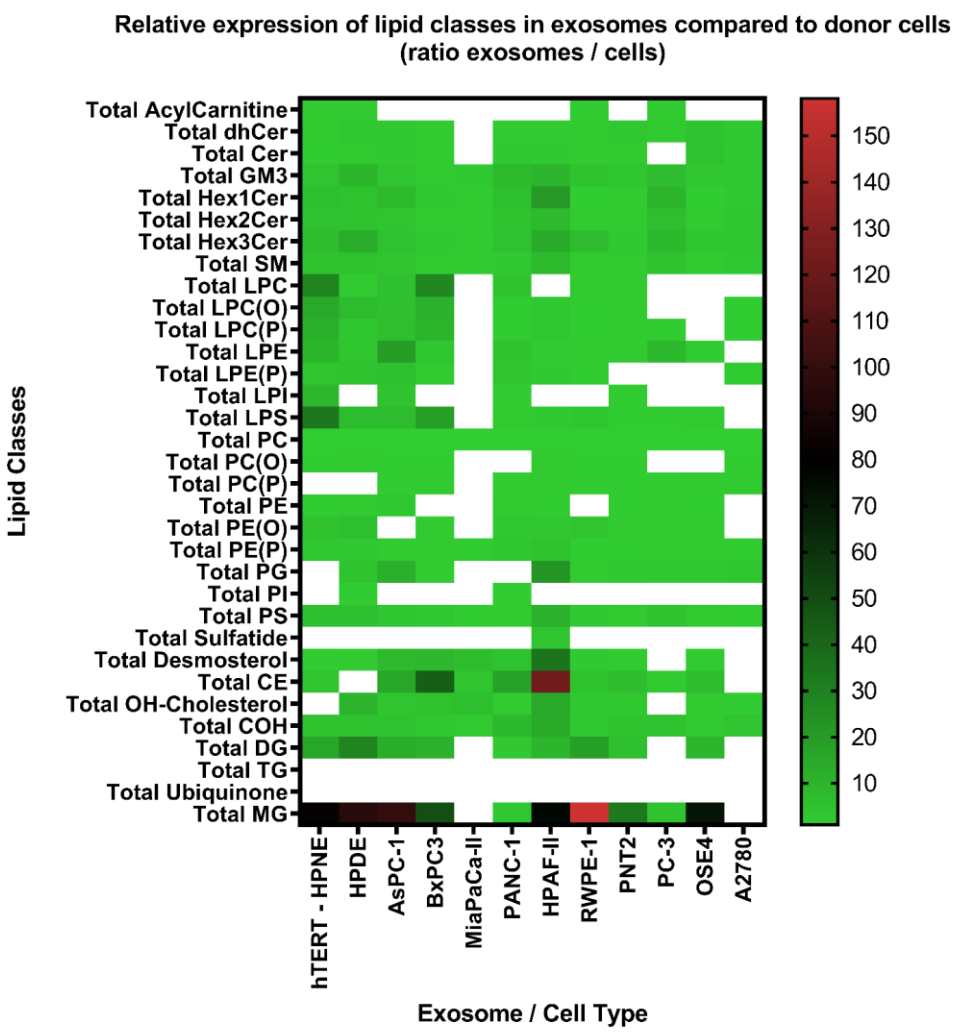
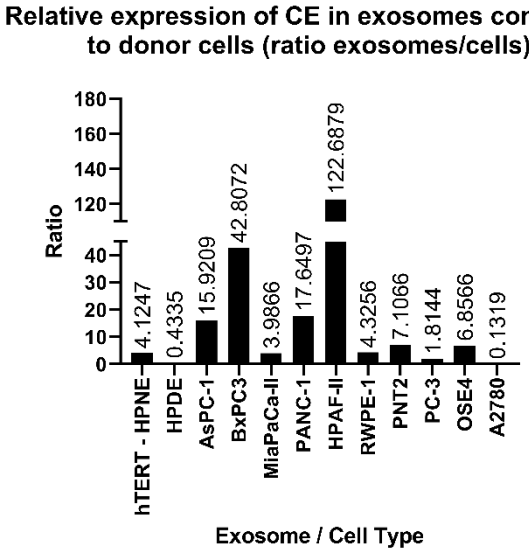
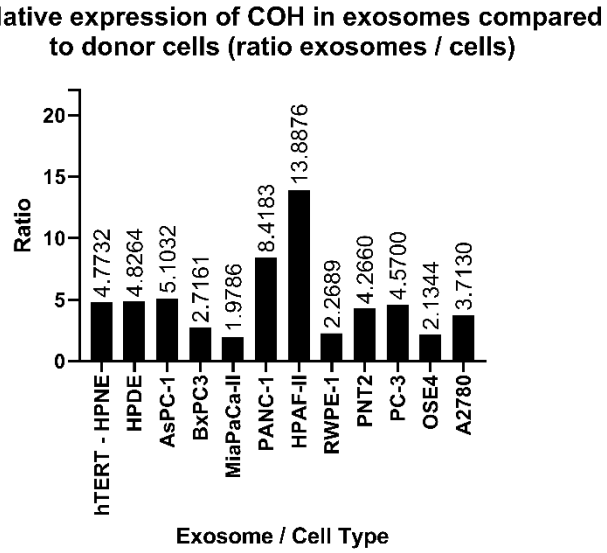


Figure 3.1 Heatmaps depicting the lipidome of pancreatic, prostate and ovarian epithelial and cancer cells, and their respective exosomes. (A) Lipidomic profile of cells samples. (B) Lipidomic profile of exosomes samples. (C) Relative expression of lipid classes in exosomes compared to donor cells (ratio exosomes / cells). For (A) data were calculated as pmol/mg of protein based on BCA assay results, then normalised to total PC to get pmol/µmol of PC, whereas for (B) whole samples were used in the extraction and data were calculated as pmol per sample, then normalised to total PC to get pmol/µmol of PC. Due to this normalisation, the values for total PC are disregarded. For (C) the corresponding cell of the heatmap is shown as white in case the ratio reflected a value <1. Heatmaps were created with GraphPad Version 8.12. For (A) and (B) data are expressed as average of n=3 independent experiments. dhCer: dihydroceramide, Cer: ceramide, GM3: G_{M3} ganglioside, Hex1Cer Hex2Cer Hex3Cer: glycosylceramides containing 1, 2 or 3 sugar moieties, SM: sphingomyelin, LPC: lysophosphatidylcholine, LPC(O): lysoalkylphosphatidylcholine, LPC(P): lysophosphatidylcholine plasmalogen, LPE: lysophosphatidylethanolamine, LPE(P): lysophosphatidylethanolamine plasmalogen, LPI: lysophosphatidylinositol, LPS: lysophosphatidylserine, PC: phosphatidylcholine, PC(O): alkylphosphatidylcholine, PC(P): phosphatidylcholine plasmalogen, PE: phosphatidylethanolamine, PE(O): alkylphosphatidylethanolamine, PE(P): phosphatidylethanolamine plasmalogen, PG: phosphatidylglycerol, PI: phosphatidylinositol, PS: phosphatidylserine, CE: cholesterol ester, COH: free cholesterol, DG: diacylglycerol, TG: triacylglycerol, MG: monoacylglycerol

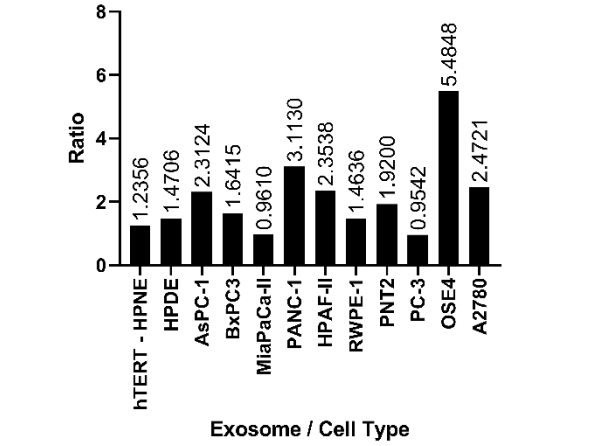
A


Relative expression of CE in exosomes compared to donor cells (ratio exosomes/cells)
Ratio
180
160
140
120
40
30
20
10
0
4.1247
0.4335
15.9209
42.8072
3.9866
17.6497
122.6879
4.3256
7.1066
1.8144
6.8566
0.1319
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Exosome / Cell Type

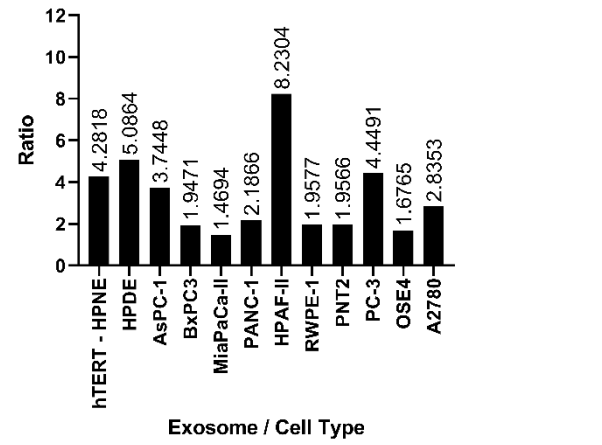
B


Relative expression of COH in exosomes compared to donor cells (ratio exosomes / cells)
Ratio
20
15
10
5
0
4.7732
4.8264
5.1032
2.7161
1.9786
8.4183
13.8876
2.2689
4.2660
4.5700
2.1344
3.7130
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Exosome / Cell Type

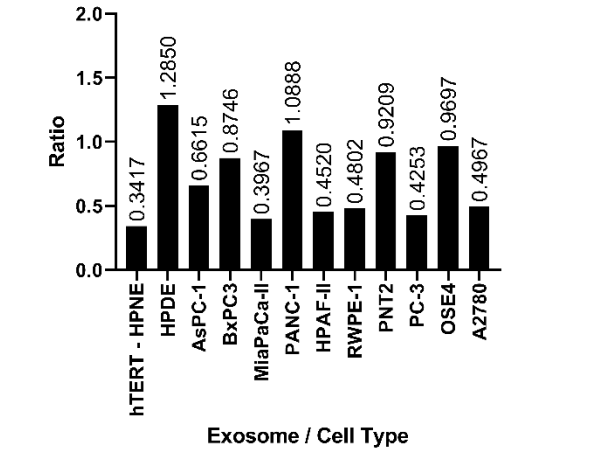
C


Relative expression of Cer in exosomes compared to donor cells (ratio exosomes / cells)
Ratio
8
6
4
2
0
1.2356
1.4706
2.3124
1.6415
0.9610
3.1130
2.3538
1.4636
1.9200
0.9542
5.4848
2.4721
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Exosome / Cell Type

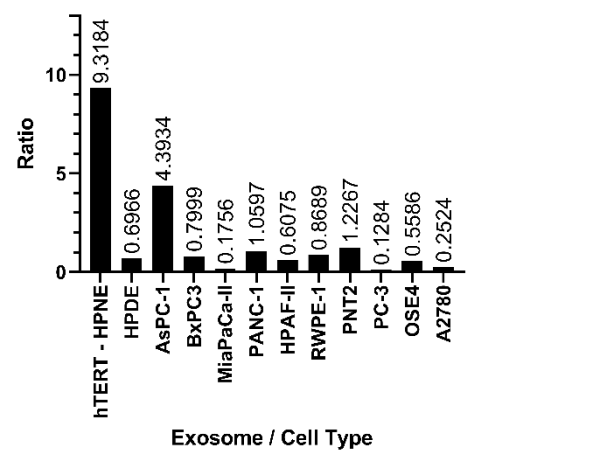
D


Relative expression of SM in exosomes compared to donor cells (ratio exosomes / cells)
Ratio
12
10
8
6
4
2
0
4.2818
5.0864
3.7448
1.9471
1.4694
2.1866
8.2304
1.9577
1.9566
4.4491
1.6765
2.8353
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Exosome / Cell Type

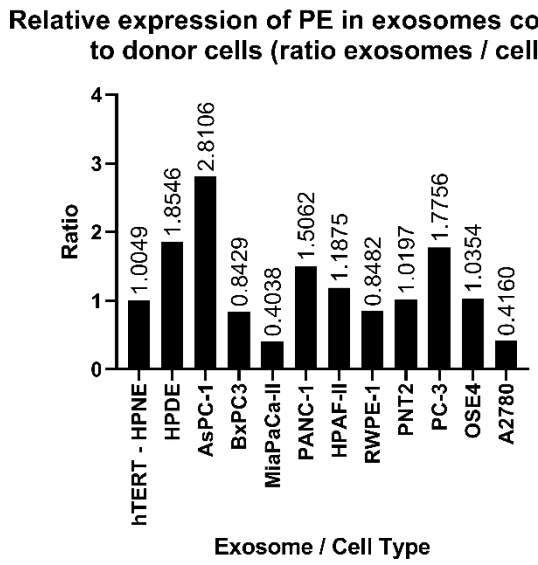
E


Relative expression of PI in exosomes compared to donor cells (ratio exosomes / cells)
Ratio
2.0
1.5
1.0
0.5
0.0
0.3417
1.2850
0.6615
0.8746
0.3967
1.0888
0.4520
0.4802
0.9209
0.4253
0.9697
0.4967
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Exosome / Cell Type

F


Relative expression of LPI in exosomes compared to donor cells (ratio exosomes / cells)
Ratio
10
5
0
9.3184
0.6966
4.3934
0.7999
0.1756
1.0597
0.6075
0.8689
1.2267
0.1284
0.5586
0.2524
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Exosome / Cell Type

G



H

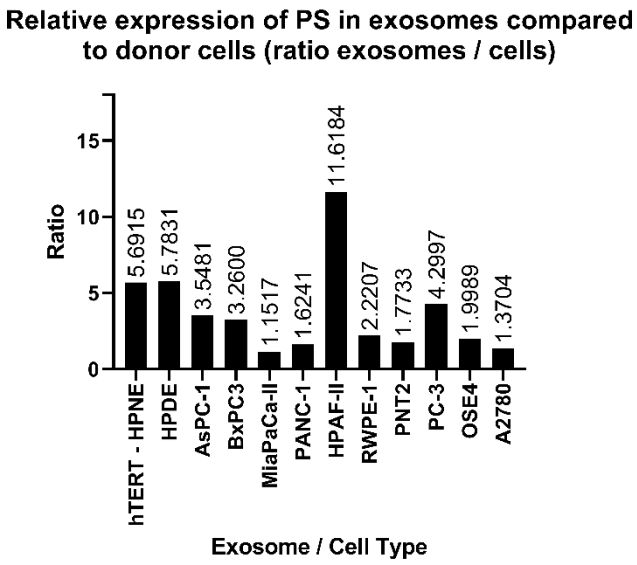
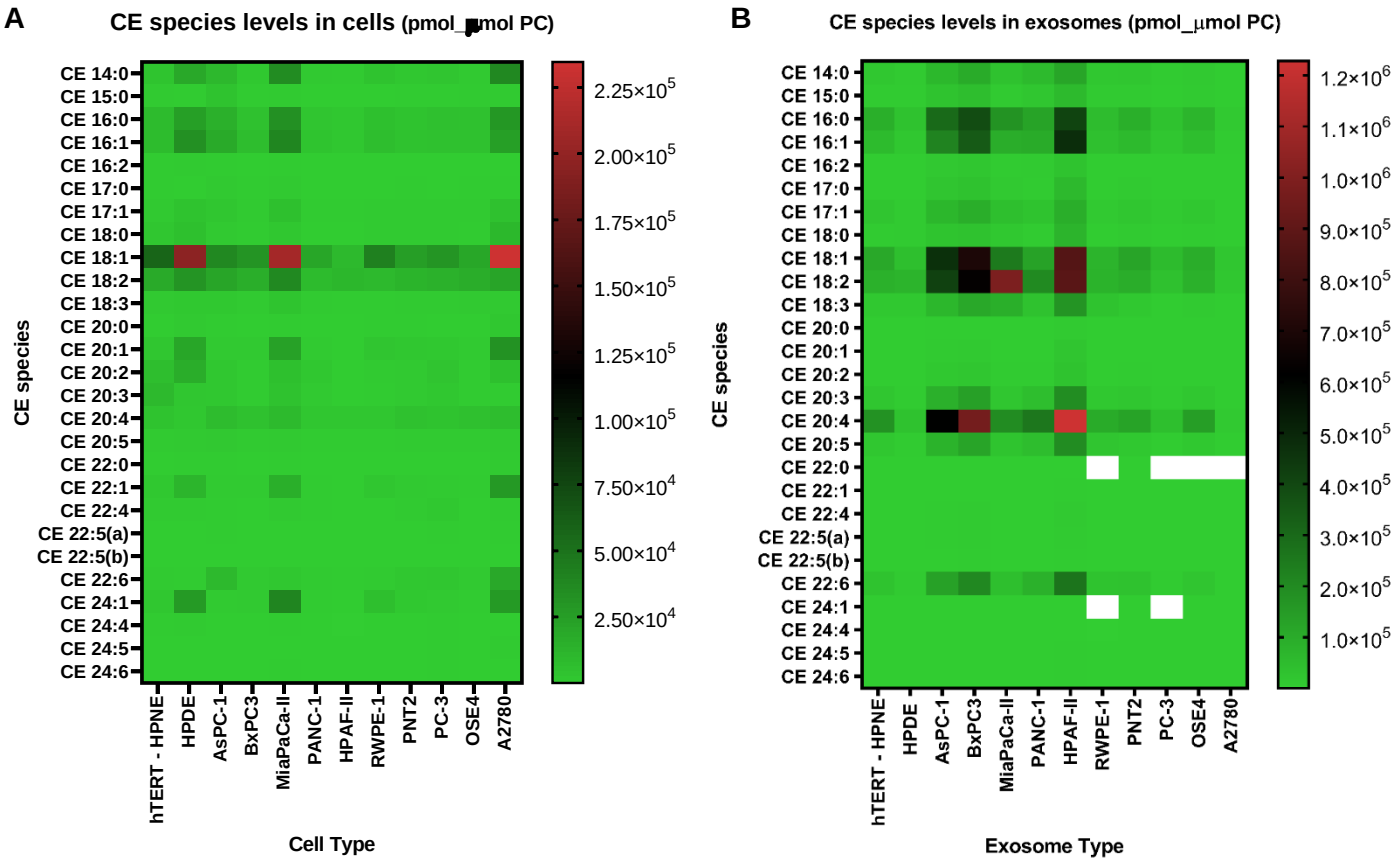


Figure 3.2 Relative expression of major lipid classes in exosomes compared to their parental cells. Ratios expressing the relative expression of (A) CE, (B) COH, (C) Cer, (D) SM, (E) PI, (F) LPI, (G) PE, (H) PS, in exosomes compared to their donor cells. Graphs were created with GraphPad Version 8.1.2.

Since the CE class showed such an interesting profile, the specific CE species levels in exosomes and cells were further analysed as shown in Figure 3.3. The predominant CE species in cells include the CE18:1, 18:2, 14:0, 16:0 and 16:1 (Figure 3.3A), while in exosomes those species along with 20:4 and 22:6 are present in higher levels (Figure 3.3B). At the same time, there seems to be a specific and selective species packaging in the exosomes; for instance BxPC-3 cells do not exhibit a notable difference among the CE species, whereas their exosomes are notably enriched in CE16:0, 16:1, 18:1, 18:2, and 20:4, the last one being of great importance as it serves as a signalling molecule and as a precursor of prostaglandins. Analogous differences are seen also in other cells / exosomes types such as HPDE or A2780, underscoring the fact that the enrichment in the CE class and specific species is unlikely to be derived from co-isolation of lipid droplets, a fact that is also supported by the low levels of TGs detected in the exosomes analysed in this study. The relative expressions of CE species in exosomes compared to their donor cells are displayed in Figure 3.3C, where the PDAC exosomes types AsPC-1, BxPC-3 and HPAF-II appear as enriched

in the majority of CE species compared to their donor cells, in contrast to the ovarian and prostate cancer-derived exosomes.


A
CE species levels in cells (pmol_µmol PC)
CE species
CE 14:0
CE 15:0
CE 16:0
CE 16:1
CE 16:2
CE 17:0
CE 17:1
CE 18:0
CE 18:1
CE 18:2
CE 18:3
CE 20:0
CE 20:1
CE 20:2
CE 20:3
CE 20:4
CE 20:5
CE 22:0
CE 22:1
CE 22:4
CE 22:5(a)
CE 22:5(b)
CE 22:6
CE 24:1
CE 24:4
CE 24:5
CE 24:6
2.25×10^5
2.00×10^5
1.75×10^5
1.50×10^5
1.25×10^5
1.00×10^5
7.50×10^4
5.00×10^4
2.50×10^4
hTERT - HPNE
HPDE
AsPC-1
BxPC3
MiaPaCa-II
PANC-1
HPAF-II
RWPE-1
PNT2
PC-3
OSE4
A2780
Cell Type
B
CE species levels in exosomes (pmol_µmol PC)
1.2×10^6
1.1×10^6
1.0×10^6
9.0×10^5
8.0×10^5
7.0×10^5
6.0×10^5
5.0×10^5
4.0×10^5
3.0×10^5
2.0×10^5
1.0×10^5
Exosome Type

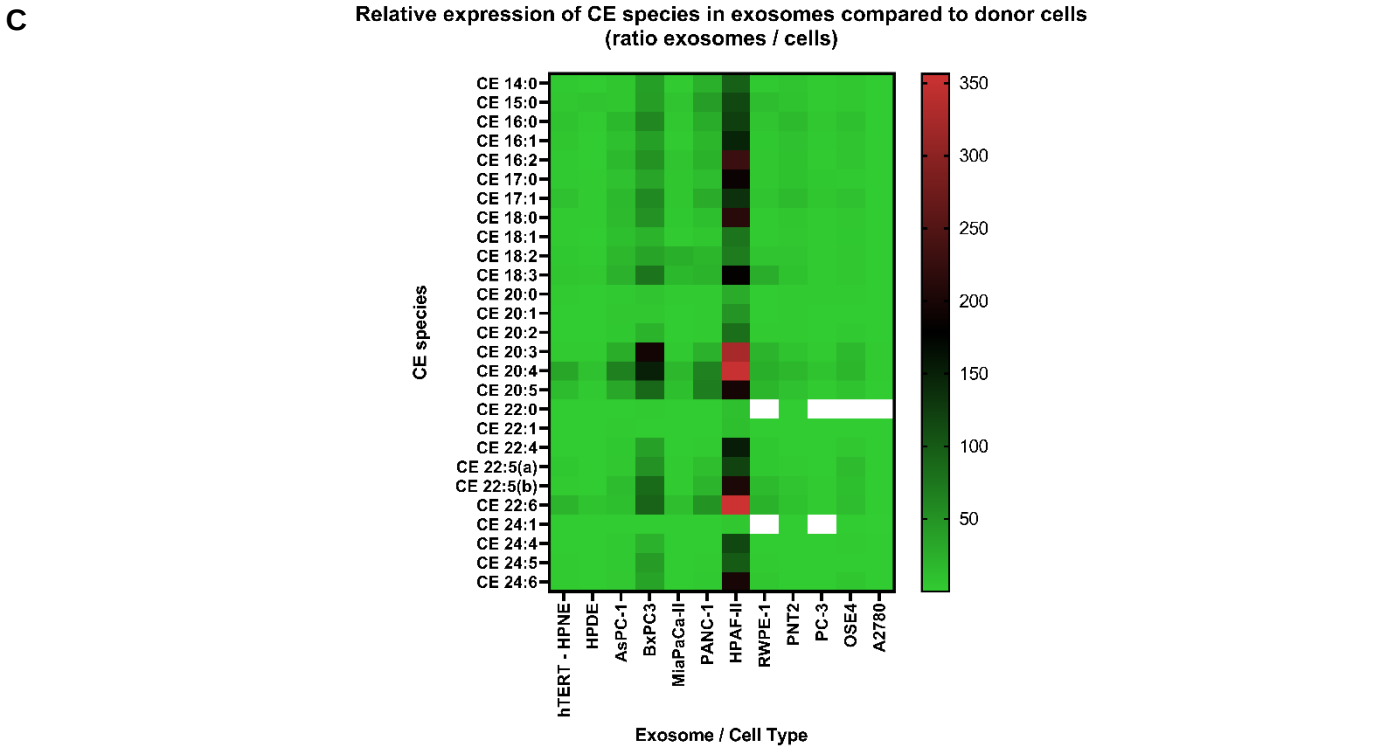

C
Relative expression of CE species in exosomes compared to donor cells
(ratio exosomes / cells)
CE species
350
300
250
200
150
100
50
Exosome / Cell Type

Figure 3.3 Heatmaps depicting the CE species of pancreatic, prostate and ovarian epithelial and cancer cells, and their respective exosomes. (A) CE species in cells. (B) CE species in exosomes. (C) Relative expression of CE species in exosomes compared to donor cells (ratio exosomes / cells).Values that could not be obtained from peak integration due to a drastic shift in retention time (they are not equal to 0), are represented by a white cell. Heatmaps were created with GraphPad Version 8.1.2. For (A) and (B) data are expressed as average of n=3 independent experiments.

3.4.2 The effect of CE depletion on T cells proliferation and subsequent cytokine expression

In order for the biological relevance of this PDAC-specific CE enrichment to be defined, experiments were performed where the PDAC cells MiaPaCa-II and PANC-1 were treated with avasimibe- a widely used acyl-CoA cholesterol acyltransferase-1 (ACAT-1) inhibitor, at a concentration of 5 μM and 10 μM for 48 h, and the derived exosomes were collected and co-incubated with $CD4^+$ and $CD8^+$ cells isolated from whole blood of healthy donors, at a concentration of 25 μg / ml following the work of (Chen, Huang et al. 2018). Co-incubation of exosomes with cells was initially performed for 3 days, a period which was not enough for the cells to proliferate (data not shown). When the incubation period was extended to 7 days the proliferation of T cells was successful, as shown in Figure 3.4. An approximately 60% and 50% of the total $CD4^+$ and $CD8^+$ cells of the untreated control proliferated, and although the proliferation of the $CD4^+$ population did not seem to be notably affected by any of the different exosome types, there was an obvious impact on the $CD8^+$ population. All exosomes types had an inhibitory effect, with MiaPaCa-II exosomes reducing T cell numbers in a statistically significant way. For $CD8^+$ cells, this effect seemed to be reversed with the use of avasimibe, however these are preliminary data and definite conclusions cannot be made at this point.

Following the confirmation of a successful proliferation assay, the supernatant of the T cells was examined for differential expression of a panel of cytokines, as shown in Figure 3.5. Both unstimulated and stimulated cells were examined (Figure 3.5B, C and 3.5D, E, respectively), with the representative gating strategy being shown in Figure 3.5A. For $CD4^+$ unstimulated cells mainly two cytokines were detected by the assay; IL-8 and MCP-1, both instigated mostly after co-incubation with MiaPaCa-II exosomes. The same trend was observed for $CD8^+$ cells, with the levels of the detected cytokines being more abundant in this case. In case of the stimulated cells, the number of cytokines detected increased, with the main cytokines standing out being IFN-γ, IL-8, IL-18, IL-23 and MCP-1. IFN-γ was expressed in both populations

(much more in $CD4^+$ than $CD8^+$), and treatment with avasimibe-exosomes seemed to lower its levels. The same pattern was repeated with the other four cytokines. A more detailed analysis of the results of the cytokine array is found in Figure 3.6A-S, where the levels of expression of each cytokine in each of the two stimulated populations, are shown in a separate graph. In this case, it was only MCP-1 which was significantly elevated in the supernatant of PANC-1 exosomes-treated $CD4^+$ cells, and treatment with avasimibe 5μM-exosomes did reduce its levels compared to the internal control (PANC-1 exosomes), but without statistically significant difference. IL-10 and IL-17A were only detected in the $CD4^+$ cells population and not in $CD8^+$, with both PANC-1 and MiaPaCa-II avasimibe-exosomes overall decreasing its levels compared to the respective non-treated PANC-1 and MiaPaCa-II exosomes and/or the main untreated T cells control. Interestingly, in regards to IL-18, avasimibe-exosomes seem to have opposite effects on the expression of the cytokine, nevertheless one of the factors contributing to such differences is the different metabolic regulation of the two populations. IL-β was only detected in low levels (10 pg / ml) in the $CD8^+$ cells, with almost all exosomes types eliminating its expression; an effect not able to be rescued by avasimibe. IL-6 exhibited very similar levels of expression in untreated $CD4^+$ and $CD8^+$ cells and in both cases PDAC exosomes instigated its expression. Overall, $CD4^+$ control cells secreted much higher levels of IFN-γ The levels of IL-8 in the supernatant of $CD4^+$ T cells was more than double than in $CD8^+$ cells, however the different types of exosomes did not seem to have an impact in any case. Finally, IL-23 was a cytokine with very low (even zero for $CD8^+$ cells) expression levels, which was instigated by MiaPaCa-II and PANC-1 exosomes, and with avasimibe-exosomes being able to somehow restrain this increase.

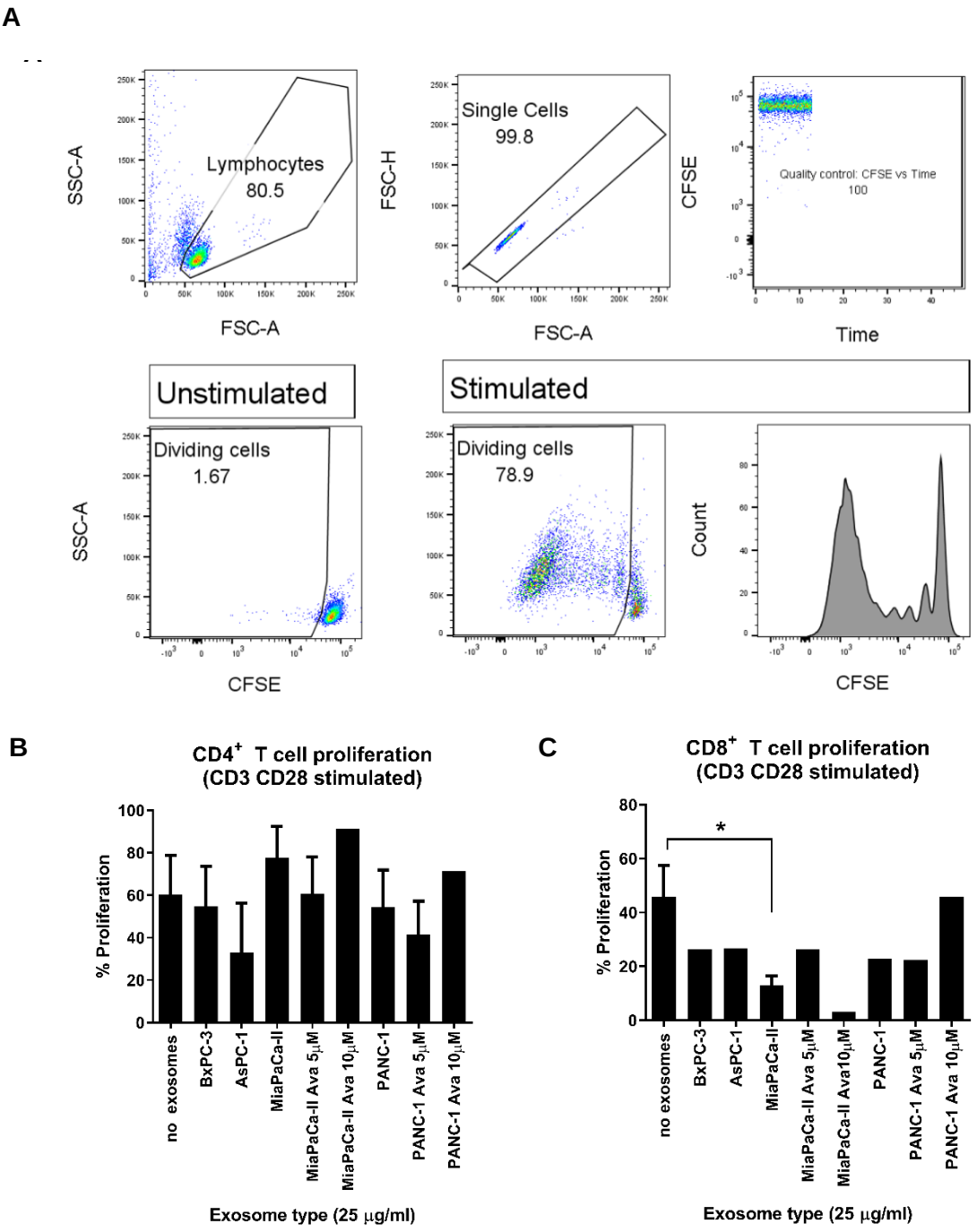
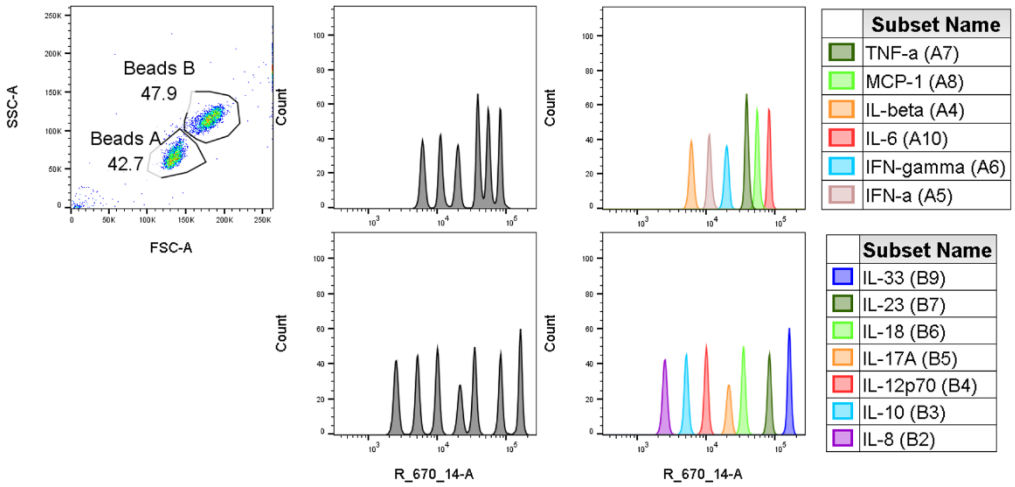


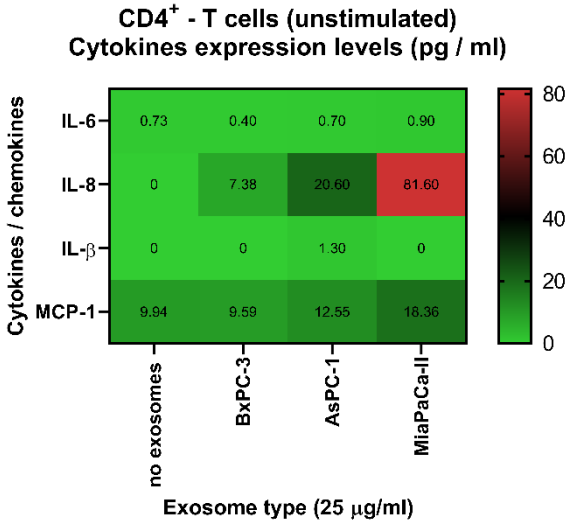
Figure 3.4 Proliferation of CFSE- labelled CD4⁺ and CD8⁺ T cells after 7 days of co-incubation with PDAC exosomes. CD4⁺ and CD8⁺ T cells were isolated from whole blood of healthy donors and incubated with 25μg/ml of PDAC exosomes in the presence of CD3 CD28 stimulus for 7 days. (A) Representative gating strategy for T- cell proliferation assay, starting from whole lymphocytes population (SSC-A vs FSC-A), to subpopulations of single cells (FSC-H vs FSC-A) and dividing cells (SSC-A vs CFSE or Count vs CFSE). A gating step of CFSE vs Time was added as quality control. (B) Percentage of proliferating CD4⁺ T cells upon different treatments with exosomes. (C) Percentage of proliferating CD8⁺ T cells upon different treatments with exosomes. Data are expressed as averages ± SEM of n≥3 independent experiments (apart from "MiaPaCa-II Ava 10 μM" and "PANC-1 Ava 10 μM", where n=1) for (B), and as averages ± SEM of n=3 independent experiments for "no exosomes" and "MiaPaCa-II" for (B), as the rest of the samples where n≤ 2. For (A), FlowJo

Version 10.6.1 was used and (B) and (C) were created with GraphPad Version 8.1.2. Statistics for (B) were performed using one-way Anova, without any statistically significant difference between samples, and for (C) using one-tailed t-test between no exosomes-MiaPaCa-II samples. *$p < 0.05$

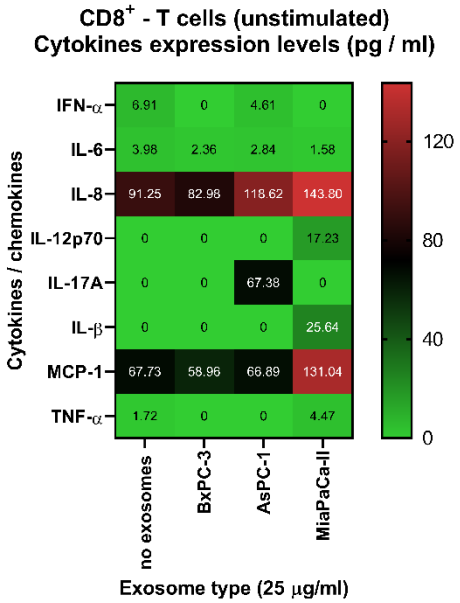
A


Beads B
47.9
Beads A
42.7
SSC-A
FSC-A
Count
R_670_14-A
Subset Name
TNF-a (A7)
MCP-1 (A8)
IL-beta (A4)
IL-6 (A10)
IFN-gamma (A6)
IFN-a (A5)
Subset Name
IL-33 (B9)
IL-23 (B7)
IL-18 (B6)
IL-17A (B5)
IL-12p70 (B4)
IL-10 (B3)
IL-8 (B2)

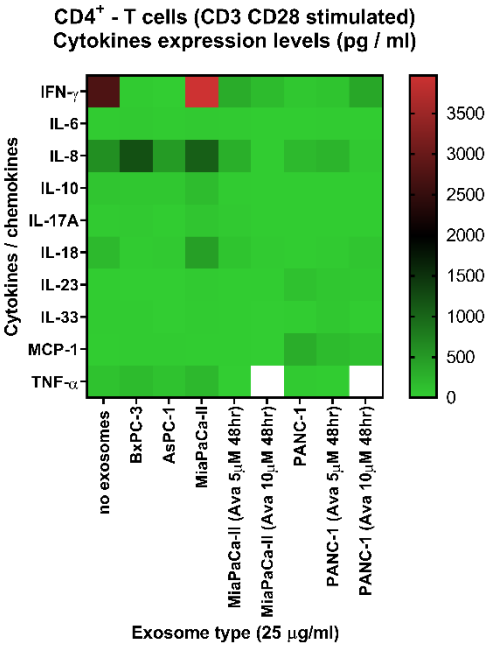
B


CD4+ - T cells (unstimulated)
Cytokines expression levels (pg / ml)
Cytokines / chemokines
IL-6
IL-8
IL-β
MCP-1
0.73 0.40 0.70 0.90
0 7.38 20.60 81.60
0 0 1.30 0
9.94 9.59 12.55 18.36
80
60
40
20
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
Exosome type (25 μg/ml)

C


CD8+ - T cells (unstimulated)
Cytokines expression levels (pg / ml)
Cytokines / chemokines
IFN-α
IL-6
IL-8
IL-12p70
IL-17A
IL-β
MCP-1
TNF-α
6.91 0 4.61 0
3.98 2.36 2.84 1.58
91.25 82.98 118.62 143.80
0 0 0 17.23
0 0 67.38 0
0 0 0 25.64
67.73 58.96 66.89 131.04
1.72 0 0 4.47
120
80
40
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
Exosome type (25 μg/ml)

D


CD4+ - T cells (CD3 CD28 stimulated)
Cytokines expression levels (pg / ml)
Cytokines / chemokines
IFN-γ
IL-6
IL-8
IL-10
IL-17A
IL-18
IL-23
IL-33
MCP-1
TNF-α
3500
3000
2500
2000
1500
1000
500
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II (Ava 5μM 48hr)
MiaPaCa-II (Ava 10μM 48hr)
PANC-1
PANC-1 (Ava 5μM 48hr)
PANC-1 (Ava 10μM 48hr)
Exosome type (25 μg/ml)

E

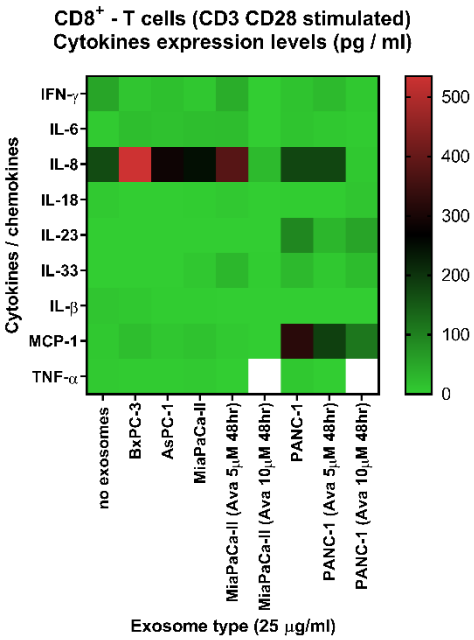
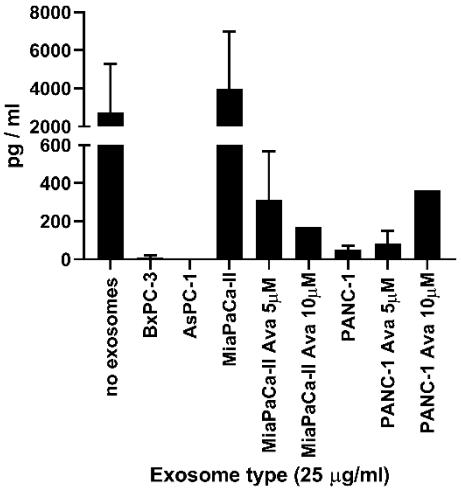
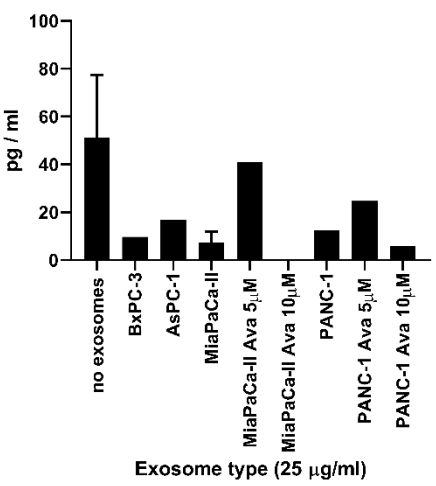

CD8+ - T cells (CD3 CD28 stimulated)
Cytokines expression levels (pg / ml)
Cytokines / chemokines
IFN-γ
IL-6
IL-8
IL-18
IL-23
IL-33
IL-β
MCP-1
TNF-α
500
400
300
200
100
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II (Ava 5μM 48hr)
MiaPaCa-II (Ava 10μM 48hr)
PANC-1
PANC-1 (Ava 5μM 48hr)
PANC-1 (Ava 10μM 48hr)
Exosome type (25 μg/ml)

Figure 3.5 Heatmaps of cytokine expression profile $CD4^+$ and $CD8^+$ T cells after 7 days of co-incubation with PDAC exosomes. $CD4^+$ and $CD8^+$ T cells were isolated from whole blood of healthy donors and incubated with 25µg/ml of PDAC exosomes in the absence (B), (C) or presence (D), (E) of CD3 CD28 stimulus for 7 days. (A) Representative gating strategy for the populations of Beads A (TNF-a, MCP-1, IL-beta, IL-6, IFN-gamma, IFN-a) and Beads B (IL-33, IL-23, IL-18, IL-17A, IL-12p70, IL-10, IL-8). (B) Cytokine profile of unstimulated $CD4^+$ T cells upon different treatments with exosomes. (C) Cytokine profile of unstimulated $CD8^+$ T cells upon different treatments with exosomes. (D) Cytokine profile of CD3 CD28 stimulated $CD4^+$ T cells upon different treatments with exosomes. (E) Cytokine profile of CD3 CD28 stimulated $CD8^+$ T cells upon different treatments with exosomes. For (B), (C) one replicate was performed; for (D) data are expressed as average of $n \geq 3$ independent experiments, apart from "MiaPaCa-II Ava 10 µM" and "PANC-1 Ava 10 µM", where n=1; for (E) data are expressed averages of n=3 independent experiments for "no exosomes" and "MiaPaCa-II", as the rest of the samples where $n \leq 2$. White boxes represent cases where a cytokine was out of the range of detection. (A) was created with FlowJo Version 10.6.1 and (B), (C), (D), (E) were created with GraphPad Version 8.1.2.

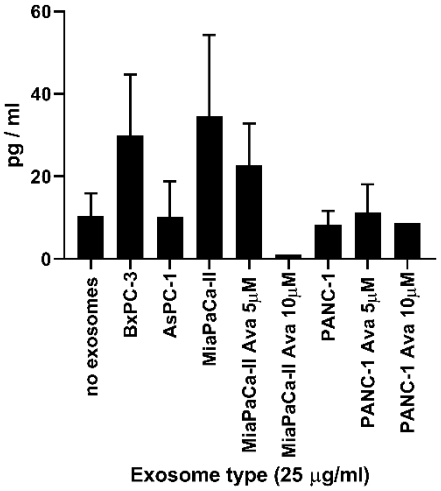
A


IFN-γ expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
8000
6000
4000
2000
600
400
200
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

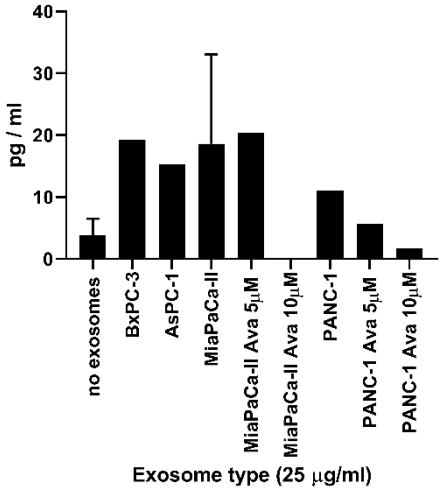
B


IFN-γ expression in CD8+ T cells
(CD3 CD28 stimulated)
pg / ml
100
80
60
40
20
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

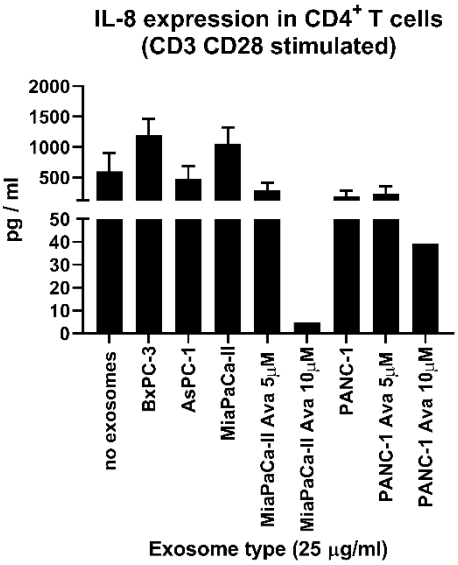
C


IL-6 expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
60
40
20
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

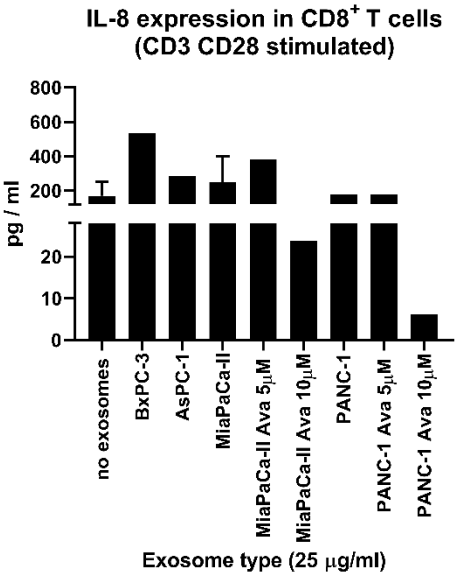
D


IL-6 expression in CD8+ T cells
(CD3 CD28 stimulated)
pg / ml
40
30
20
10
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

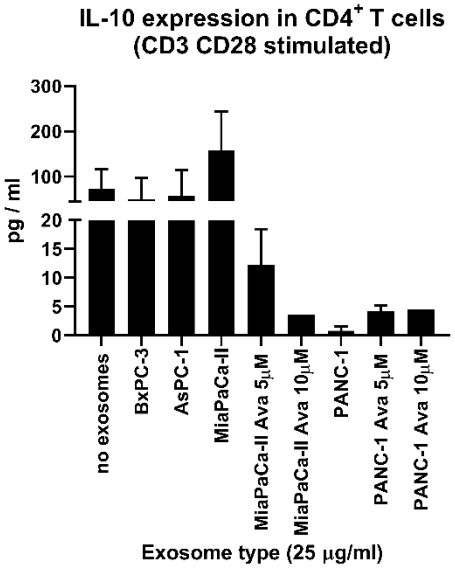
E


IL-8 expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
2000
1500
1000
500
50
40
30
20
10
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

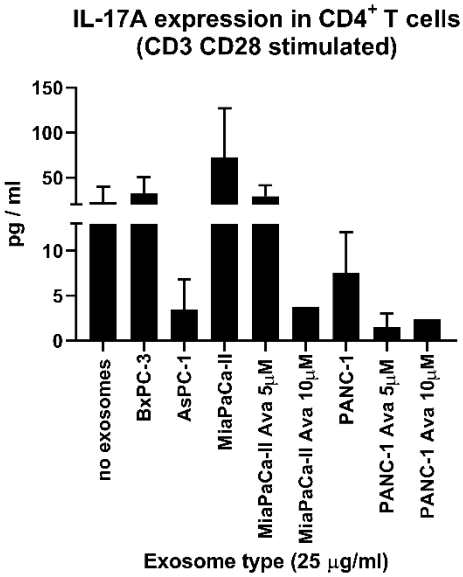
F


IL-8 expression in CD8+ T cells
(CD3 CD28 stimulated)
pg / ml
800
600
400
200
20
10
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

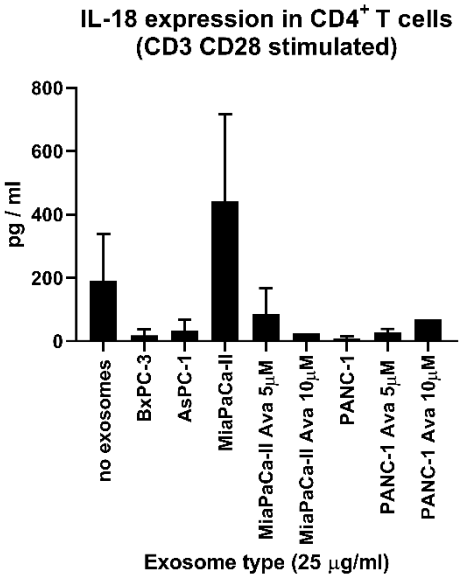
G


IL-10 expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
300
200
100
20
15
10
5
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

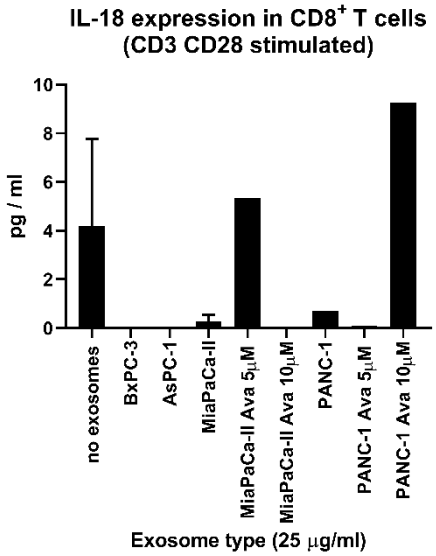
H


IL-17A expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
150
100
50
10
5
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

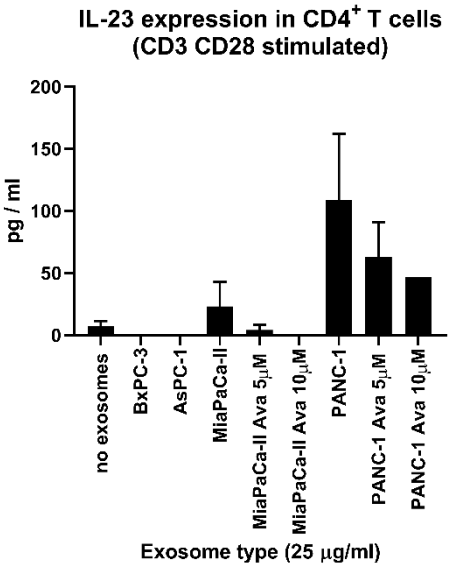
I


IL-18 expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
800
600
400
200
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

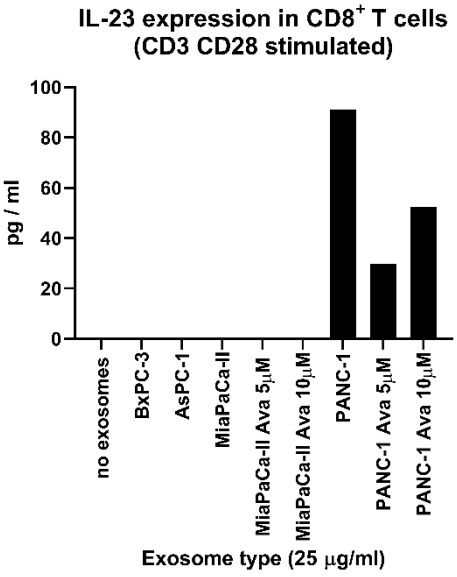
J


IL-18 expression in CD8+ T cells
(CD3 CD28 stimulated)
pg / ml
10
8
6
4
2
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

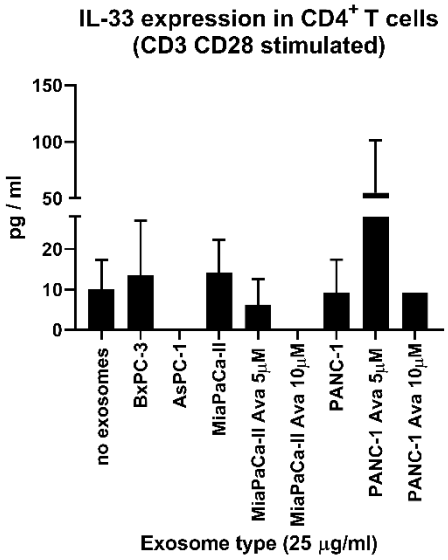
K


IL-23 expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
200
150
100
50
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

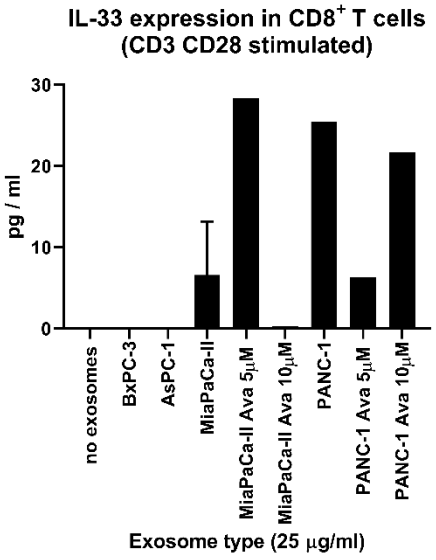
L


IL-23 expression in CD8+ T cells
(CD3 CD28 stimulated)
pg / ml
100
80
60
40
20
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

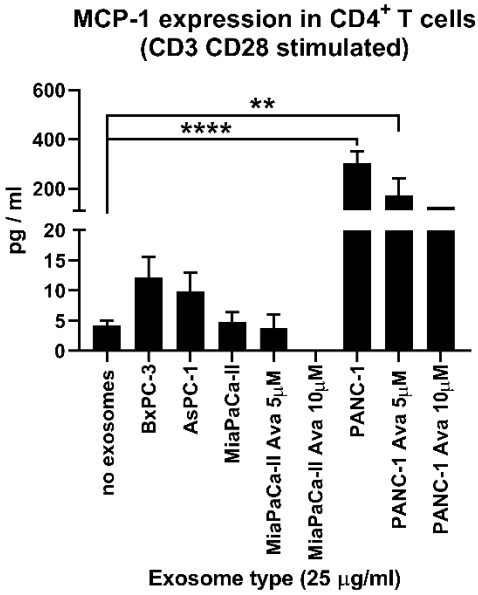
M


IL-33 expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
150
100
50
20
10
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

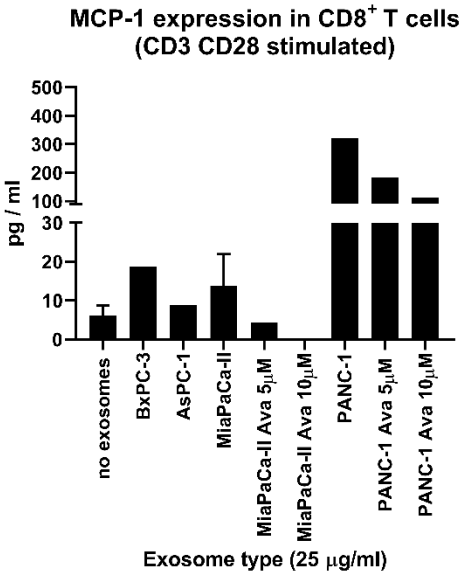
N


IL-33 expression in CD8+ T cells
(CD3 CD28 stimulated)
pg / ml
30
20
10
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

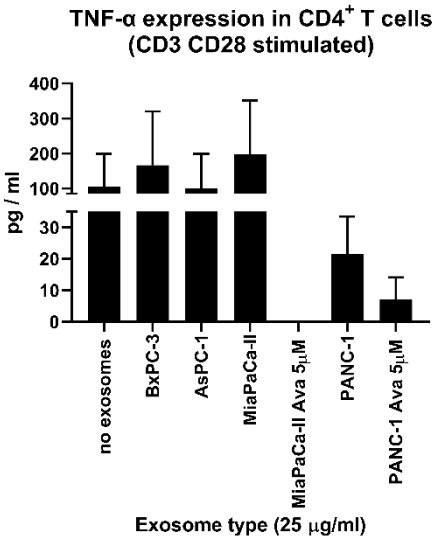
O


MCP-1 expression in CD4+ T cells
(CD3 CD28 stimulated)
**
****
pg / ml
600
400
200
20
15
10
5
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

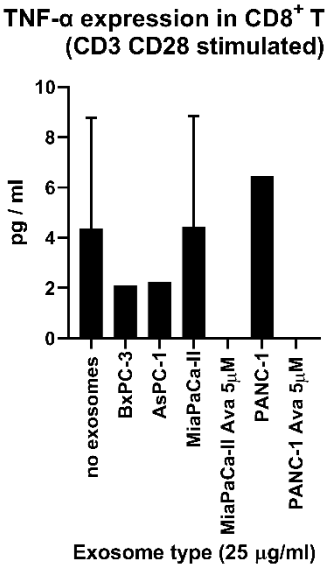
P


MCP-1 expression in CD8+ T cells
(CD3 CD28 stimulated)
pg / ml
500
400
300
200
100
30
20
10
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
MiaPaCa-II Ava 10μM
PANC-1
PANC-1 Ava 5μM
PANC-1 Ava 10μM
Exosome type (25 μg/ml)

Q


TNF-α expression in CD4+ T cells
(CD3 CD28 stimulated)
pg / ml
400
300
200
100
30
20
10
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
PANC-1
PANC-1 Ava 5μM
Exosome type (25 μg/ml)

R


TNF-α expression in CD8+ T cells
(CD3 CD28 stimulated)
pg / ml
10
8
6
4
2
0
no exosomes
BxPC-3
AsPC-1
MiaPaCa-II
MiaPaCa-II Ava 5μM
PANC-1
PANC-1 Ava 5μM
Exosome type (25 μg/ml)

S

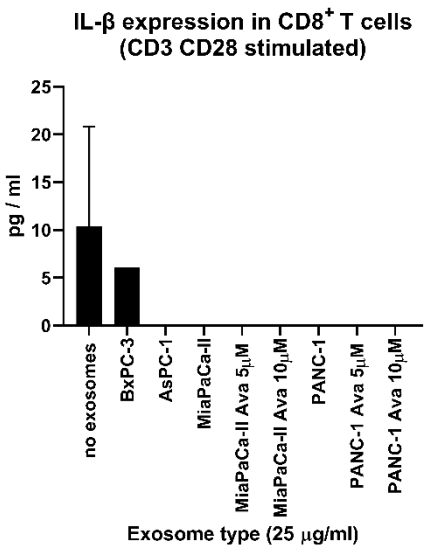


Figure 3.6 Cytokines expression in supernatants of CD3 CD28 stimulated CD4⁺ and CD8⁺ T cells after 7 days of co-incubation with PDAC exosomes. (A) IFN-γ expression levels in CD4⁺ T cells. (B) IFN-γ expression levels in CD8⁺ T cells. (C) IL-6 expression levels in CD4⁺ T cells. (D) IL-6 expression levels in CD8⁺ T cells. (E) IL-8 expression levels in CD4⁺ T cells. (F) IL-8 expression levels in CD8⁺ T cells. (G) IL-10 expression levels in CD4⁺ T cells. (H) IL-17A expression levels in CD4⁺ T cells. (I) IL-18 expression levels in CD4⁺ T cells. (J) IL-18 expression levels in CD8⁺ T cells. (K) IL-23 expression levels in CD4⁺ T cells. (L) IL-23 expression levels in CD8⁺ T cells. (M) IL-33 expression levels in CD4⁺ T cells. (N) IL-33 expression levels in CD8⁺ T cells. (O) MCP-1 expression levels in CD4⁺ T cells. (P) MCP-1 expression levels in CD8⁺ T cells. (Q) TNF-α expression levels in CD4⁺ T cells. (R) TNF-α expression levels in CD8⁺ T cells. (S) IL-β expression levels in CD8⁺ T cells. For CD4⁺ T cells, data are expressed as averages ± SEM of n≥3 independent experiments (apart from "MiaPaCa-II Ava 10 μM" and "PANC-1 Ava 10 μM", where n=1). One-way Anova with Tukey's post hoc test was used for statistical analysis, ** $p \leq 0.01$, **** $p \leq 0.0001$. For CD8⁺ T cells, t-test was performed between no exosomes (untreated) control and MiaPaCa-II-exosomes, as they were the only conditions with n=3. No statistically significant difference was observed for between samples. For (A-S) GraphPad Version 8.1.2 was used.

3.5 Discussion

When cancer arises the immune system detects the malignant cells and acts towards their elimination, however this process can result to the natural selection of tumour cells that grow resistant, survive and proliferate under these conditions (Vesely, Kershaw et al. 2011). The phenomenon in which the immune system switches from having anti-tumorigenic properties to supporting a pro–tumorigenic state is called immunoediting and involves three stages. Initially, the immune system successfully detects the tumour and eliminates it (elimination), nevertheless at a later stage its capacity is limited to restricting the tumour growth (equilibrium). In this second stage an immunoselection of resistant tumour cells takes place, inevitably leading to the final stage, which is the immune system's override and subsequent clinical manifestation of the disease (escape) (Dunn, Old et al. 2004).

The immune system consists of two branches, the innate and the adaptive one, which involve distinct cellular components. The innate immune system shows an acute response to foreign pathogens and comprises of cells which are pre-existent in the body, such as macrophages, NKs, DCs, mast cells and granulocytes. Neutrophils, a subcategory of the latter, secrete cytokines which further promote the immune response process (Cassatella 1999), whereas macrophages phagocytose dead cells and debris at the site of inflammation. Upon maturation, macrophages can polarize into either M1 or M2 macrophages and have pro- or anti-inflammatory properties, respectively (Hao, Lü et al. 2012). Within the tumour microenvironment, the two types of immunosuppressive macrophages that arise are the myeloid-derived suppressor cells (MDSCs) which can induce the suppressor T cells (Tregs) (Huang, Pan et al. 2006), and the tumour–associated macrophages (TAMs) which trigger T-lymphocyte cell death through the apoptotic pathway, among others (Saio, Radoja et al. 2001). The number of the circulating NK cells in the blood is positively correlated with longer survival without the incidence of metastasis, as these cells are known for their ability to eliminate their targets- including tumour cells, through the initiation of programmed cell death (Smyth, Hayakawa et al. 2002). DCs connect the innate with the adaptive branch, as upon activation they transform into APCs and in turn activate B- and T-cells (Janeway, Travers et al. 2005).

The adaptive immunity comprises of B- and T–lymphocytes and counteracts the foreign body that has prevailed over the innate response and poses a threat to the host. The T-lymphocytes are divided into subcategories based on the presence or not of CD8 or CD4 antigens on their surface. Both T regulatory and T helper lymphocytes

express CD4 on their surface, with the first being responsible for the restriction of autoimmune responses, and the latter being able to mediate $CD8^+$ cytotoxic T-lymphocytes activation and subsequent elimination of foreign pathogens. Moreover, $CD4^+$ lymphocytes can express cytokines which trigger B-lymphocytes and macrophages secretion. These capabilities allow $CD4^+$ immune cells to regulate responses across both the adaptive and the innate branch of the immune system (Janeway, Travers et al. 2001). B-lymphocytes are responsible for the creation of immune response memory, they are antibody secreting cells and activate the cascade once they detect a pathogen that has previously been encountered (Alberts, Bray et al. 1994).

As has previously been mentioned, chronic pancreatitis is often a pre–PDAC condition characterized by macrophages, mast cells and B- and T-lymphocytes infiltrating the pancreas and being linked to fibrosis, and by the presence of T-regulatory lymphocytes acting as immune suppressors in the circulation of patients. Common examples of the malfunction of the immune system in PDAC include absence or dysfunction of immune cells, presence of specific immune cells with tumour-promoting functions and the action of immune suppressive cells (Inman, Francis et al. 2014). *In vivo* studies in PDAC mouse models describe early stage immunosurveillance escape, with MDSCs and Tregs being the prevalent cell type. Inactivated $CD8^+$ cells and MDSCs are inversely correlated, underscoring the immunosuppressive role of the latter (Clark, Hingorani et al. 2007). Despite being undetectable in healthy human tissue, this cell type comprises almost 70% of the pancreatic stroma infiltrating leucocytes and is significantly increased in metastatic states compared to localised tumours, therefore relating to disease staging (Diaz-Montero, Salem et al. 2009, Porembka, Mitchem et al. 2012). $CD4^+$ Tregs comprise the largest population of T- lymphocytes in the tumour stroma as well as in blood and tissue specimens of patients (Liyanage, Moore et al. 2002) and their numbers are correlated with progression of premalignant states (such as PanINs and IPMN) to PDAC, metastasis, disease staging and survival (Inman, Francis et al. 2014). Cancer cells are shown to overexpress ligands for the Treg specific receptor CCR5, and interruption of this intercellular communication results in reduced tumour burden (Tan, Goedegebuure et al. 2009). In summary, although the functions of these immune cells are not considered as tumour promoting in a direct manner, they successfully attenuate a major obstacle to tumour progression (Dunn, Bruce et al. 2002). Cells that have a more immediate tumour promoting effect are mast cells detected in high numbers in PDAC, which express molecules known for instigating and sustaining

pancreatic tumour growth such as fibroblast and vascular endothelial growth factors (FGF and VEGF, respectively) (Yamazaki, Nagao et al. 1997, Tsuzuki, Carreira et al. 2001). Mast cells are selectively located at the boarder of the tumour, playing a key role in tissue degradation and invasion (Cai, Yang et al. 2011). Macrophages also support the tumour via secretion of cytokines, regulated upon activation normal T cell expressed and secreted (RANTES) and MMP-9 (Liou, Döppler et al. 2013). The main immune cell type exhibiting defects in the pancreatic cancer setting is the one of DCs, the maturation of which is repressed following exposure to tumour secreted interleukins (ILs), TGF-β and mucins. DCs in PDAC patients are not only found in low levels but are also defective in stimulating other immune cells (Inman, Francis et al. 2014). Similarly, NK cells have very limited activity in patients, reflecting the restriction imposed on the immune system (Aparicio-Pagés, Verspaget et al. 1991).

The major characteristic of PDAC is desmoplasia, a stroma which may even be vaster than the actual epithelial component of the tumour and consists of a number of different cell types such as stellate cells, fibroblasts, immune and endothelial cells as well as activated PSCs (Rasheed, Matsui et al. 2012). The latter have been shown to promote epithelial–to–mesenchymal transition (EMT) and express cytokines and growth factors (Erkan, Adler et al. 2012), while they prevent $CD8^+$ cells from reaching the tumour by physically adhering to them (Ene–Obong, Clear et al. 2013). Last but not least, PSCs and mast cells have been shown to be able to activate one another, therefore sustaining a feedback loop where the stromal environment supports immunosuppression and vice versa (Ma, Hwang et al. 2013).

Taking into consideration the complex immunosuppressive microenvironment of PDAC and the disease's highly demanding metabolic need, we performed a detailed analysis of the lipidome profile of PDAC cells and their respective exosomes and we found a surprising accumulation of CE specifically in the exosomes. A similar trend was not detected in the lipidome of prostate and ovarian cancer cells and their derived exosomes. In light of this finding, we decided to investigate the biological meaning of this CE accumulation, so we treated PDAC cells with the ACAT-1 inhibitor avasimibe and co-incubated $CD8^+$ and $CD4^+$ T cells with the derived exosomes, in order to see potential effects on proliferation and subsequent cytokine expression.

In a recent study, analysis of human pancreatic tissues revealed that CE is enriched in the malignant specimens compared to the normal ones, and more detailed Raman spectroscopy depicted CE18:1 and CE18:2 as the two more predominant species. Parallel examination of the ACAT-1 levels in BxPC-3, MiaPaCa-II, AsPC-1, PANC-1

and HPDE6 cell lines revealed higher protein expression in MiaPaCa-II and PANC-1 compared to the rest, with the predominant CE species being CE18:1 (Li, Gu et al. 2016). Indeed, according to our data the most abundant CE species in MiaPaCa-II and PANC-1.

Apart from CE accumulation, COH synthesis plays an important role in pancreatic cancer, however it has been shown that disruption of the biosynthetic pathway of COH did not deliver the expected beneficial results in PDAC (Corcos and Le Jossic-Corcos 2013). Indeed, a recent study targeting the cholesterol uptake rather than its synthesis, showed remarkable results. Pancreatic cancer cells overexpress the low-density lipoprotein receptor (LDLR) and its silencing -apart from reducing the COH uptake, resulted in lower intracellular CE levels and reduced proliferation and survival rates of PDAC cells via ERK1/2 inactivation, which most probably was a consequence of phosphatases activity rather than pathway inhibition. Last but not least, LDLR ablation also resulted in PDAC cells re-sensitisation to gemcitabine (Guillaumond, Bidaut et al. 2015). Cellular COH content has also been shown to affect immune cells (Molnár, Swamy et al. 2012) and manipulation of the CE content plays a big role as well. Upon activation $CD8^+$ T cells increase their intracellular and membrane COH content, as well as their ACAT-1 levels. Interestingly, ACAT-1 downregulation both pharmacologically or genetically instigates $CD8^+$ T cells proliferation specifically, and enhances their antitumorigenic properties, resulting in increased cytotoxicity due to the formation of a more robust immunological synapse, and higher expression of interferon-γ (IFN-γ). At the same time, ACAT-1 depletion was accompanied by increased COH biosynthesis, which added to the antitumorigenic phenotype (Yang, Bai et al. 2016). Similar effects were observed in later studies with chimeric antigen receptor-modified T cells (CART cells) (Zhao, Li et al. 2018).

Apart from exosomes, cytokines are also key players of intercellular communication, and in our study we examined a panel of inflammatory cytokines. Interferons comprise of three family types and IFN-γ belongs to type II; the only one involved in the immune reactions in case of cancer occurrence (Shankaran, Ikeda et al. 2001). It is comprised of two subunits and it can fortify downstream signalling cascades by binding to two receptors at a time, and possibly stimulating receptors of IFNs type I (Ealick, Cook et al. 1991, Takaoka, Mitani et al. 2000). IFN-γ expression is stimulated by other cytokines such as IL-18 and mitogenic signals, while it can be produced by different cell populations including T cells and NK cells, and to a smaller scale by APCs and B cells. The main pathway through which it exerts its functions is the JAK/STAT, where

ultimately STAT1 activates interferon-stimulated genes (ISGs) which contain special sequences in their promoters, namely gamma-activated sites (GAS). An example of such a gene is the interferon-regulatory factor 1 (IRF1) which controls the interferon-stimulated response elements (ISREs) and therefore a wide range of cell death-related cascades. This underscores the crucial role of STAT1 in IFN-γ–mediated responses, and how STAT1-deficient tumours can evade immune surveillance. Often tumour cells manage to avoid immune surveillance by reducing their responsiveness to IFN-γ signalling and activating relevant inhibitory molecules and alternative pro-tumorigenic pathways (Castro, Cardoso et al. 2018)**.** Although IFN-γ is known for its anti-tumorigenic properties, studies have shown that it can also exert the exact opposite function. Late stage ovarian cancer patients receiving IFN-γ in combination with chemotherapy showed markedly shorter overall survival time, compared to those receiving chemotherapy alone, during a phase III clinical trial (Alberts, Marth et al. 2008). This unexpected effect can be explained by the results of a recent study reporting that $CD8^+$ T cells-secreted IFN-γ induces PD-L1 expression on the surface of ovarian cancer cells (Abiko, Matsumura et al. 2015). Similar results were also obtained in cases of gastric cancer, where also the implication of the JAK/STAT pathway was confirmed (Mimura, Teh et al. 2018).

Interestingly, in our case we observed that co-incubation with avasimibe-exosomes reduced the levels of IFN-γ released by T cells. However, it has to be noted here that in the aforementioned studies T cells were directly treated with the drug, whereas in our case we co-incubated T cells with exosomes released from avasimibe-treated PDAC cells, so the mechanism of action might be different. A connection between cholesterol metabolism and IFN-γ signalling was recently established through studies on Helicobacter pylori (H pylori), which can surprisingly reside in the gastric environment despite the host's inflammatory response against it. Through the Cgt enzyme, H pylori can deplete the host's cells from COH and redistribute it in its own membrane. The attacked cells suffer lipid rafts disruption and since lipid rafts serve as assembly sited for the two subunits of IFN-γ, the downstream signalling involving JAK/STAT1 is abolished (Morey, Pfannkuch et al. 2018). IFN-γ has also been shown to increase ACAT-1 expression and therefore promote CE accumulation, and at the same time prevent COH efflux (Panousis and Zuckerman 2000). This ACAT-1 upregulation is achieved via IFN-γ–dependent STAT1 dimerization, nucleus translocation and binding to the a GAS site present in the ACAT-1 promoter region (Yang, Duan et al. 2001). Given this information, we can propose that by increasing IFN-γ secretion, MiaPaCa-II exosomes-treated $CD4^+$ T cells able to increase ACAT-

1 levels and therefore CE levels in neighbouring cells. CE and COH have a bi-directional relationship and increase in CE would automatically mean COH increase, which would result in higher cell membrane rigidity and inhibition of proliferation. Of course, CE accumulation has a profound effect on the exosome-treated T cell itself; our preliminary results show that MiaPaCa-II and PANC-1 exosomes reduce T cell proliferation compared to non-treated controls, an effect which seems to be reversed upon avasimibe treatment. Moreover, it is known that IL-18 stimulates IFN-γ production (Nakamura, Okamura et al. 1989), and our results for the $CD4^+$ cells and the effects of the MiaPaCa-II- exosomes (treated / non-treated) are in accordance to this.

IL-18 is constitutively expressed by PDAC cells and is considered to exert anti-tumorigenic functions. It is initially expressed as a biologically inactive molecule which becomes active upon caspase-1 action. The chemotherapeutic drug 5-fluorouracil (5-FU) induces apoptotic cascades in pancreatic cancer cells, leading to the release of the mature IL-18 molecule (Carbone, Rodeck et al. 2005). The mature IL-18 can be bound and therefore regulated by the IL-18 binding protein (IL-18BP), which is possible to interfere with the molecules anti-tumorigenic effects. Nevertheless, this seems to not be the case as IL-18BP was found to be expressed in lower levels in malignant tissue compared to healthy one, which can be seen as a way of protecting the pancreas from a potential IL-18 induced inflammation with severe effects. However, elevated levels of this cytokine in PDAC patients' serum is linked to unfavourable survival outcome. Patients undergoing therapy with gemcitabine, which has minimal impact on the IL-18 levels, exhibited more prolonged survival compared to patients receiving gemcitabine in combination with 5-FU or oxaliplatin. This might be explained by the fact that IL-18 can instigate invasion via MMPs production and ECM degradation, and also target Fas-expressing immune cells by upregulating its respective ligand on tumour cells (Carbone, Vizio et al. 2009). Whereas systemic and sole IL-18 elevation is pro-tumorigenic, local release by CART cells has been shown to be accompanied by IL-12 expression and support the function of T cells (Vidal-Vanaclocha, Mendoza et al. 2006, Chen, Rui et al. 2012). In addition to this, IL-18 is shown to increase the numbers of B regulatory cells- a population which is immunosuppressive and keeps a fine balance between healthy states and autoimmune diseases or even malignancy, and at the same time promote PD-1 expression on their surface. A combined inhibition of this cytokine and the PD-L1 / PD-1 axis showed promising results in regards to *in vivo* pancreatic tumours inhibition, and has been proposed as an appealing therapeutic target (Zhao, Shen et al. 2018).

IL-8 expression has been shown to enhance the proliferation of pancreatic and liver cancer cells and mediate the enhanced expression of sialyl-Lewis antigens on their surface, allowing them to adhere to cells of endothelial origin and therefore establish a dissemination route (Miyamoto, Shimizu et al. 1998). Its receptor, the C-X-C chemokine receptor type 1 (CXCR1), is a GPCR found to be overexpressed in PDAC and has been correlated with cancer hallmarks such as invasion and metastasis, as well as chemoresistance. In addition to those, its correlation to cancer stemness was recently demonstrated, and it has been linked to sphere formation, metastasis to the lymph nodes and poor survival. Those effects were shown to be reversible upon blockade of the receptor (Chen, Fan et al. 2014). The IL-8 mRNA is known to be stabilized by a protein complex comprised of HuR and the cellular retinoic acid binding protein II (CRABP-II), of which the latter is significantly elevated in PDAC cases. This stabilization sustains the expression of MMPs, leading to lymph node metastasis (Yu, Parameswaran et al. 2017). Interestingly, E-LDL uptake has been shown to increase the cellular CE content as well as the IL-8 expression in endothelial cells (Suriyaphol, Fenske et al. 2002), and in agreement to those findings, we observed a dose-dependent reduction in IL-8 concentrations in both $CD4^+$ and $CD8^+$ T cells upon treatment with exosomes derived from MiaPaCa-II and PANC-1 avasimibe treated cells, supporting a link between CE and IL-8 levels.

IL-23 is upregulated in a number of tumours and supresses immune surveillance. It has been shown to inhibit $CD8^+$ T cell infiltration and upregulate MMP9, therefore mediating invasion. Its inhibition reverses these phenomena, and *in vivo* data show that tumours are unable to grow in hosts lacking either IL-23 receptors or being incapable of generating this cytokine endogenously (Langowski, Zhang et al. 2006). It has been speculated that its mechanism of action could be via IL-12p70; downregulation of the first coincides with upregulation of the latter, subsequent IFN-γ production and enhancement of the NK cells cytotoxic activity (Teng, Andrews et al. 2010). The fact that $CD8^+$ T cells co-incubated with avasimibe-treated PANC-1 exosomes dose-dependently decrease the levels of their secreted IL-23 can have potential clinical impact, as it can restore the capacity of cytotoxic lymphocytes to infiltrate the tumour and eliminate it.

The monocyte chemoattractant protein-1 or otherwise known as C-C Motif Chemokine Ligand 2 (MCP-1 or CCL2, respectively) was the only one among 25 cytokines found to be specifically elevated in PDAC patients who had cachexia

manifestation and was proposed to be further examined for its biomarker potential (Talbert, Lewis et al. 2018). In our case, MCP-1 was the only cytokine that gave a statistically significant difference between different treatments in $CD4^+$ T cells.

CHAPTER 4

Chapter 4: Targeting PDK1 in pancreatic cancer

This chapter is displayed in the form of three peer-reviewed articles

Publication 2

Emmanouilidi, A., & Falasca, M. (2017). 3-Phosphoinositide-Dependent Kinase 1 (PDK1). *Encyclopedia of Signaling Molecules*, 1-4.

Publication 3

Emmanouilidi, A., & Falasca, M. (2017). Targeting PDK1 for chemosensitization of cancer cells. *Cancers*, *9*(10), 140.

Publication 4

Emmanouilidi, A., Fyffe, C. A., Ferro, R., Edling, C. E., Capone, E., Sestito, S., ... & Maffucci, T. (2019). Preclinical validation of 3-phosphoinositide-dependent protein kinase 1 inhibition in pancreatic cancer. *Journal of Experimental & Clinical Cancer Research*, *38*(1), 191.

4.1 Articles synopsis

4.1.1 Background

The 3-phosphoinositide-dependent kinase-1 (PDK1) is comprised of an N- and C-terminal domain, with the latter being the pleckstrin homology (PH) domain responsible for phosphatidylinositol lipids binding. PDK1 is translocated to the plasma membrane upon PI3K activation, and activates Akt. A Ser241 *trans*-autophosphorylation allows for the proteins' constitutive activation. Aberrant PDK1 expression facilitates cancer occurrence; the protein is known for its involvement in cancer cells invadopodia formation and its control over collective and mesenchymal cell invasion. PDK1 is also involved in chemoresistance in acute myeloid leukaemia, ovarian and breast cancer among others, and it is able to exert its functions in a PI3K/Akt independent manner, via different pathways including the PDK1-PLK1-MYC, PDK1-YAP/Hippo and PDK1-SGK axes. The aim of the following study was to decipher the mechanism of action of PDK1 in pancreatic cancer.

4.1.2 Experimental design in brief

A panel of PDAC cell lines was selected for the pharmacological inhibition and genetic downregulation of PDK1, and its subsequent effect on anchorage-dependent and –independent growth as well as the downstream signalling pathway were assessed. The impact of a PDK1 inhibitor on tumour volume was tested *in vivo* and a simultaneous inhibition of PDK1 and the PI3K isoform p110γ was carried out *in vitro* in order to see if proliferation was affected.

4.1.3 Results in brief

Both types of PDK1 downregulation efficiently reduced PDAC cell growth *in vitro*, compared to non-malignant epithelial cells and/or untreated controls. A PDK1 inhibitor, namely MP7, was able to restrict the growth of the tumour in xenografts, without exhibiting adverse effects. PDK1 inhibition was shown to reduce Akt and FoxO1/FoxO3a phosphorylation, and interestingly it also suppressed SGK3 and NDRG1 phosphorylation, indicating a role of the PDK1/SGK3 axis in PDAC. SGK3 downregulation resulted in reduced cell numbers, suggesting that a part of the PDK1 effect on PDAC growth is exerted through SGK3. Finally, inhibition of both PDK1 and p110γ showed enhanced potential in inhibiting PDAC cell growth, compared to administration of each treatment solely.

4.1.4 Concluding remarks in brief

Given the crucial role of PDK1 in cell homeostasis, any disruption could have severe effects. KRAS being an undruggable target makes PDK1 is an appealing alternative; nevertheless an inhibitor is yet not clinically available. We demonstrated that PDK1 hampers PDAC growth *in vitro* and *in vivo*, partly through its downstream effectors SGK3 and NDRG1. Moreover, combinations of PI3K p110γ and PDK1 inhibitors at suboptimal concentrations exhibit greater potential in inhibiting PDAC cell growth.

Publication 2

1R20

▶ Regulator of G-Protein Signaling 1 (RGS1)

23 kDa Photoreceptor Cell-Specific Protein

▶ Recoverin

27/28 kDa Stress Protein

▶ HspB1

2A9

▶ S100A6

30 kDa Adipocyte Complement-Related Protein

▶ Adiponectin

3-Phosphoinositide-Dependent Kinase 1 (PDK1)

Aikaterini Emmanouilidi and Marco Falasca
School of Biomedical Sciences, Metabolic Signalling Group, Curtin University, Faculty of Health Sciences, Perth, WA, Australia

Synonyms

3-phosphoinositide-dependent protein kinase 1; PDK1; PDPK1; PDPK2P; PRO0461

Historical Background

The 3-phosphoinositide-dependent kinase-1 (PDK1) is a well-studied member of the AGC kinase subfamily which is implicated in many physiological functions and when dysregulated can play a role in cancer, among other pathological conditions. The AGC kinases can act on tyrosine (Tyr), serine (Ser), or threonine (Thr) residues of their substrates and comprise of 60 members, for most of which there are a number of splice variants isoforms. For the majority of the AGC kinases, phosphorylation of the T-loop in the catalytic domain and the hydrophobic motif in the non-catalytic region leads to activation of the kinase. Apart from these two highly conserved motifs, the turn motif can also be phosphorylated in several members of the ACG kinases family. In regard to interaction with their substrates, the specificity of the kinases is determined by the residues in the peptide binding site. PDK1 specifically contains a 100 amino acids long pleckstrin homology (PH) domain which binds to phosphatidylinositol lipids. Generally, AGC kinases can be activated by various extracellular stimuli (Calleja et al. 2014).

PDK1 Structure and Function

The origin of PDK1 is estimated 2.3 billion years ago and its maintenance and conservation throughout the eukaryotic species suggest that it is crucial for survival. Indeed, PDK1 knock-out mice are not viable and die during the embryonic development. The actual PDK1 protein is 556 amino acids long and is comprised of two main domains: an N-terminal kinase domain and a C-terminal pleckstrin homology (PH) domain. The kinase domain itself can be subdivided into an N-terminal and C-terminal region comprising the PDK1-interacting fragment (PIF)-binding pocket and the T-loop (Gagliardi et al. 2015). PDK1 had initially been known for its role in glucose metabolism since, following the binding of insulin to its receptor and subsequent phosphoinositide 3-kinase (PI3K) activation, it would localize to the plasma membrane together with Akt and activate it. Akt would then inhibit via

phosphorylation the glycogen synthase kinase 3 (GSK3), and glucose storage would be facilitated (Cohen et al. 1997).

PDK1 Subcellular Localization and Activation and Deactivation Sites

PDK1 is characterized as a master regulator of numerous AGC kinases and understanding how the kinase itself is regulated, as well as how it interacts and modulates its substrates both spatially and temporally, is of great interest. PDK1 is one of the major components through which the PI3K/phosphatase and tensin homologue deleted on chromosome 10 (PTEN) signaling pathway regulates various cellular processes. PDK1 is found to be constitutively active within the cell due to a Ser241 *trans*-autophosphorylation in the activation segment residue, and it has also been found in the nucleus, with phosphorylation on a noncatalytic site (Ser396) inhibiting the nuclear export regardless of growth factors stimulation. Different mechanisms contribute to the distribution of PDK1 within the cells, including anchoring of the protein to the cytosol by binding to soluble inositol phosphates, and over time various cases of acute PDK1 regulation have emerged, such as homodimerization and subcellular localization. For this kinase, phosphorylation on Ser/Ther as well as Tyr sites has been reported. Among major PDK1 phosphorylation sites are Ser 25, 241, 393, 396, and 410, but only Ser241 is essential for PDK1 to exert its activity. Interestingly, this particular site is also not accessible by phosphatases and thus it is resistant to protein phosphatase 2A (PP2A) activity. Notably, although Ser241 autophosphorylation is essential for PDK1 activation, it does not suffice; full activation is accomplished upon Thr513 *trans*–autophosphorylation within the PH domain, in the simultaneous presence of phosphatidylinositol 3,4,5 trisphosphate (Ptdlns(3,4,5)P3). This highlights the regulatory role of the PH domain – Ptdlns(3,4,5)P3 interaction in the activation of PDK1. Other PDK1 worth-mentioned phosphorylation sites are Ser160, which is PI3K-dependent and stabilizes the protein's active conformation, Ser501, which is PKCθ-dependent and abolishes the proteins' activity upon platelet-derived growth factor or insulin stimulation, and, last but not least, Thr354 and Ser398 and 394 which in cooperation decrease PDK1 activity. The proximity of these negative-regulating phosphorylation sites to formerly described activation sites leads to the notion that there is a mutual exclusion governing positive and negative regulative mechanisms.

Apart from phosphorylation, another way of enhancing and stabilizing PDK1 activity is binding to regulators or other substrates. For instance, the interaction between Src protein and PDK1 gets stabilized by the binding of heat-shock protein 90 (HSP90) to the latter, which furthermore increases the kinase's activation. Notably, both HSP90 and PDK1 are found in elevated levels in various cancers, leading to the conclusion that stabilizing PDK1 is very important in the cancer context (Calleja et al. 2014).

Apart from anchoring to the plasma membrane, PDK1 can be found predominantly in the cytoplasm but also in the nucleus, due to the existence of a nuclear export signal (NES). Retainment of PDK1 in the nucleus can be the result of Leu380/Phe383 mutation, and it is critical for tumorigenesis since, once in there, PDK1 inhibits the forkhead box O3A (FOXO3A) and the c-Jun N-terminal kinase (JNK) – thus promoting proliferation and survival, respectively, and increases intranuclear phospho-Akt. All these events are correlated with solid tumor progression and anchorage-independent growth in in vivo mouse models (Kikani et al. 2012).

PH Domain and PIF Pocket in PDK1 Function

Through its C-terminal PH domain which interacts mostly with Ptdlns(3,4,5)P3, PDK1 is able to localize at the plasma membrane together with Akt, in this way phosphorylating the latter at Thr308 in its activation segment, after it has changed conformation following interaction with Ptdlns(3,4,5)P3 or phosphatidylinositol 3,4-bisphosphate (Ptdlns(3,4)P2). In in vivo

models there have been cases where the activation segment of Akt was still phosphorylated even when PDK1 was mutated in a way that it could not interact with phosphoinositides, leading to the notion that there might be additional components facilitating PDK1-Akt communication, such as the five repressor element under dual repression-binding protein 1 (FREUD1) or the growth factor receptor-bound protein 14 (GRB14) (Pearce et al. 2010). GRB14 has been reported to modulate PDK1 compartmentalization together with the insulin receptor which is localized in the plasma membrane (King and Newton 2004). Via a docking site in the kinase domain, the PIF pocket, PDK1 is also able to interact with substrates lacking a PH domain, such as p70 ribosomal S6 kinase (S6K) and serum-and glucocorticoid-induced protein kinase (SGK) – once their hydrophobic motifs are phosphorylated, and also members of the protein kinase C (PKC) family. Thanks to these distinct mechanisms, PDK1 is able to coordinate the regulation of its downstream targets and activate them independently (Pearce et al. 2010). Interestingly, a novel PDK1 target that did not belong to the AGC kinases family was recently revealed. More specifically, a study demonstrated that phospholipase Cγ1 (PLCγ1) activation is controlled by PDK1, and experiments involving chemical inhibition of PDK1 as well as genetic silencing of both PDK1 and PLCγ1 demonstrated that the two molecules act on the same pathway and are a prerequisite for cancer cell invasion. Therefore, PDK1 can create a bridge between PI3K signaling pathway and PLCγ1, in which both PDK1 and PLCγ1 would translocate at the membrane following PI3K activation and form a protein complex (Arteaga et al. 1991; Sala et al. 2008; Maurer et al. 2009; Raimondi et al. 2012). Notably, both PLCγ and PDK1 are found to be overexpressed in breast cancer especially in case of metastatic disease.

PDK1 and Cancer Invasion

As aforementioned, dysregulation of the AGC kinases leads to pathologic conditions, and studies have reported PDK1 alterations in both genetic and protein levels in human malignancies. For instance, whole gene or specific locus amplification have been reported in breast (Maurer et al. 2009) and prostate carcinoma, respectively, and protein overexpression has been reported in melanoma, esophageal squamous cell carcinoma, and acute myeloid leukemia. Moreover, this protein is involved in different hallmarks of tumor invasion (Gagliardi et al. 2015). To begin with, PDK1 is involved in the formation of protrusive structures in cancer cells called invadopodia. It is the PI3K pathway that triggers the formation of these structures and more specifically the p110α subunit which activates the cascade through PDK1 and Akt. In fact, this pathway is not only pivotal for invadopodia formation but also for their functionality and ability to facilitate cancer dissemination by degrading the basal membrane and the extracellular matrix (Murphy and Courtneidge 2011; Yamaguchi et al. 2011; Kung et al. 2012). Secondly, it was shown to activate ROCK1 kinase and in that way modulating amoeboid invasion, in which case when cancer cells invade a healthy tissue they simply incorporate between the fibers instead of degrading the extracellular matrix (Sahai and Marshall 2003; Pinner and Sahai 2008). Finally, PDK1 is implicated in the so-called mesenchymal and collective type of cancer invasion through activating MRCKα. In this case, tumor cells degrade the extracellular matrix and adhere to its components (Gagliardi et al. 2014).

Summary

To conclude, PDK1 is a very old and highly preserved kinase, implicated in physiological and pathological conditions and with a crucial role in multiple signal transduction pathways. Recent findings suggest that PDK1 loss-of-function or impairment of its activity can restrain cancer progression and induce apoptosis, and interestingly, in vivo work on pancreatic ductal adenocarcinoma driven by an oncogenic K-Ras mutation showed that PDK1 conditional ablation could confer normal life expectancy. This

emphasizes the importance of the K-Ras/PI3K/PDK1 axis in this specific type of cancer and makes PDK1 an intriguing target for therapeutic intervention (Eser et al. 2013). Since K-Ras is considered to be an "undruggable" target, alternative ways of targeting this specific pathway have been explored, such as PDK1 inhibition. Another alternative way for impairing this axis is to target microRNAs. For instance, miR-375, which is upstream of many important oncogenes, has been found to be downregulated in many cancers including pancreatic cancer. Overexpression of this microRNA is shown to suppress the levels of transcription of *pdk1*, as well as its protein levels (reviewed in Ferro and Falasca 2014). Despite the research carried out up to date, there are still many aspects that need to be further elucidated, such as the precise spatial and temporal PDK1 regulation, as well as the development of effective and specific inhibitors.

References

Arteaga CL, Johnson MD, Todderud G, Coffey RJ, Carpenter G, Page DL. Elevated content of the tyrosine kinase substrate phospholipase C-gamma 1 in primary human breast carcinomas. Proc Natl Acad Sci USA. 1991;88(23):10435.

Calleja V, Laguerre M, de Las Heras-Martinez G, Parker PJ, Requejo-Isidro J, Larijani B. Acute regulation of PDK1 by a complex interplay of molecular switches. Biochem Soc Trans. 2014;42(5):1435.

Cohen P, Alessi DR, Cross DAE. PDK1, one of the missing links in insulin signal transduction? FEBS Lett. 1997;410:3–10. 1 The previous Datta Lectures were given by: F. Melchers 1997(1st, 1986), N. Sharon (2nd, FEBS Lett. 217 (1987) 145–157)C.

Eser S, Reiff N, Messer M, Seidler B, Gottschalk K, Dobler M, et al. Selective requirement of PI3K/PDK1 signaling for kras oncogene-driven pancreatic cell plasticity and cancer. Cancer Cell. 2013;23(3):406–20.

Ferro R, Falasca M. Emerging role of the KRAS-PDK1 axis in pancreatic cancer. World J Gastroenterol. 2014;20(31):10752.

Gagliardi PA, di Blasio L, Puliafito A, Seano G, Sessa R, Chianale F, et al. PDK1-mediated activation of MRCKα regulates directional cell migration and lamellipodia retraction. J Cell Biol. 2014;206(3):415–34.

Gagliardi PA, Di Blasio L, Primo L. PDK1: A signaling hub for cell migration and tumor invasion. BBA – Rev Cancer. 2015;1856(2):178–88.

Kikani CK, Verona EV, Ryu J, Shen Y, Ye Q, Zheng L, et al. Proliferative and antiapoptotic signaling stimulated by nuclear-localized PDK1 results in oncogenesis. Sci Signal. 2012;5(249):ra80.

King CC, Newton AC. The adaptor protein Grb14 regulates the localization of 3-phosphoinositide-dependent kinase-1. J Biol Chem. 2004;279(36):37518–27.

Kung M-L, Tsai H-E, Hu T-H, Kuo H-M, Liu L-F, Chen S-C, et al. Hepatoma-derived growth factor stimulates podosome rosettes formation in NIH/3T3 cells through the activation of phosphatidylinositol 3-kinase/Akt pathway. Biochem Biophys Res Commun. 2012;425(2):169–76.

Maurer M, Su T, Saal LH, Koujak S, Hopkins BD, Barkley CR, et al. 3-phosphoinositide–dependent kinase 1 potentiates upstream lesions on the phosphatidylinositol 3-kinase pathway in breast carcinoma. Cancer Res. 2009;69(15):6299–306.

Murphy DA, Courtneidge SA. The 'ins' and 'outs' of podosomes and invadopodia: characteristics, formation and function. Nat Rev Mol Cell Biol. 2011;12(7):413–26.

Pearce LR, Komander D, Alessi DR. The nuts and bolts of AGC protein kinases. Nat Rev Mol Cell Biol. 2010;11(1):9.

Pinner S, Sahai E. PDK1 regulates cancer cell motility by antagonising inhibition of ROCK1 by RhoE. Nat Cell Biol. 2008;10(2):127–37.

Raimondi C, Chikh A, Wheeler AP, Maffucci T, Falasca M. A novel regulatory mechanism links PLCγ1 to PDK1. J Cell Sci. 2012;125(Pt 13):3153.

Sahai E, Marshall CJ. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. Nat Cell Biol. 2003;5(8):711–9.

Sala G, Dituri F, Raimondi C, Previdi S, Maffucci T, Mazzoletti M, et al. Phospholipase Cγ1 is required for metastasis development and progression. Cancer Res. 2008;68(24):10187–96.

Yamaguchi H, Yoshida S, Muroi E, Yoshida N, Kawamura M, Kouchi Z, et al. Phosphoinositide 3-kinase signaling pathway mediated by p110α regulates invadopodia formation. J Cell Biol. 2011;193(7):1275–88.

3-Phosphoinositide-Dependent Protein Kinase 1

► 3-Phosphoinositide-Dependent Kinase 1 (PDK1)

4F2 Heavy Chain

► SLC3A2

Publication 3

cancers

MDPI

Review

Targeting PDK1 for Chemosensitization of Cancer Cells

Aikaterini Emmanouilidi and Marco Falasca *

Metabolic Signaling Group, School of Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth 6102, Western Australia, Australia; a.emmanouilidi@postgrad.curtin.edu.au
* Correspondence: marco.falasca@curtin.edu.au; Tel.: +61-089-266-9712

Academic Editor: Samuel C. Mok
Received: 21 September 2017; Accepted: 19 October 2017; Published: 24 October 2017

Abstract: Despite the rapid development in the field of oncology, cancer remains the second cause of mortality worldwide, with the number of new cases expected to more than double in the coming years. Chemotherapy is widely used to decelerate or stop tumour development in combination with surgery or radiation therapy when appropriate, and in many cases this improves the symptomatology of the disease. Unfortunately though, chemotherapy is not applicable to all patients and even when it is, there are many cases where a successful initial treatment period is followed by chemotherapeutic drug resistance. This is caused by a number of reasons, ranging from the genetic background of the patient (innate resistance) to the formation of tumour-initiating cells (acquired resistance). In this review, we discuss the potential role of PDK1 in the development of chemoresistance in different types of malignancy, and the design and application of potent inhibitors which can promote chemosensitization.

Keywords: PDK1; phosphoinositides; PI3K; chemotherapy; chemoresistance

1. Introduction

1.1. The Journey to PDK1 Discovery

The 3-phosphoinositide-dependent protein kinase 1 (PDK1) was identified in 1997, in a strenuous attempt by a number of research groups to elucidate the insulin signaling pathway. Around 1990 it had been observed that within seconds of the interaction of insulin with its receptor, the phosphoinositide 3-kinases Class 1A (PI3K Class 1A) would be recruited to the plasma membrane and would mediate the generation of phosphatidylinositol (3,4,5)-trisphosphate (PtdIns(3,4,5)P_3) from phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P_2). During the same period, protein kinase B (PKB/Akt) was discovered by a number of research groups and it was shown that within just one-minute following cell stimulation, insulin could trigger the activation of Akt in a PI3K-dependent manner. The phosphorylation motif of Akt present in its substrates was determined and many proteins were shown to possess it and get activated following insulin stimulation, however the theory that another kinase—part of the PI3K pathway was responsible for these phosphorylations was still being investigated. In 1996, the interaction between Akt and PtdIns(3,4,5)P_3 came to light, yet the activation mechanism of the first remained unknown. Studies showed that following insulin stimulation, Akt underwent phosphorylation at Thr308 (activation loop) and Ser473 (hydrophobic motif), with nonsynonymous substitution of either of the two leading to more than 85% reduction of Akt activation, and that membrane recruitment was required for phosphorylation of both residues (reviewed in [1]). It was not until 1997 that a protein responsible for Akt phosphorylation at Thr308 was detected, purified and cloned. This was the 3-phosphoinositide-dependent protein kinase 1 (PDK1), a protein which interacted with PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 through its pleckstrin homology (PH) domain and exhibited a

dramatic increase in its ability to activate Akt in the presence of these phosphoinositides. The necessity of these phosphoinositides lies in the facts that they change the configuration of Akt in a way that the Thr308 residue is much more approachable for PDK1, and that they enable the simultaneous localization of both PDK1 and Akt in the membrane, allowing for the first to phosphorylate the latter [2,3]. PDK1 was later shown to phosphorylate more members of the AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family, including the serum-and glucocorticoid-induced protein kinases (SGKs), the ribosomal protein S6 kinase beta-1 (S6K1) and the protein kinase C (PKC) (reviewed in [1]). The next step was to elucidate the mechanism behind the regulation of PDK1, which possesses five constantly phosphorylated Ser residues that remain unaffected by the action of insulin. One of them, Ser241, is a prerequisite for PDK1 activity and it seems like the protein gets auto-phosphorylated on that residue [4]. After many observations it was proposed that PDK1 is constitutively active and that phosphoinositides are responsible for converting its substrate into a form susceptible to phosphorylation by PDK1 [1].

1.2. Physiological Roles of PDK1 and Its Correlation with Malignancies

The physiological importance of PDK1 was clear in knockout mice which exhibited lethality at the second embryonic week (E9.5) [5]. Lethality at the embryonic stage was also shown in knock-in models with mutations of either the PDK1-interacting fragment (PIF) pocket [6] or the PH domain [7]. To overcome the issue of embryonic lethality, any interventions performed in the mouse models would now be tissue-specific. Muscle and liver PDK1$^{-/-}$ mice would only be viable for up to 4 months, and would suffer heart failure, and glucose intolerance followed by liver failure, respectively [8–10]. Pancreas PDK1$^{-/-}$ mice would be viable but diabetic [11], whereas the models created with conditional knock-in tissue-specific strategies would exhibit different phenotypes ranging from hyperinsulinemia, glucose intolerance and smaller body size, depending on the mutation type [12,13]. The studies performed on hypomorphic mice revealed a number of roles of PDK1, including the management of gastric acid secretion levels [14] and the stimulation of the Na^+/H^+ exchanger via the serum- and glucocorticoid-inducible kinase 1 (SGK1) and the consequent regulation of the transport of electrolytes in the intestine [15]. Decreased PDK1 activity leads to the distortion of amino acid transport in the jejunum and increased amino acid excretion in the urine, implying defective renal reabsorption and decreased transport of amino acids in the kidneys and the intestine.

The PDK1 protein is a member of the AGC kinases and it is encoded by the PDPK1 gene, located at 16p13.3. This specific locus amplification has been correlated with poor survival prognosis in breast cancer, and has also been detected in lung and prostate cancer [16,17]. In breast cancer in particular, PDK1 is the means by which upstream lesions such as *PTEN*, *PIK3CA* and *ERBB2* boost their signal output and reach to Akt, rendering the cells resistant to PI3K pathway inhibitors [18]. Increasing evidence suggests that PDK1 plays a pivotal role in cell migration [19], while it is able to control cell motility via ROCK1 and has a significant effect in three-dimensional (3D) environments [20]. It is interesting that whereas in monolayer cell culture the downregulation of PDK1 seems to have no effect, its inhibition in 3D environments decreases cancer cell invasion in an Akt-dependent manner, suggesting that this could be a target to counteract cancer invasiveness (reviewed in [19,21,22]). The PI3K pathway is often deregulated in malignancies and exhibits high PtdIns(3,4,5)P3 levels. Being part of the pathway PDK1 is also implicated in cancer, in the majority of the cases though it is the overexpression that leads to pathologic conditions, rather than mutations. For instance, increase in gene copy number and protein overexpression have been reported in breast cancer and acute myeloid leukaemia, among other malignancies [23]. During melanoma initiation, PDK1 is the intermediate for the PKC regulation by the tissue inhibitor of metalloproteinase-1 (TIMP1), and in later stages of progression and metastasis, it promotes resistance to *anoikis* [24]. In non-small cell lung cancer, glutamine shortage leads to the PDK1/Akt axis activation, which in turn promotes metastasis [25]. Tissue sample analysis of patients with hepatocellular carcinoma who underwent surgical resection but where not submitted to any other type of therapy, revealed that the PDK1 mRNA levels were

the most potent factor regarding time to recurrence (TTR) prediction and were also correlated with decreased overall survival rate [26].

Due to the great matter of attention drawn to PI3K and Akt as the major molecules in order to target many aspects of cancer, the role of PDK1 in cancer has been overlooked. However, the ability of the latter to act independently of them two, has revamped the focus on this molecule and its pharmacological targeting [7]. Indeed, the ability of PDK1 to drive tumorigenesis in an Akt-independent manner has been recently demonstrated. More specifically, Vasudevan et al. demonstrated that in the context of existing *PIK3CA* mutations, Akt activity is deficient and SGK3 serves as the main PDK1 effector [27]. Mutations in *PIK3CA* seem to be a late event in tumorigenesis, and they mostly result in increase of the kinase activity, allowing for *PIK3CA* to act as an oncogene. Two significant hotspot mutations of *PIK3CA* occur in the helical domain in exon 9 (E545K) and the kinase domain in exon 20 (H1047R) [28,29]. Cells harbouring such *PIK3CA* mutations exhibit higher levels of phosphorylated PDK1, and these specific mutations seem to partially control the recruitment of PDK1 on the membrane. This can be attributed to a function that is not related to the kinase activity of PIK3CA; for instance maintenance of adaptor-proteins that facilitate membrane recruitment of PDK1 irrespectively of the PH domain. The *PIK3CA* mutant cells highly depend on SGK3 for their survival, and it is possible that PI3K exerts its signaling through the endosomes, where SGK3 localizes and subsequently engages PDK1. The study concluded that tumours with *PIK3CA* mutations exhibit Akt dependency when there is manifestation of defective feedback regulation or phosphate and tensin homolog (PTEN) signaling, whereas they exert Akt- independent signaling and they recruit SGK3 when such deficiencies are absent [27]. In addition to this, another study showed that although breast cancer cells growth is independent of PDK1, their ability for tumour initiation in vitro relies on it, regardless of their PIK3CA burden. *p53* and *K-RAS* mutated cells are more prone to inhibition of PDK1 than *PIK3CA* mutated ones, but their growth in 3D conditions remains uninfluenced by Akt inhibition, in contrast to the ones with mutated *PIK3CA*. Kinase activity of the molecule is a prerequisite for 3D growth, but the same does not apply for the PH domain, thus leading to the conclusion that Akt is not an effector molecule in the tumorigenic activity of PDK1. In line with this, constitutively active Akt was not able to redeem for PDK1 loss in regards to 3D growth and its chemical or genetic inhibition did not alter the PDK1 hyperactivation outcome [30].

Lately, many cases have been reported where PDK1 is directly or indirectly implicated in chemoresistance, with its inhibition resulting in re-sensitization of cancer cells to chemotherapeutic agents, and this is going to be the topic of discussion in the following sections.

2. PDK1 in Chemoresistance

2.1. Ovarian Cancer

Ovarian cancer has a high mortality among women and the PI3K pathway has been shown to be the main pathway exhibiting aberrant expression and deregulation, affecting the progression of the disease [31]. The importance of PDK1 in ovarian cancer is underscored by an immunohistological analysis of the expression of the peroxisome proliferator-activated receptor β (PPARβ) and PDK1 in healthy ovary tissue, benign tumours and many malignant subtypes. In this analysis, although PPARβ staining was positive in healthy and benign conditions, PDK1 was absent in 90% of the healthy tissue specimens. Only one healthy specimen and less than half of the benign ones exhibited weak PDK1 expression, whereas all malignant as well as borderline tumours were stained positively. These findings strengthen the notion of the oncogenic capacity of PDK1 in ovarian cancer [32]. Very recently, Moxley et al. demonstrated that PDK1 is implicated in ovarian cancer via an alternative splicing product of the Ron receptor tyrosine kinase, named short-form Ron (sfRon) [33]. This protein is absent from normal ovarian tissue but is strongly expressed in malignant ovarian tumours, especially in one of the most lethal types, the high-grade serous one. Overexpression of sfRon in OVCAR3 cells resulted in a more aggressive phenotype both in vitro and in vivo, induced epithelial-to-mesenchymal transition

(EMT) reflected by vimentin and N-cadherin upregulation and E-cadherin reduction, and instigated the PDK1 signaling pathway, which was depicted by increase of pSer241-PDK1 and pThr308-/pSer473 AKT [33]. In agreement to the group's previous studies, this model revealed that sfRon was dependent on PI3K signaling to induce EMT, one of the most crucial steps of the pathway being plasma membrane recruitment of PDK1, and this PI3K signaling effects were AKT/mTOR-independent [34]. Notably, PDK1 has previously been held accountable for EMT initiation in gallbladder cancer [35]. The fact that OVCAR3-sfRon cells had a very firm PDK1 expression compared to the absence of the protein's expression in control OVCAR3 cells, reflects the importance of PDK1 in driving aggressiveness in ways that are not correlated to AKT, and that focus should be given on testing inhibitors of PDK1 alone or in combination with Ron inhibitors, especially in this type of ovarian cancer [33].

Epithelial ovarian carcinoma (EOC), one of the most dismal malignancies among women, exhibits chemoresistance via increase of phosphorylated Akt levels, which is achieved by stabilization of the PDK1 protein by the collagen type XI aplha1 (COL11A1). COL11A1-PDK1 binding sequesters the latter and protects it from degradation via the ubiquitin (Ub)-proteasome pathway (UPP), which is normally induced by paclitaxel and cisplatin, thus conferring resistance to chemotherapy [36]. COL11A1 is physiologically known to be essential for the cartilage, since its presence allows for differentiation of the chondrocytes and formation of the collagen fibrils [37]. Its mRNA levels are significantly elevated in chemoresistant EOC cell lines, compared to chemosensitive ones and is found to play a role in paclitaxel and cisplatin resistance. Studies on A2780CP70 (cisplatin-resistant) and A2780 (cisplatin-naïve) cells indicated that both paclitaxel and cisplatin- but not doxorubicin or gemcitabine, induced COL11A1 levels dose- and time-dependently, whereas no similar activity was observed with the naïve cell line. In parallel, the genetic modification of *COL11A1* had a clear impact on the half maximal inhibitory concentration (IC_{50}) of the chemotherapeutic drugs, as its knockdown increased the responsiveness of the A2780CP70 cells to cisplatin and paclitaxel, and its overexpression in the naïve cell line decreased it. Further experimental work revealed that both drugs triggered the interaction between the *COL11A1* promoter and the CCAAT/Enhancer Binding Protein Beta (c/EBPβ) [36]. Interestingly, the Akt pathway increases the c/EBPβ expression [38], and chemical inhibition of the phosphatidylinositol 3-kinase (PI3K) inhibits the aforementioned interaction and promotes chemosensitivity in A2780 cells. Notably, genetic downregulation of *COL11A1* was found to attenuate the expression of the Akt pathway components such as phospho-Akt and PDK1, which led to the notion that COL11A1 increased the stability of PDK1 protein. Indeed, *COL11A1* silencing promoted PDK1 degradation through the proteasome pathway and immunoprecipitation assays indicated that both cisplatin and paclitaxel strengthened the COL11A1-PDK1 binding, whereas PDK1 knockdown in COL11A1- overexpressing cells was able to reduce c/EBPβ and phospho-Akt levels [36].

In the context of sensitivity to chemotherapeutic drugs, a specific PDK1 inhibitor termed 2-*O*-benzyl-myo-inositol 1,3,4,5,6-pentakisphosphate (2-*O*-Bn-InsP_5), had an additive effect in combination with rapamycin and was able to enhance the effect of paclitaxel on SKOV-3 cancer cells. This compound inhibited PDK1 with an IC_{50} of 26.5 nM and reduced phosphoThr308-Akt both in vitro and in vivo, while it was also capable of in vitro impeding mTOR in the low micromolar range, raising the question of whether it could be further investigated as a dual inhibitor [39].

A very interesting example of the redundant pathways adapted by cancer cells to promote their survival in different aspects was very recently demonstrated by Gocher et al. Previous studies have mentioned that Akt can be activated by Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CAMKK2) in ovarian, prostate and embryonic kidney cells, however Gocher et al. addressed the question of whether ovarian cancer resistance to platinum was a consequence of a combined action of CAMKK and the canonical PI3K pathway. Indeed, CAMKK2 was shown to regulate the phosphorylation of Akt at both the threonine and serine residues in ovarian cancer cells, and combined downregulation of the CAMKK2 and PDK1 proteins had comparable effects on Akt phosphorylation, as did pharmacological inhibition of CAMKK2 and PI3K. This provides significant information that CAMKK2 is able to sustain Akt phosphorylation when the PI3K pathway is inhibited. There could be a

number of explanations for the simultaneous existence of these two pathways for the regulation of Akt. One possibility could be that the low availability of growth factors in the hypoxic tumour environment was insufficient for complete activation of the PI3K pathway, so CAMKK2 was an alternative way to compensate and sustain tumour growth [40]. Alternatively, it is possible that growth factors exploit the calcium-dependent pathway to modulate the Akt activation levels, as in the case of phospholipase Cε (PLCε), explained in [41].

It is worth mentioning that in cell lines derived from serous epithelial ovarian cancer, Yes-associated protein 1 (YAP) acts as an oncogene implicated in various cancer characteristics. Transfection of cells with pSer147-YAP led to significant boost of their colony formation capacity, in contrast to cells transfected with wild type protein or a mutant with a defective WW domain and at the same time allowed these cells to surpass contact inhibition in monolayer culture and increased their invasion and migration potential in transwell assays. Moreover, pSer147-YAP shielded ovarian cancer cells against Taxol and cisplatin treatment, and in ovarian cancer patients there was an association between nuclear sequestration of the protein and reduced progression free survival (PFS) [42]. PDK1 plays a very crucial and fine-balanced role in pSer147-YAP related activities, which will be extensively discussed in Section 3.

2.2. Breast Cancer

Breast cancer is the most common cancer type affecting women worldwide and amplification of the *PDK1* genetic locus is correlated with low survival rates. One fifth of breast tumours exhibit *PDK1* amplification, with increase of the phosphorylated protein (pSer241-PDK1) levels being a frequent event in this malignancy, which is also present in more than three fourths of metastatic cases. Both pharmacological and genetic inhibition of PDK1 in vitro have demonstrated its significance for all steps of breast cancer progression, and in vivo experiments display its role in growth and metastasis [43]. Anti-oestrogen therapies apply on more than half of breast cancer cases, due to the expression of oestrogen receptor α (ERα) which automatically categorizes them as oestrogen signaling dependent. Therapy with tamoxifen is among the most widely used, however almost half of the patients with an early onset, as well as all patients with metastasis, develop resistance to it. In 2009, Iorns et al. performed both siRNA and compound screening to identify a molecular pathway as a good candidate for enhancing sensitivity to tamoxifen, with all findings pointing to the PDK1 pathway. More specifically, the nucleoside derivative triciribine was found to be a potent molecule- inhibitor of PDK1 and was used in further studies which suggested that counteracting the pathway in question could in fact sensitize breast cancer cells not only to tamoxifen but to also to other types of endocrine therapy such as fulvestrant (ICI 182780). Moreover, genetic silencing of *PDK1* in combination with tamoxifen result in G1 cell cycle arrest and upregulation of p21Cip1. This was the first study to demonstrate the suitability of PDK1 as a target towards restoration of sensitivity to tamoxifen [44]. Soon after this, the aforementioned compound 2-*O*-Bn-InsP$_5$, was also shown to reduce the survival of breast cancer cells in vitro in combination with tamoxifen, in a more effective manner than the two drugs did separately. The same was observed when it was administered in conjugation with curcumin and paclitaxel [39]. As for PDK1, there is a possibility that it is able to tune signaling cascades initiated by the oestrogen receptor (ER), thanks to its control over the forkhead box O (Foxo) (discussed in [45]).

In addition to these, OSU-03012, a derivative of celecoxib, a cyclooxygenase-2 (COX2) inhibitor, which accounts for PDK1 but not COX2 inhibition [46], managed to sensitize breast cancer cell lines to tamoxifen therapy independently of their ER status and the ER downstream pathway. OSU-03012 was able to counteract the tamoxifen-induced phosphorylation of Akt in MCF-7 cells, and to some extent in MDA-MB-231 cells, and restore cell responsiveness to therapy. It is also notable that daily treatment of MDA-MB-231 tumours in nude mice with a combination of tamoxifen and OSU-03012 resulted in 50% suppression of tumour growth [47]. This compound exerts its PDK1 inhibitory activity (IC_{50} of 5 μM) by competing the adenosine triphosphate (ATP) binding and in parallel it hampers $p70^{S6K}$ and Akt activation. Further details, as well as chemical structure and properties can be found in [46].

It is interesting that despite the fact that PDK1 is overexpressed in a number of related cancer cell lines and patients' samples [48,49], the current norm for treatment focuses on different components of the pathway, such as the epidermal growth factor receptor-2, and there is limited ongoing research on the evaluation of PDK1 as target. Meanwhile, elevated phospho-PDK1 was correlated to insusceptibility to gemcitabine-mediated cell death, compared to elevated phospho- Akt, and *PDK1* silencing rendered MCF-7 cells more responsive to the drug, than did Akt silencing. These observations highlight the suitability of PDK1 as a focus point in order to restore chemosensitivity, especially in regard to gemcitabine, in breast cancer cells [50]. The enhanced gemcitabine resistance conferred by PDK1 in contrast to Akt1, can be partially attributed to the fact that PDK1 acts relatively earlier in the pathway and its activation affects a wider range of molecules, including protein kinase Cα (PKCα) which has been reported to be overexpressed concomitantly with PDK1 and is implicated in gemcitabine resistance [51,52]. In addition to that, there is only one phosphorylation site within the activation loop that is required for PDK1 to become active, and the protein can be also found in a constitutively activated form. On the contrary, complete Akt1 activation is more complicated and requires for a series of phosphorylations to take place [50]. All together, these findings underscore the action of PDK1 via an Akt-independent mechanism.

A recent study concluded that PDK1 plays a role in triple negative breast cancer (TNBC) chemoresistance. More specifically, this type of cancer exhibits aberrant levels of fatty acid-binding protein 5 (FABP5) which through retinoic acid (RA) delivery is known to enhance the peroxisome proliferator-activated receptor β/δ (PPARβ/δ) at the transcriptional level and activate the vascular endothelial growth factor-A (VEGF-A) and PDK1. Curcumin was able to render TNBC cells sensitive to retinoid therapy, by restricting the FABP5 expression and reducing RA-induced *PDK1* transcription; with the exact mechanism connecting RA and PDK1 yet to be elucidated [53]. The effects of curcumin on breast, as well as pancreatic and prostate cancer cells have been shown to be enhanced when curcumin is combined with 2-*O*-Bn-InsP_5, a specific PDK1 inhibitor [39].

2.3. Acute Myeloid Leukaemia

Acute myeloid leukaemia (AML) is another type of cancer with dismal prognosis due to lack of responsiveness to chemotherapy and common relapse, accountable for which are leukaemia stem cells (LSCs). Upon leukaemia patients' chemotherapy, a small LSCs population that exhibited resilience and managed to survive, starts to proliferate and turns into the dominant population, paving the way for disease relapse [54]. The characteristics of LSCs such as quiescence, low division rate and resistance to therapy, state them an intractable target; yet a recent study indicated PDK1 as a LSCs survival regulator and thus an appealing target for therapy [55]. Almost half of AML sufferers with poor prognosis exhibit overexpression of PDK1 [56] and hematopoietic malignancies including AML often exhibit inactivation of PTEN and Akt activation, which is strongly correlated with poor prognosis. This Akt activation is in part due to aberrant regulation of other signaling pathways such as Bcl-2-associated death promoter (BAD) and *p53* [57–60]. As abovementioned, recent studies revealed that *PDK1* deletion not only had a positive effect on the lifespan of an MLL-AF9 mouse model and reduced LSCs incidence following secondary transplantation, but also significantly enhanced *p53* and *Bax* expression [55], both of which are known for their pro-apoptotic properties [61]. In the clinical setting, tumours with high levels of *p53* are considered more responsive to chemotherapy, whilst *Bax* downregulation is linked to both limited drug sensitivity and decreased survival [62,63]. Another interesting observation of the same study was that *PDK1* deletion was coincident with lower *Stat5* levels, raising the notion that this might be the main LSCs maintenance pathway [55], since Stat5 is constitutively active in many types of leukaemia [64].

2.4. PDK1 and Chemoresistance in Multiple Types of Cancer

Metadherin (MTDH) is an oncogene thought to affect many cancer related pathways such as PI3K/AKT and Wnt, and its downregulation was shown to increase the chemosensitivity of many

tumours such as prostate and breast cancer to 5-fluorouracil (5-FU), paclitaxel and doxorubicin; the exact mechanism though had remained unknown. A recent study came to unravel this mechanism of action, demonstrating that MTDH protects cancer cells by interfering with the cell cycle checkpoints and initiating pro-survival cascades. MTDH downregulation resulted in caspase-3 and -8 mediated endometrial cancer cell death, following treatment with tumour necrosis factor-α-related apoptosis-inducing ligand (TRAIL) and the LBH589 histone deacetylase (HDAC) inhibitor. MTDH depletion coincides with a decline in PDK1 phosphorylation which renders the cell prone to apoptosis via the invigoration of Bim expression. A protein of interest in this case is galectin-1, which has a strong presence in a number of tumour types and is implicated in the PI3K pathway. Knockdown of this protein was shown to avert PtdIns(3,4,5)P_3 increase following stimulation with insulin growth factor 1 (IGF1) in glioblastoma cells, and this study denoted that MTDH-induced galectin-1 increase might result in an increase of PtdIns(3,4,5)P_3 levels and activation of the PI3K cascade. Overall, these data exhibit that MTDH constitutes a target for more effective chemotherapy, with PDK1 and Bim playing key role in this procedure [65].

Whilst the constitutively active PDK1 does not undergo any further activation derived from mitogenic signals [3], its activity is modulated by interactions with other proteins-modulators; for instance the 14-3-3 protein which reduces its activity [66], the heat shock protein 90 (Hsp90) which protects it from the activity of the proteasome [67], and the tumour suppressor candidate 4 (TUSC4), which was found to be implicated in cancer cells chemosensitivity. More specifically, TUSC4 negatively regulates the PDK1 downstream cascade, as it is able to form a ternary complex with Src and PDK1 and attenuate the latter's Src-induced tyrosine sites phosphorylation; thus, hampering the S6K and Akt activation. Cells modulated to express TUSC4 were found to be more responsive to chemotherapeutic drugs including doxorubicin, taxol, cDDP and VP-16, than their control counterparts. This underscores the significance of PDK1 inhibition in overcoming resistance to anticancer drugs [68].

PDK1 can be also implicated in chemoresistance through another PDK1-binding protein, the tongue cancer resistance-related protein-1 (TCRP1), the levels of which are found to be increased in a number of cancers including pancreatic and ovarian ones [69]. TCRP1 is correlated with cisplatin resistance in lung cancer [70] and oral squamous cell carcinoma resistance to radiation [71]. Aberrant expression of this protein induces phosphorylation of PDK1 in a PTEN and PI3K-independent manner; TCRP1 binds to PDK1 via two amino-acid sequences (T109-A124 and R93-S107) and it is speculated to promote 14-3-3/PDK1 dissociation, leaving PDK1 in a monomer configuration that not only has the ability of auto-phosphorylation, but can also phosphorylate its downstream target, Akt. Nonetheless, further studies need to be carried out concerning the TCRP1-mediated PDK1 phosphorylation. TCRP1 was shown to induce cell transformation via PDK1 activation, and chemical (OSU-03012) or genetic downregulation of the latter reversed this phenomenon [69]. A summary of the aforementioned pathways where PDK1 is implicated can be found in Figure 1.

3. PDK1 Oncogenic Signaling in Chemoresistance: Beyond AKT

3.1. PDK1-PLK1-MYC Axis

Recently, there have been a number of reports showing that in some cancer cases PDK1 acts independently of the PI3K pathway to exert its oncogenic properties [27,72,73]. PDK1 has been shown to act through another route, that of Polo-like kinase 1 (PLK1)-MYC [74]. *MYC* is a well-studied oncogene, with its respective protein being involved in the ability of cancer cells, as well as stem cells, to self-renew [75], and was lately proved to be PDK1-dependent in order to induce HEK cells transformation. PDK1 triggers the phosphorylation of PLK1, which is also upregulated in many cancers, and the latter interacts with MYC, phosphorylates it and results in its accumulation in cancer cells (Figure 2). MYC-driven breast cancer is shown to be more responsive to PDK1/PLK1 inhibitors than is MYC-independent one, and taking into consideration that a MYC inhibitor is not currently available in the clinic, targeting the PDK1-PLK1-MYC axis reveals a new potential therapeutic

approach against MYC-induced cancers. It is also likely that chemoresistance will be affected as well, since inhibition of either PDK1 or PLK1 resulted in depletion of the $CD44^{+}/CD24^{-/low}$ stem cell-like populations in the MDA-MB-231 cell line, a phenomenon not observed when the PI3K-AKT pathway was impaired [74].

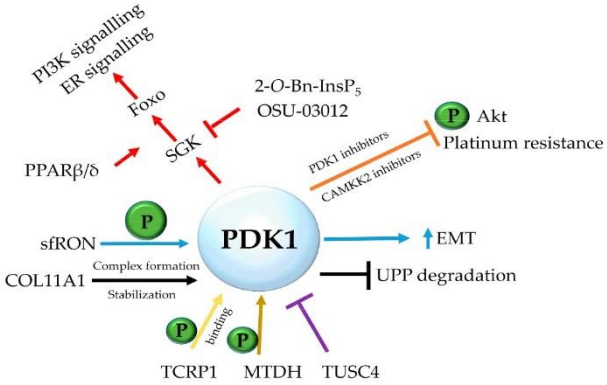


Figure 1. Major signaling pathways in chemoresistance and PDK1 implication. PDK1 is implicated in a number of signaling pathways and cancers. Complex formation with COL11A1 results in PDK1 stabilization and resistance to ubiquitin (Ub)-proteasome pathway degradation in epithelial ovarian carcinoma, its instigation by sfRON leads to EMT in high-grade serous ovarian cancer, whereas PDK1 inhibition combined with CAMKK2 inhibition leads to reduction of phospho-Akt levels and decreased platinum resistance in ovarian cancer. Sensitization of breast cancer cells to drugs such as tamoxifen and paclitaxel is correlated with the effect and sustaining of PI3K and ER signaling through SGK1/3 and Foxo1/3 isoforms, where components such as PPARβ/δ have a stimulating effect, and PDK1 inhibitors such as 2-*O*-Bn-$InsP_5$ and OSU-03012 have proven to be effective. In other types of cancer, proteins such as MTDH and TCRP1 are shown to directly or indirectly activate PDK1, and the tumour suppressor TUSC4 is shown to form a complex with it and negatively regulate the signaling cascade.

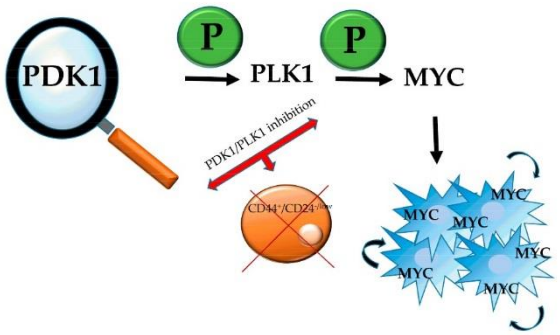


Figure 2. The PDK1-PLK1-MYC axis. PDK1, via PLK1 phosphorylation and subsequent MYC phosphorylation, results in the sequestration of the latter in cancer cells. MYC is known for its ability to promote self-renewal of cancer cells as well as stem cells, and therefore inhibition of either of the first two components of the axis leads to depletion of cancer stem-like cells.

3.2. PDK1-YAP/Hippo Pathway Axis

The Salvador/Warts/Hippo (SWH) pathway, alternatively named Hippo signaling pathway, was initially discovered and studied in Drosophila, where it coordinates organ size. The main core of the cascade is comprised of the tumour suppressor proteins Warts (Wts), Salvador (Sav), Hippo (Hpo) and Mob-as-tumour-suppressor (Mats) and mutations leading to loss of function of any of them results in an increased proliferation- or hippopotamus-like phenotype. The respective mammalian homologues are the Lats1/Lats2, Sav1 or WW45, Mst1/Mst2 and MOBKL1A/1B, which shape a conserved cassette that responds upon high cell density signals, and inactivates YAP by phosphorylation. More specifically, the kinase complex formed by Sav1 and Mst/HIPPO phosphorylates Lats kinase, which targets the Ser127-YAP and results in its restriction to the cytoplasmic compartment. Inactivation of the pathway leads in YAP nuclear translocation, where is acts as a transcriptional activator for genes related with proliferation (reviewed in [76]). This activation is mediated by TEA domain transcription factors (TEADs) and it has been demonstrated that the promoter of the connective tissue growth factor (CTGF) gene is bound by the YAP-TEAD1 complex in MCF10A and NIH-3T3 cells. In cancer cells overexpressing YAP, knockdown of either of the aforementioned complex components had a major impact on the CTGF mRNA levels, and knockdown of the CTGF itself abrogated cell growth [77]. In MCF10A serum starved cells which have reached the point of contact-inhibition, YAP is not detected in the nucleus, however this phenomenon is quickly reversed by epidermal growth factor (EGF) treatment. EGF acts via the Hippo pathway by inhibiting Lats, and therefore reduces Ser127 phosphorylation of YAP, promoting its recruitment in the nucleus and allowing it to exert its transcriptional activity. Treatments with inhibitors of PI3K, PDK1 and its downstream effectors revealed that it was only the first two kinases that were involved in YAP phosphorylation and nuclear accumulation, therefore it is the PI3K-PDK1 signal that links EGFR with the SWH pathway. Further experiments showed that signals unrelated to EGFR, such as horse serum or LPA, could initiate the PI3K cascade and cause nuclear accumulation of YAP, suggesting that signals upstream of this pathway are able to inhibit the Hippo cascade. PDK1 forms a complex with the core SWH pathway proteins (Sav1, Mst, Lats), which can be disrupted following EGF treatment. This dissociation can be prevented with the use of PDK1 and PI3K inhibitors. Analysis of the complex showed that since PDK1 lacks the domains needed to directly interact with all the components of the HIPPO pathway, Sav1 is the mediator molecule. Sav1 relates to PDK1 via its 145–162 residues and allows for the PDK1-Lats and PDK1-Mst interactions via its WW and SARAH domains, respectively. The proposed model connecting PDK1, YAP and EGF signaling was that in growth factor signaling absence, PDK1 is found in the cytoplasm in the form of a complex with the Hippo components, leading to phosphorylation and cytoplasmic retention of YAP. Cell stimulation with growth factors leads to plasma membrane recruitment of PDK1 and subsequent disruption of the complex, resulting to Lats inactivation, pSer147-YAP levels reduction and accumulation of the dephosphorylated protein in the nucleus, where it acts as a transcriptional activator for growth controlling genes [78] (Figure 3).

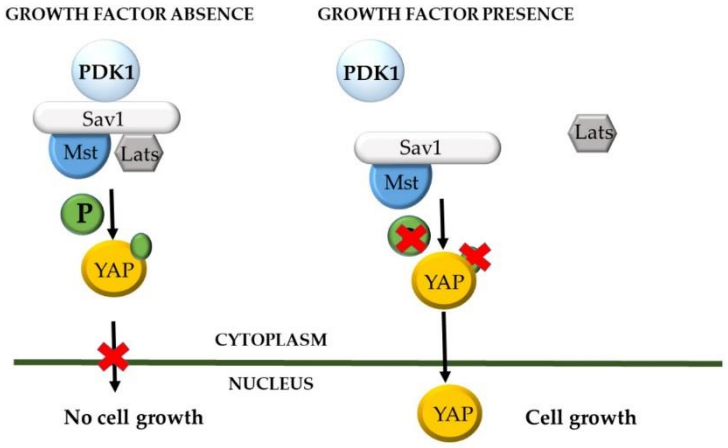


Figure 3. PDK1 and the Salvador/Warts/Hippo (SWH) pathway. When epidermal growth factor receptor (EGFR) signaling is active, PDK1 is recruited to the cell membrane and the core components of the HIPPO pathway are not forming a complex, thus YAP protein is in its inactive form and can insert the nucleus and act as a transcription factor for growth-promoting genes. On the opposite, in the absence of growth stimuli, a cytosolic complex is formed between the core proteins of the HIPPO pathway and PDK1, which phosphorylates YAP and renders it in the cytoplasm, excluding its nuclear entry.

3.3. *PDK1-SGK Axis*

Aberrant expression of the PI3K/AKT/mTOR pathway is a frequent phenomenon in breast cancer and it is the result of a PIK3CA mutation, which corresponds to the PI3K p110 catalytic subunit, and more specifically to isoform α. Regardless of the development of specific PI3Kα inhibitors, not all tumours are responsive and the molecular mechanisms sustaining this resistance need to be defined. Even upon complete PI3K/AKT inhibition, there is a remaining mTORC1 activity which allows cancer cells to overcome PI3Kα- inhibitors treatment [79–83]. The classic pathway by which PI3K triggers mechanistic target of rapamycin complex 1 (mTORC1) activity, is initiated by G-protein-coupled receptors (GPCRs) or RTKs. As mentioned in previous sections, AKT is phosphorylated by PDK1 but can only exert its full activity following phosphorylation by mTORC2. Once fully active, AKT can inhibit the tuberous sclerosis complex (TSC) by phosphorylating it in four serine and one threonine residues (S1132, S1130, S981, S939 and T1462). The TSC acts as a GTPase activating protein (GAP) for the Ras homolog enriched in brain (Rheb) GTPase, and its inhibition results in activation of the latter, which in turn activates mTORC1. Consequently, AKT phosphorylates the inhibitory subunit 40-kDa proline-rich (PRAS40) causing it to dissociate from the complex, rendering mTORC1 accessible to substrates (extensively reviewed in [84]). Yet, this seems to not be the case in tumours resistant to PI3Kα inhibition, as recent research has shown that PDK1 is a new player actively underpinning this resistance. Experiments using breast cancer cell lines carrying PI3Kα mutations and therefore resistant to the specific PI3Kα- inhibitor BYL719, revealed that the observed residual mTORC1 activity was PtdIns(3,4,5)P_3- and thus AKT- independent, and stands in need of both PIF-binding pocket and kinase activity of PDK1. Results from simultaneous PI3Kα and PDK1 inhibition using BYL719 and GSK2334470 suggested that resistant cells were acquiring a transcriptional activity dependent on forkhead box proteins O (FOXOs), and more specifically on FOXO3 which showed robust nuclear sequestration upon dual treatment [85]. While in the presence of growth stimuli the 14-3-3 proteins

restrain FOXO proteins in the cytoplasm, their cessation causes dephosphorylation and nuclear transport of the proteins in question, leading to expression of pro-apoptotic genes [86]. Despite the fact that FOXO1/3 have been identified as targets of AKT, its complete inhibition does not seem to result in the expected FOXO3 nuclear translocation in BYL719 resistant cells and so, researchers focused on the detection of a protein that would comprise a member of the AGC kinases, associate with the PDK1 PIF-binding pocket and be dependent on its catalytic activity, while it would concomitantly affect mTORC1 and FOXOs activity in an AKT unbiased manner. Transcriptomic analysis of a number of breast cancer cell lines with different BYL719-resistance status pointed out to SGK1, since the phosphorylation levels of its target N-Myc Downstream Regulated 1 (phosphoNDRG1) were significantly elevated compared to total protein in resistant cell lines [85]. Although SGK1 and pNDRG1 levels vary accordingly in vivo, and AKT has been shown to target NDRG1 in vitro [87,88] as well as in vivo in the case of mouse models with PDK1 K465E knock-in mutation [89], the significance of SGK1 in this mechanism was confirmed by the fact that pNDRG1 levels were not affected by BYL719 treatment in resistant cells. On the contrary, it was the combination of both the GSK2334470 and BYL719 inhibitors that sufficed to reduce this phosphorylation. The outcomes of this study showed that pharmacological targeting of SGK1 was a realistic and achievable objective and that complete inhibition of mTORC1 requires dual targeting of SGK1 and AKT. Apart from these, another novelty was the discovery that in fact it is the phosphorylation of TSC2 by SGK1 that is responsible for this residual mTORC1 activation. It should be kept in mind that although AKT and SGK1 may both be under the control of PDK1 and mTORC2, what makes a big difference is that AKT contains a PH domain which renders it dependent on plasma membrane recruitment, whereas SGK1 can be active even when PtdIns(3,4,5)P_3 is unavailable. The partial reduction in SGK1 activity that follows PI3Kα inhibition in resistant cell lines, can be explained by the fact that PtdIns(3,4,5)P_3 can affect mTORC2 in a mammalian stress-activated protein kinase interacting protein 1 (mSIN1)-dependent manner. There also exist other intracellular mTORC2 pools with ambivalent localization and different dependency on growth factors status, which can explain this observation [85].

Apart from SGK1, SGK3 also holds a key role in melanoma resistance to PI3K/Akt inhibition [90]. *BRAF* mutations, and especially $BRAF^{V600E}$, are an early and very frequent event in melanoma. This specific mutation promotes PTEN silencing to sustain the progress from a benign to a malignant state. It was recently shown that independently of their Akt and PTEN status, melanoma cells are prone to PI3K or $BRAF^{V600E}$ inhibition, and even more sensitive to a dual inhibition. Noticeably, mTORC1/2 inhibition was able to apprehend cell proliferation comparably to $BRAF^{V600E}$ or PI3K inhibition. It was further demonstrated that PI3K and $BRAF^{V600E}$ were able to control mTORC1 activity in an AKT-independent manner [91]. Following these studies, another group showed that SGK3 together with PDK1 are implicated in melanomas harbouring BRAF mutations and wild type PTEN, which in fact account for more than half the cases of melanomas occurrence. SGK3, being a substrate of PDK1, acts as its mediator, and inhibition of either of the two kinases leads to cell cycle arrest at G1 phase. Simultaneous inhibition of PDK1 and PI3K/mTOR or the proteasome exhibits synergism and has a higher impact on melanoma proliferation [90]. Due to the important role of Akt in the maintenance of cancer cells, it would be a natural consequence that following extended Akt or PI3K Class I inhibition, the cells would urge to recompense for that loss. Indeed, a very efficient strategy undertaken by malignant cells is the upregulation of SGK3, which has a high level of overlapping targets with Akt and can be activated by hVps34 in a PI3K Class I-independent manner. hVps34 acts by producing PtdIns(3)P, to which SGK3 can bind via its PX domain and be subsequently phosphorylated and activated by PDK1, and it is worth mentioning that SGK1 and SGK2 lack a PX domain and depend on PI3K for their activation. TSC2 phosphorylation and the resulting SGK3 and mTORC1 activation can comprise an Akt-independent cascade responsible for the occurrence of chemoresistance [92].

3.4. Inhibitors of PDK1

Since its discovery in 1997, PDK1 has attracted the interest of the research community and a number of patents have been established by different companies and institutions, including Merck & Co. (Kenilworth, NJ, USA), Boehringer Ingelheim International GmbH (Ingelheim am Rhein, Germany), GlaxoSmithKline (Brentford, UK), Novartis International AG (Basel, Switzerland), Biogen Idec Inc. (Cambridge, MA, USA), Sunesis Pharmaceuticals (South San Francisco, CA, USA), Wyeth (now Pfizer Inc., Manhattan, NY, USA), Ohio State University, University of London and University of Bath [93]. It is interesting that in a commentary article, Alessi—a leading scientist in the PDK1 research field—and Peifer specifically emphasize that "just because PDK1 is not on many researchers' radars does not mean it is not a key anti-cancer target" [45]. It is true that the research community focuses on different elements of the pathway such as mTOR and Akt, nevertheless inhibition of PDK1 specifically has an advantage over them, due to the fact that as a key regulator it controls an impressive number of other kinases including Akt, SGK and ribosomal S6K. Moreover, it is now well-established that PDK1 activates a unique signaling pathway distinct from the canonical Akt- and PI3K-dependent pathways. The fact that the protein exists in a single isoform makes it an even more attractive target for inhibition in malignant conditions [94]. An extensive overview on PDK1 inhibitors until 2008, explains in detail how the ATP binding site of PDK1 has been used as a scaffold to create the majority of the inhibitors and analyses the mechanism of action of all the known categories, from small molecule inhibitors (bisindolmaleimides, LY333531, LY317615, UCN-01, substituted thieno[3,2-*c*]pyridine-7-carboxamides, indolinones, pyridinonyl-PDK1 inhibitors, *N*- phenylpyrimidin-2-amines, 4-heterocycloalkyl-2-aminopyrimidines, diazepinones) to tetracyclic imidazophenanthrenones (imidazo[4,5-*c*]quinolones, pyrrole derivatives, quinazolines, celecoxib derivatives, 4-aryl-7-azaindoles, 3,5-diaryl-7-azaindoles, pyrrolo[2,3-*d*]pyrimidines, pyrazole[1,5-*α*] pyrimidines, triazolo[1,5-*α*]pyrimidines, pyrazolylbenzimidazoles, indazoles, dibenzo[c,f][2,7]-naphthyridines), 3-hydroxyanthranilic acid and activators/modulators of PDK1 [94]. Apart from the aforementioned PDK1 inhibitors, one more compound has been recently identified, the inositol 1,3,4,5,6-pentakisphosphate ($InsP_5$), termed 2-*O*-Bn-$InsP_5$ (Figure 4A), which exerts anti-tumour and pro-apoptotic functions, while at the same time renders cancer cells prone to chemotherapeutic drugs such as tamoxifen and curcumin. Its mechanism of action is proposed to take place through binding of the PH domain of PDK1, which results in retention of the latter in the cytosol, thus hampering phosphorylation and activation of Thr308 of Akt [39,95]. The antineoplastic effects of $InsP_5$ were validated in vivo, where administration of the drug in an ovarian cancer xenograft resulted in growth inhibition akin to cisplatin [96]. Moreover, derivatives of 2-oxindole (OXIDs) (Figure 4B), have been shown to disrupt the PDK1/Akt pathway and comprise good candidates for non-small cell lung cancer treatment [97], and these results initiated studies for the synthesis and assessment of new derivatives within the same family, which gave rise to promising candidates for the targeting of glioblastoma multiforme (GBM) [98].

Recently, a very potent PDK1 inhibitor termed GSK2334470 (Figure 4C) has been developed by GlaxoSmithKline and has shown remarkable specificity towards PDK1 ($IC_{50} \approx 10$ nM), when tested among almost a hundred protein kinases together with members of the AGC-kinase family. This compound was shown to inhibit the phosphorylation of the SGK T-loop and the NDRG1 protein as well as the endogenous S6K1 phosphorylation on the Thr229 of the T-loop, following IGF1 stimulation. Though it was less efficient in decreasing Akt activity compared to PI3K inhibitors, GSK2334470 had a great impact on components such as GSK3 and FoxO. This can be explained by the fact that both PDK1 and Akt contain PH domains which enable them to bind PtdIns(3,4,5)*P3* and co-localize on the surface of the membrane, remarkably increasing chances of interaction. Thus, even the slightest fraction of PDK1 escaping GSK2334470 inhibition would be sufficient to activate Akt, which is in agreement with the observation that cytosolic Akt lacking PH domain (ΔPH-Akt) was more potently inhibited that the full-length protein. Another notion is that PDK1 anchored to the plasma membrane is less accessible to the drug than the cytosolic one. It is important to remember that Akt controls a number of functions

crucial for the cells' survival, and its inhibition could have severe side effects. Since there are also other pathways driving tumorigenesis, which are independent of Akt but require SGK, the evaluation of a compound like GSK2334470 would be of great clinical importance [99]. A structure-based explanation of the moderate effect of this inhibitor on Akt phosphorylation levels is the reverse allosteric effect, where the PDK1-substrate interaction mediated by the PIF-pocket, is regulated by the ATP- binding site (reviewed in [100]). The binding of a compound to the PIF-pocket would probably stabilize the protein conformation regardless of its activation status at that point in time, and it is speculated to have higher specificity than ATP- binding site targeted molecules. This was demonstrated in vitro, where such molecules were shown to be able to inhibit S6K phosphorylation and signaling, but did not affect Akt (reviewed in [101,102]). Last but not least, following observations in conditional knock-in animal models expressing a mutated PDK1 PH domain, where Akt was exhibiting partial activation [13], a later model proposed that PDK1 is able to interact with pSer473-Akt via the PIF-pocket, and can therefore be independent of PtdIns(3,4,5)*P3* binding. In fact, distortion of the PIF-dependent mechanism was shown to render Akt more susceptible to PDK1 inhibitors such as GSK2334470 [103].

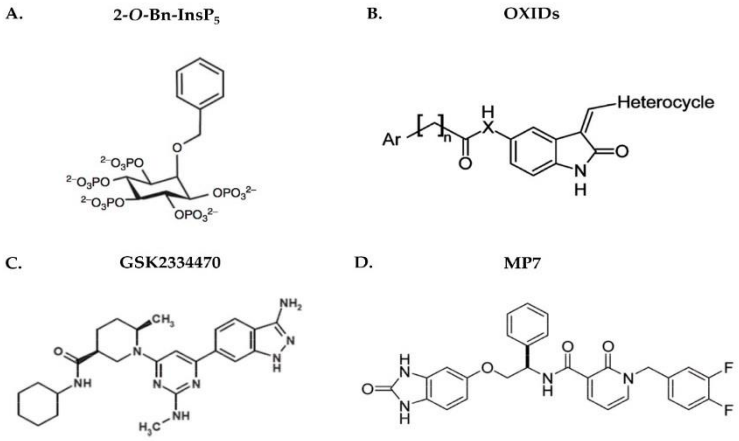


Figure 4. Structures of commonly used PDK1 inhibitors. (**A**) 2-*O*-Bn-InsP$_5$ (M. Falasca Laboratory); (**B**) OXIDs (S. Rapposelli Laboratory); (**C**) GSK2334470 (GlaxoSmithKline); (**D**) MP7 (Merck).

Currently, preclinical testing of GSK2334470 in a number of cancer cases is showing encouraging results. Advanced breast cancer (ER$^+$) is initially treated with CDK4/6 inhibitors, such as palbociclib, and anti-estrogens, but a number of patients fail to benefit from this therapy due to acquired or de novo resistance. The use of GSK2334470 was shown to act synergistically with CDK4/6 inhibitors to promote apoptosis and inhibit cell proliferation, while at the same time it was able to restore chemosensitivity in cells resistant to palbociclib [104]. The same compound has been shown to negatively affect the proliferation of multiple myeloma (MM) cell lines and overcome their resistance to dexamethasone, leaving the non-malignant cells unaffected [105]. Nevertheless, it was found that the responsiveness of the cells to this inhibitor was proportionally correlated to the expression status of PTEN, supporting the idea that following loss of PTEN, sole PDK1 inhibition does not suffice to hamper cancer progression [73]. In this case, although pSer241-PDK1 and pSer2448-mTOR levels were decreased following GSK2334470 administration, cells exhibiting low PTEN expression maintained the pSer473-AKT and pSer2481-mTOR levels. Restoration of PTEN levels resulted in decreased levels of these phosphoproteins and therefore inhibition of their activity, depicting a novel perspective in

the mechanisms of MM resistance to PDK1 inhibition. The mTOR cascade has been proposed as an appropriate target in MM, but the existing major barrier is that inhibition of mTORC1/C2 causes increase in phosphorylation of IGFR-1 in MM cells, and blocks apoptosis. Interestingly, GSK2334470 pro-apoptotic effects cannot be reversed by IGFR-1 upregulation and when combined with PP242 (an inhibitor of mTORC1/C2) it is cytotoxic for both chemosensitive and chemoresistant MM cells. Collectively, this evidence emphasizes the pro-apoptotic effects of the combination of PDK1 and mTOR inhibition in MM, independently of the PTEN expression levels [105].

Recently, Sunesis Pharmaceuticals has disclosed data regarding two promising PDK1 inhibitors, SNS-229 and SNS-510, which seem to be effective in haematological malignancies resistant to Akt and PI3K inhibition. In contrast with GSK2334470, these inhibitors act via intervention in the PIF-pocket and disruption of substrate binding, and have shown auspicious in vitro and in vivo preliminary results, and superiority over GSK2334470 [106,107].

PDK1 inhibition has also been shown to be effective as part of a multitargeted therapy regime. In regards to GBM, combination of MP7 (Figure 4D) (characterized in [108]) and alisertib which are PDK1 and Aurora Kinase A (AurA) inhibitors respectively, exhibited higher potential of reducing both the proliferation of GBM cells and their respective tumorspheres, than sole administration of each agent. A recently discovered OXID-pyridonyl derivative named SA16 was shown to be a dual PDK1/AurA pathway inhibitor which successfully reduces cell proliferation and invasiveness, while inducing the differentiation and subsequent apoptosis of GMB stem-like cells, therefore reducing their population and pointing towards a solution to GBM chemoresistance [109].

4. Conclusions

Due to the crucial role PDK1 holds regarding the cell physiology, it would be reasonable to argue that apart from the medical benefit, PDK1 inhibition would have multiple side effects. This protein is a crucial regulator of signaling pathways so important, that any deregulation would have detrimental effects. Apprehension would gradually lead to type II diabetes [110], whereas overstimulation would result in excessive proliferation and malignant conditions [111]. Nevertheless, animal studies showed that mice with only 10% of the *PDPK1* function exhibited a normal phenotype and when crossed with $PTEN^{+/-}$ mice, tumour formation capacity was disrupted [112]. Interestingly, there is currently a number of PI3K inhibitors used in the clinical setting, which have not fulfilled the initial expectations though. This could be due to inadequate effect on the final target or to the fact that more molecules of the same pathway need to be co-targeted. This is where PDK1 inhibitors can be introduced and have a synergistic effect, counteracting invasion, metastasis and chemoresistance [113]. In breast cancer, it was recently shown that PDK1 inhibition was able to re-sensitize *PIK3CA*-mutant cells to PI3Kα inhibitors through suppression of SGK1 and reduction of mTORC1 activity [85]. The PI3K pathway holds an important role in embryonic stem cell fate, with the main effector being PDK1 [114], and in addition to the implication of the latter in cancer stem cells maintenance mentioned in previous sections, it has been shown that dual inhibition of checkpoint kinase 1 (CHK1) and PDK1 effectively eliminates glioblastoma stem-like cells responsible for resistance to therapy and tumour recurrence [115]. Given the crucial role of cancer stem cells in the course of malignancies and their impact on the efficiency of therapies and the overall survival of the patients, application of the existing knowledge to target PDK1 and continuous efforts for the discovery of new molecules-inhibitors, are of utmost importance.

The significance of PDK1 lies in the fact that in terms of targeted therapy, it can replace undruggable molecules such as KRAS. *KRAS* is mutated in approximately 90% of pancreatic cancer cases but all efforts for its direct targeting have proven unfruitful. Since PDK1 comprises the most crucial effector downstream of KRAS, it is the best alternative for targeted therapy in this type of cancer. More specifically, in pancreatic cancer, *KRAS* mutations result in a constitutively active protein which upregulates the PI3K/Akt cascade; the suppression of which is not feasible due to loss of *PTEN* in earlier stages of the disease (reviewed in [116]). Interestingly, Eser et al. demonstrated that KRAS downstream effectors exhibit tissue-specificity and provided evidence that PI3K/PDK1 is a

suitable target in pancreatic ductal adenocarcinoma (PDAC) [117]. Additionally, it seems that a specific micro-RNA, miR-375 is the missing link connecting these two proteins and its expression is significantly different in normal versus pancreatic cancer tissues, correlating also with PDK1 expression levels (reviewed in [116]). Moreover, emerging evidence is pointing towards a role of PDK1 in the tumour microenvironment. PDK1 is necessary for the T-cell receptor to convey its signal, for the maturation of the T-cell, migration of endothelial cells and neutrophils chemotaxis among others, however it was shown that it is pivotal for tumour angiogenesis, which is one of the hallmarks of cancer, as well as proliferation and progression (reviewed in [118]).

Due to the key role of PDK1 in a number of cellular functions, it would be reasonable to argue whether apart from the delivery of beneficial effects, PDK1 inhibition could cause serious side effects to the subjects in clinical trials. The answer to this question comes via a plethora of preclinical studies in a variety of animal models for diverse diseases. Early studies in a transgenic adenocarcinoma of the mouse prostate (TRAMP) model showed that following treatment with OSU-03012, metastasis occurrence was minimized, as well as lobe proliferation, and in fact all prostate lobes exhibited a weight reduction. Nevertheless, other compound-related in vivo effects were observed such as weight loss which could not be counteracted by food intake, and compromise of the Type II skeletal myofibers [119]. On the contrary, OSU-03012 was able to cross the blood-brain barrier and exhibit good drug tolerability and effective tumor growth inhibition in schwannoma xenograft mouse models [120]. In prion and Alzheimer's disease studies, different mouse models treated with the BX912 PDK1 inhibitor, acquired ameliorated cognitive skills, social behavior and memory, and scored higher in multiple tasks, comparing to control mice. Further benefits included improved motor function, prolonged survival and decreased levels of PrP^{Sc} in the brain. However, one model suffered BX912 toxicity at day 350- therefore mitigating the beneficial effects of the inhibitor regarding Alzheimer's disease, but not undermining the overall outcomes of the study [121]. *In vivo* studies have shown that GSK2334470 in combination with PP242, an mTORC1/C2 inhibitor, was able to significantly abrogate tumor growth in xenograft multiple myeloma mouse models, comparing to sole administration of the inhibitors [105]. Preclinical studies of SNS-229 and SNS-510 in CD/1 mice, carried out by Sunesis Pharmaceuticals, have shown satisfying oral bioavailability (>90%) and pharmacokinetic properties (retrieved from https://www.sunesis.com/pap_pdk1.php), and no side effects were reported from either of the studies. In addition to these, nude mice treated with BX-320 exhibited no observable side effects, and the number of treated animals that died was equal to the control ones, with deaths being attributed to the dosing regimen rather than toxicity of the inhibitor [122]. Overall, it can be concluded that PDK1 inhibition does not result in any serious side effects and can be well-tolerated, a fact that can pave the way to Phase I clinical trials.

For the reason that Akt plays a multifaceted role in normal and pathologic cellular conditions, it has been the centre of attention for therapeutic strategies development, overlooking other members of the AGC family. PDK1 has been an emerging and promising target for anti-cancer therapies, which through both an Akt-dependent and independent manner is implicated in many aspects of carcinogenesis. Application of the most potent inhibitors on tumour models will reveal an integrated picture of the PDK1 abrogation potential in carcinogenesis (discussed in [21]). In parallel with this, PDK1 could serve well in the field of cancer biomarker discovery, and more specifically act as a pharmacodynamic (PD) biomarker, in order to measure the biological activity of a protein-target, following treatment with an inhibitor- candidate. For instance, during kinase drug discovery, the levels of phosphorylation sites on the kinases- targets are measured and quantified, instead of the levels of the phosphorylation levels of the protein- substrates, for the reason that the latter can be affected by pathway cross-talk and compensatory mechanisms. In the case of PDK1, the Thr^{513} and Ser^{410} phospho-residues constitute targets for PDK1 inhibitor molecules and their dephosphorylation levels reflect the potency of the inhibitor. In fact, PDK1 inhibitors type I, which compete for the ATP-binding site were shown to have an impact on these sites, but not on $pSer^{241}$ which resides in the T-loop, as was shown by immunoaffinity precipitation- mass spectrometry (IAP-MS). Supporting these findings,

the PDK1 *bona fide* readouts such as $pRSK^{Ser221}$ and $pAKT^{Thr308}$ were not representative of the PDK1 inhibition in prostate cancer cells [123]. There is currently little literature information regarding the two aforementioned PDK1 phosphorylation sites and their effect on the kinase activity, and further studies would shed more light on this matter [4,124]. $PhosphoSer^{241}$ PDK1 has also been identified and served as a candidate PD biomarker for the prediction of the efficacy of a DGF-out PDK1 inhibitor named compound 7 [108].

To conclude, we have here presented a thorough literature review assessing the value of PDK1 in chemosensitization of cancer cells, analyzed Akt-dependent and independent pathways, and reported the available inhibitors up to date, while providing information on the preclinical validation of these inhibitors and the studies outcome, as well as the potential role of PDK1 as a pharmacodynamic biomarker.

Acknowledgments: The authors acknowledge the infrastructure and staff support provided by Curtin Health Innovation Research Institute (CHIRI), School of Biomedical Sciences and Faculty of Health Sciences, Curtin University. Work in the Falasca lab is supported by Avner Pancreatic Cancer Foundation and Keith & Ann Vaughan Pancreatic Cancer Fund. A.E. is supported by AB Analitica.

Author Contributions: Aikaterini Emmanouilidi and Marco Falasca are equally contributing authors.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

3D	Three dimensional
5-FU	5-fluorouracil
AML	acute myeloid leukaemia
ATP	adenosine triphosphate
AurA	Aurora Kinase A
BAD	Bcl-2-associated death promoter
c/EBPβ	CCAAT/Enhancer Binding Protein Beta
CAMKK2	Ca^{2+}/calmodulin-dependent protein kinase kinase 2
cDDP	cis-diamminedichloroplatinum(II)/cisplatin/cisplatinum
COL11A1	collagen type XI aplha1
COX2	cyclooxygenase-2
CTGF	connective tissue growth factor
EGF	epidermal growth factor
EMT	epithelial-to-mesenchymal transition
EOC	epithelial ovarian carcinoma
ERα	oestrogen receptor α
FABP5	fatty acid-binding protein 5
Foxo	forkhead box O
GAP	GTPase activating protein
GBM	glioblastoma multiforme
GPCR	G-protein-coupled receptor
HDAC	histone deacetylase
Hpo	Hippo
Hsp90	heat shock protein 90
IC_{50}	half maximal inhibitory concentration
IGF1	insulin growth factor 1
K-RAS	Kirsten Rat Sarcoma Viral Oncogene Homolog
Lats	Large Tumor Suppressor Kinase 1
LPA	lysophosphatidic acid
LSC	leukaemia stem cells
Mats	Mob-as-tumour-suppressor
MLL-AF9	mixed lineage leukemia-ALL1- fused gene from chromosome 9 protein
MM	Multiple myeloma
MOBKL1A/1B	Mps one binder kinase activator-like 1A
mRNA	messenger ribonucleic acid
mSIN1	mammalian stress-activated protein kinase interacting protein 1
MTDH	metadherin

mTOR	mammalian/mechanistic target of rapamycin
MYC	v-myc myelocytomatosis viral oncogene homolog (avian)
NDRG1	N-Myc Downstream Regulated 1
nM	nanomolar
p21Cip1	cyclin-dependent kinase inhibitor 1
PDAC	pancreatic ductal adenocarcinoma
PDK1	3-phosphoinositide-dependent protein kinase 1
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIF	PDK1-interacting fragment
PKB/Akt	protein kinase B
PKC	protein kinase C
PLCε	phospholipase Cε
PLK1	Polo-like kinase 1
PPAR β	peroxisome proliferator-activated receptor β
PRAS40	inhibitory subunit 40-kDa proline-rich
PtdIns(3,4,5)P_3	phosphatidylinositol (3,4,5)-trisphosphate
PtdIns(4,5)P_2	phosphatidylinositol (4,5)-bisphosphate
PTEN	phosphate and tensin homolog
RA	retinoic acid
RTK	receptor tyrosine kinase
Rheb	Ras homolog enriched in brain
S6K1	Ribosomal protein S6 kinase beta-1
Sav	Salvador
Ser	serine
sfRon	short-form Ron
SGK	Serum and glucocorticoid-induced protein kinase
Src	V-Src Avian Sarcoma (Schmidt-RuppinA-2) Viral Oncogene
SWH	Salvador/Warts/Hippo
TCRP1	tongue cancer resistance-related protein-1
TEADs	TEA domain transcription factors
Thr	threonine
TIMP1	tissue inhibitor of metalloproteinase-1
TNBC	triple negative breast cancer
TRAIL	tumour necrosis factor-α-related apoptosis-inducing ligand
TSC	tuberous sclerosis complex
TUSC4	tumour suppressor candidate 4
UPP	ubiquitin (Ub)-proteasome pathway
VEGF-A	vascular endothelial growth factor-A
VP-16	Etoposide Phosphate
Wts	Warts
YAP	Yes-associated protein 1
μM	micromolar

References

1. Alessi, D. Discovery of PDKI, one of the missing links in insulin signal transduction. *Biochem. Soc. Trans.* **2001**, *29*, 1–14. [CrossRef] [PubMed]
2. Alessi, D.R.; James, S.R.; Downes, C.P.; Holmes, A.B.; Gaffney, P.R.; Reese, C.B.; Cohen, P. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. *Curr. Biol.* **1997**, *7*, 261–269. [CrossRef]
3. Alessi, D.R.; Deak, M.; Casamayor, A.; Caudwell, F.B.; Morrice, N.; Norman, D.G.; Gaffney, P.; Reese, C.B.; MacDougall, C.N.; Harbison, D. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): Structural and functional homology with the Drosophila DSTPK61 kinase. *Curr. Biol.* **1997**, *7*, 776–789. [CrossRef]

4. Casamayor, A.; Morrice, N.A.; Alessi, D.R. Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: Identification of five sites of phosphorylation in vivo. *Biochem. J.* **1999**, *342*, 287–292. [CrossRef] [PubMed]
5. Lawlor, M.A.; Mora, A.; Ashby, P.R.; Williams, M.R.; Murray-Tait, V.; Malone, L.; Prescott, A.R.; Lucocq, J.M.; Alessi, D.R. Essential role of PDK1 in regulating cell size and development in mice. *EMBO J.* **2002**, *21*, 3728–3738. [CrossRef] [PubMed]
6. Collins, B.J.; Deak, M.; Arthur, J.S.; Armit, L.J.; Alessi, D.R. In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J.* **2003**, *22*, 4202–4211. [CrossRef] [PubMed]
7. McManus, E.J.; Collins, B.J.; Ashby, P.R.; Prescott, A.R.; Murray-Tait, V.; Armit, L.J.; Arthur, J.S.; Alessi, D.R. The in vivo role of PtdIns(3,4,5)P3 binding to PDK1 PH domain defined by knockin mutation. *EMBO J.* **2004**, *23*, 2071–2082. [CrossRef] [PubMed]
8. Mora, A.; Davies, A.M.; Bertrand, L.; Sharif, I.; Budas, G.R.; Jovanović, S.; Mouton, V.; Kahn, C.R.; Lucocq, J.M.; Gray, G.A. Deficiency of PDK1 in cardiac muscle results in heart failure and increased sensitivity to hypoxia. *EMBO J.* **2003**, *22*, 4666–4676. [CrossRef] [PubMed]
9. Alfonso, M.; Lipina, C.; Tronche, F.; Sutherland, C.; Alessi, D.R. Deficiency of PDK1 in liver results in glucose intolerance, impairment of insulin-regulated gene expression and liver failure. *Biochem. J.* **2005**, *385*, 639–648.
10. Okamoto, Y.; Ogawa, W.; Nishizawa, A.; Inoue, H.; Teshigawara, K.; Kinoshita, S.; Matsuki, Y.; Watanabe, E.; Hiramatsu, R.; Sakaue, H. Restoration of glucokinase expression in the liver normalizes postprandial glucose disposal in mice with hepatic deficiency of PDK1. *Diabetes* **2007**, *56*, 1000–1009. [CrossRef] [PubMed]
11. Hashimoto, N.; Kido, Y.; Uchida, T.; Asahara, S.-i.; Shigeyama, Y.; Matsuda, T.; Takeda, A.; Tsuchihashi, D.; Nishizawa, A.; Ogawa, W. Ablation of PDK1 in pancreatic [beta] cells induces diabetes as a result of loss of [beta] cell mass. *Nat. Genet.* **2006**, *38*, 589–593. [CrossRef] [PubMed]
12. Bayascas, J.R.; Sakamoto, K.; Armit, L.; Arthur, J.S.C.; Alessi, D.R. Evaluation of approaches to generation of tissue-specific knock-in mice. *J. Biol. Chem.* **2006**, *281*, 28772–28781. [CrossRef] [PubMed]
13. Bayascas, J.R.; Wullschleger, S.; Sakamoto, K.; García-Martínez, J.M.; Clacher, C.; Komander, D.; Van Aalten, D.M.; Boini, K.M.; Lang, F.; Lipina, C. Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance. *Mol. Cell. Biol.* **2008**, *28*, 3258–3272. [CrossRef] [PubMed]
14. Rotte, A.; Bhandaru, M.; Ackermann, T.F.; Boini, K.M.; Lang, F. Role of PDK1 in regulation of gastric acid secretion. *Cell. Physiol. Biochem.* **2008**, *22*, 725–734. [CrossRef] [PubMed]
15. Sandu, C.; Artunc, F.; Palmada, M.; Rexhepaj, R.; Grahammer, F.; Hussain, A.; Yun, C.; Alessi, D.R.; Lang, F. Impaired intestinal NHE3 activity in the PDK1 hypomorphic mouse. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2006**, *291*, G868–G876. [CrossRef] [PubMed]
16. Muranen, T.A.; Greco, D.; Fagerholm, R.; Kilpivaara, O.; Kämpjärvi, K.; Aittomäki, K.; Blomqvist, C.; Heikkilä, P.; Borg, Å.; Nevanlinna, H. Breast tumors from CHEK2 1100delC-mutation carriers: Genomic landscape and clinical implications. *Breast Cancer Res.* **2011**, *13*, R90. [CrossRef] [PubMed]
17. Choucair, K.A.; Guérard, K.-P.; Ejdelman, J.; Chevalier, S.; Yoshimoto, M.; Scarlata, E.; Fazli, L.; Sircar, K.; Squire, J.A.; Brimo, F. The 16p13. 3 (PDPK1) genomic gain in prostate cancer: A potential role in disease progression. *Transl. Oncol.* **2012**, *5*, 453–460. [CrossRef] [PubMed]
18. Maurer, M.; Su, T.; Saal, L.H.; Koujak, S.; Hopkins, B.D.; Barkley, C.R.; Wu, J.; Nandula, S.; Dutta, B.; Xie, Y. 3-Phosphoinositide-dependent kinase 1 potentiates upstream lesions on the phosphatidylinositol 3-kinase pathway in breast carcinoma. *Cancer Res.* **2009**, *69*, 6299–6306. [CrossRef] [PubMed]
19. Di Blasio, L.; Gagliardi, P.A.; Puliafito, A.; Primo, L. Serine/Threonine Kinase 3-Phosphoinositide-Dependent Protein Kinase-1 (PDK1) as a key regulator of cell migration and cancer dissemination. *Cancers* **2017**, *9*, E25. [CrossRef] [PubMed]
20. Pinner, S.; Sahai, E. PDK1 regulates cancer cell motility by antagonising inhibition of ROCK1 by RhoE. *Nat. Cell Biol.* **2008**, *10*, 127–137. [CrossRef] [PubMed]
21. Raimondi, C.; Falasca, M. Targeting PDK1 in cancer. *Curr. Med. Chem.* **2011**, *18*, 2763–2769. [CrossRef] [PubMed]
22. Gagliardi, P.A.; di Blasio, L.; Primo, L. PDK1: A signaling hub for cell migration and tumor invasion. *Biochim. Biophys. Acta* **2015**, *1856*, 178–188. [CrossRef] [PubMed]
23. Bayascas, J.R. PDK1: The major transducer of PI 3-kinase actions. In *Phosphoinositide 3-Kinase in Health and Disease*; Springer: Berlin, Germany, 2010; pp. 9–29.

24. Toricelli, M.; Melo, F.H.; Hunger, A.; Zanatta, D.; Strauss, B.E.; Jasiulionis, M.G. Timp1 promotes cell survival by activating the PDK1 signaling pathway in melanoma. *Cancers* **2017**, *9*, 37. [CrossRef] [PubMed]
25. Wang, Y.; Fu, L.; Cui, M.; Wang, Y.; Xu, Y.; Li, M.; Mi, J. Amino acid transporter SLC38A3 promotes metastasis of non-small cell lung cancer cells by activating PDK1. *Cancer Lett.* **2017**, *393*, 8–15. [CrossRef] [PubMed]
26. Wang, J.; Liu, F.; Ao, P.; Li, X.; Zheng, H.; Wu, D.; Zhang, N.; She, J.; Yuan, J.; Wu, X. Correlation of PDK1 expression with clinicopathologic features and prognosis of hepatocellular carcinoma. *Onco Targets Ther.* **2016**, *9*, 5597–5602. [PubMed]
27. Vasudevan, K.M.; Barbie, D.A.; Davies, M.A.; Rabinovsky, R.; McNear, C.J.; Kim, J.J.; Hennessy, B.T.; Tseng, H.; Pochanard, P.; Kim, S.Y. AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer. *Cancer Cell* **2009**, *16*, 21–32. [CrossRef] [PubMed]
28. Samuels, Y.; Wang, Z.; Bardelli, A.; Silliman, N.; Ptak, J.; Szabo, S.; Yan, H.; Gazdar, A.; Powell, S.M.; Riggins, G.J. High frequency of mutations of the PIK3CA gene in human cancers. *Science* **2004**, *304*, 554. [CrossRef] [PubMed]
29. Samuels, Y.; Diaz, L.A.; Schmidt-Kittler, O.; Cummins, J.M.; DeLong, L.; Cheong, I.; Rago, C.; Huso, D.L.; Lengauer, C.; Kinzler, K.W. Mutant PIK3CA promotes cell growth and invasion of human cancer cells. *Cancer Cell* **2005**, *7*, 561–573. [CrossRef] [PubMed]
30. Gagliardi, P.A.; di Blasio, L.; Orso, F.; Seano, G.; Sessa, R.; Taverna, D.; Bussolino, F.; Primo, L. 3-phosphoinositide-dependent kinase 1 controls breast tumor growth in a kinase-dependent but Akt-independent manner. *Neoplasia* **2012**, *14*, 719–731. [CrossRef] [PubMed]
31. Dobbin, Z.C.; Landen, C.N. The importance of the PI3K/AKT/MTOR pathway in the progression of ovarian cancer. *Int. J. Mol. Sci.* **2013**, *14*, 8213–8227. [CrossRef] [PubMed]
32. Ahmed, N.; Riley, C.; Quinn, M.A. An immunohistochemical perspective of PPAR beta and one of its putative targets PDK1 in normal ovaries, benign and malignant ovarian tumours. *Br. J. Cancer* **2008**, *98*, 1415–1424. [CrossRef] [PubMed]
33. Moxley, K.M.; Wang, L.; Welm, A.L.; Bieniasz, M. Short-form Ron is a novel determinant of ovarian cancer initiation and progression. *Genes Cancer* **2016**, *7*, 169–181. [CrossRef] [PubMed]
34. Liu, X.; Zhao, L.; Derose, Y.S.; Lin, Y.C.; Bieniasz, M.; Eyob, H.; Buys, S.S.; Neumayer, L.; Welm, A.L. Short-Form Ron Promotes Spontaneous Breast Cancer Metastasis through Interaction with Phosphoinositide 3-Kinase. *Genes Cancer* **2011**, *2*, 753–762. [CrossRef] [PubMed]
35. Lian, S.; Shao, Y.; Liu, H.; He, J.; Lu, W.; Zhang, Y.; Jiang, Y.; Zhu, J. PDK1 induces JunB, EMT, cell migration and invasion in human gallbladder cancer. *Oncotarget* **2015**, *6*, 29076–29086. [CrossRef] [PubMed]
36. Wu, Y.-H.; Chang, T.-H.; Huang, Y.-F.; Chen, C.-C.; Chou, C.-Y. COL11A1 confers chemoresistance on ovarian cancer cells through the activation of Akt/c/EBPβ pathway and PDK1 stabilization. *Oncotarget* **2015**, *6*, 23748–23763. [CrossRef] [PubMed]
37. Li, Y.; Lacerda, D.; Warman, M.; Beier, D.; Yoshioka, H.; Ninomiya, Y.; Oxford, J.; Morris, N.; Andrikopoulos, K.; Ramirez, F. A fibrillar collagen gene, Col11a1, is essential for skeletal morphogenesis. *Cell* **1995**, *80*, 423–430. [CrossRef]
38. Nakamura, A.; Naito, M.; Tsuruo, T.; Fujita, N. Freud-1/Aki1, a novel PDK1-interacting protein, functions as a scaffold to activate the PDK1/Akt pathway in epidermal growth factor signaling. *Mol. Cell. Biol.* **2008**, *28*, 5996–6009. [CrossRef] [PubMed]
39. Falasca, M.; Chiozzotto, D.; Godage, H.; Mazzoletti, M.; Riley, A.; Previdi, S.; Potter, B.; Broggini, M.; Maffucci, T. A novel inhibitor of the PI3K/Akt pathway based on the structure of inositol 1, 3, 4, 5, 6-pentakisphosphate. *Br. J. Cancer* **2010**, *102*, 104–114. [CrossRef] [PubMed]
40. Gocher, A.M.; Azabdaftari, G.; Euscher, L.M.; Dai, S.; Karacosta, L.G.; Franke, T.F.; Edelman, A.M. Akt activation by Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2) in ovarian cancer cells. *J. Biol. Chem.* **2017**, *292*, 14188–14204. [CrossRef] [PubMed]
41. Lemmon, M.A.; Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* **2010**, *141*, 1117–1134. [CrossRef] [PubMed]
42. Zhang, X.; George, J.; Deb, S.; Degoutin, J.L.; Takano, E.A.; Fox, S.B.; Bowtell, D.D.; Harvey, K.F. The Hippo pathway transcriptional co-activator, YAP, is an ovarian cancer oncogene. *Oncogene* **2011**, *30*, 2810–2822. [CrossRef] [PubMed]
43. Fyffe, C.; Falasca, M. 3-Phosphoinositide-dependent protein kinase-1 as an emerging target in the management of breast cancer. *Cancer Manag. Res.* **2013**, *5*, 271–280. [PubMed]

44. Iorns, E.; Lord, C.J.; Ashworth, A. Parallel RNAi and compound screens identify the PDK1 pathway as a target for tamoxifen sensitization. *Biochem. J.* **2009**, *417*, 361–371. [CrossRef] [PubMed]
45. Peifer, C.; Alessi, D.R. New anti-cancer role for PDK1 inhibitors: Preventing resistance to tamoxifen. *Biochem. J.* **2009**, *417*, e5–e7. [CrossRef] [PubMed]
46. Zhu, J.; Huang, J.-W.; Tseng, P.-H.; Yang, Y.-T.; Fowble, J.; Shiau, C.-W.; Shaw, Y.-J.; Kulp, S.K.; Chen, C.-S. From the cyclooxygenase-2 inhibitor celecoxib to a novel class of 3-phosphoinositide-dependent protein kinase-1 inhibitors. *Cancer Res.* **2004**, *64*, 4309–4318. [CrossRef] [PubMed]
47. Weng, S.-C.; Kashida, Y.; Kulp, S.K.; Wang, D.; Brueggemeier, R.W.; Shapiro, C.L.; Chen, C.-S. Sensitizing estrogen receptor-negative breast cancer cells to tamoxifen with OSU-03012, a novel celecoxib-derived phosphoinositide-dependent protein kinase-1/Akt signaling inhibitor. *Mol. Cancer Ther.* **2008**, *7*, 800–808. [CrossRef] [PubMed]
48. Fry, M.J. Phosphoinositide 3-kinase signalling in breast cancer: How big a role might it play? *Breast Cancer Res.* **2001**, *3*, 304–312. [CrossRef] [PubMed]
49. Lin, H.; Hsieh, F.; Song, H.; Lin, J. Elevated phosphorylation and activation of PDK-1/AKT pathway in human breast cancer. *Br. J. Cancer* **2005**, *93*, 1372–1381. [CrossRef] [PubMed]
50. Liang, K.; Lu, Y.; Li, X.; Zeng, X.; Glazer, R.I.; Mills, G.B.; Fan, Z. Differential roles of phosphoinositide-dependent protein kinase-1 and akt1 expression and phosphorylation in breast cancer cell resistance to Paclitaxel, Doxorubicin, and gemcitabine. *Mol. Pharmacol.* **2006**, *70*, 1045–1052. [CrossRef] [PubMed]
51. Ali, S.; Aranha, O.; Li, Y.; Pettit, G.R.; Sarkar, F.H.; Philip, P.A. Sensitization of human breast cancer cells to gemcitabine by the protein kinase C modulator bryostatin 1. *Cancer Chemother. Pharmacol.* **2003**, *52*, 235–246. [CrossRef] [PubMed]
52. Zeng, X.; Xu, H.; Glazer, R.I. Transformation of mammary epithelial cells by 3-phosphoinositide-dependent protein kinase-1 (PDK1) is associated with the induction of protein kinase Cα. *Cancer Res.* **2002**, *62*, 3538–3543. [PubMed]
53. Thulasiraman, P.; McAndrews, D.J.; Mohiudddin, I.Q. Curcumin restores sensitivity to retinoic acid in triple negative breast cancer cells. *BMC Cancer* **2014**, *14*, 724. [CrossRef] [PubMed]
54. Meads, M.B.; Gatenby, R.A.; Dalton, W.S. Environment-mediated drug resistance: A major contributor to minimal residual disease. *Nat. Rev. Cancer* **2009**, *9*, 665–674. [CrossRef] [PubMed]
55. Hu, T.; Li, C.; Zhang, Y.; Wang, L.; Peng, L.; Cheng, H.; Wang, W.; Chu, Y.; Xu, M.; Cheng, T. Phosphoinositide-dependent kinase 1 regulates leukemia stem cell maintenance in MLL-AF9-induced murine acute myeloid leukemia. *Biochem. Biophys. Res. Commun.* **2015**, *459*, 692–698. [CrossRef] [PubMed]
56. Zabkiewicz, J.; Pearn, L.; Hills, R.K.; Morgan, R.G.; Tonks, A.; Burnett, A.K.; Darley, R.L. The PDK1 master kinase is over-expressed in acute myeloid leukemia and promotes PKC-mediated survival of leukemic blasts. *Haematologica* **2014**, *99*, 858–864. [CrossRef] [PubMed]
57. Cheong, J.W.; Eom, J.I.; Maeng, H.Y.; Lee, S.T.; Hahn, J.S.; Ko, Y.W.; Min, Y.H. Phosphatase and tensin homologue phosphorylation in the C-terminal regulatory domain is frequently observed in acute myeloid leukaemia and associated with poor clinical outcome. *Br. J. Haematol.* **2003**, *122*, 454–456. [CrossRef] [PubMed]
58. Kornblau, S.M.; Womble, M.; Qiu, Y.H.; Jackson, C.E.; Chen, W.; Konopleva, M.; Estey, E.H.; Andreeff, M. Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. *Blood* **2006**, *108*, 2358–2365. [CrossRef] [PubMed]
59. Grandage, V.; Gale, R.; Linch, D.; Khwaja, A. PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kB, MAPkinase and p53 pathways. *Leukemia* **2005**, *19*, 586–594. [CrossRef] [PubMed]
60. Datta, S.R.; Dudek, H.; Tao, X.; Masters, S.; Fu, H.; Gotoh, Y.; Greenberg, M.E. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **1997**, *91*, 231–241. [CrossRef]
61. Miyashita, T.; Krajewski, S.; Krajewska, M.; Wang, H.G.; Lin, H.; Liebermann, D.A.; Hoffman, B.; Reed, J.C. Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* **1994**, *9*, 1799–1805. [PubMed]
62. Lowe, S.W.; Bodis, S.; McClatchey, A.; Remington, L.; Ruley, H.E.; Fisher, D.E.; Housman, D.E.; Jacks, T. p53 status and the efficacy of cancer therapy in vivo. *Science* **1994**, *266*, 807–810. [CrossRef] [PubMed]

63. Prokop, A.; Wieder, T.; Sturm, I.; Ebmann, F.; Seeger, K.; Wuchter, C.; Ludwig, W.; Henze, G.; Dörken, B.; Daniel, P. Relapse in childhood acute lymphoblastic leukemia is associated with a decrease of the Bax/Bcl-2 ratio and loss of spontaneous caspase-3 processing in vivo. *Leukemia* **2000**, *14*, 1606–1613. [CrossRef] [PubMed]
64. McCubrey, J.; Steelman, L.; Abrams, S.; Bertrand, F.; Ludwig, D.; Bäsecke, J.; Libra, M.; Stivala, F.; Milella, M.; Tafuri, A. Targeting survival cascades induced by activation of Ras/Raf/MEK/ERK, PI3K/PTEN/Akt/mTOR and Jak/STAT pathways for effective leukemia therapy. *Leukemia* **2008**, *22*, 708–722. [CrossRef] [PubMed]
65. Meng, X.; Brachova, P.; Yang, S.; Xiong, Z.; Zhang, Y.; Thiel, K.W.; Leslie, K.K. Knockdown of MTDH sensitizes endometrial cancer cells to cell death induction by death receptor ligand TRAIL and HDAC inhibitor LBH589 co-treatment. *PLoS ONE* **2011**, *6*, e20920. [CrossRef] [PubMed]
66. Sato, S.; Fujita, N.; Tsuruo, T. Regulation of kinase activity of 3-phosphoinositide-dependent protein kinase-1 by binding to 14–3-3. *J. Biol. Chem.* **2002**, *277*, 39360–39367. [CrossRef] [PubMed]
67. Fujita, N.; Sato, S.; Ishida, A.; Tsuruo, T. Involvement of Hsp90 in signaling and stability of 3-phosphoinositide-dependent kinase-1. *J. Biol. Chem.* **2002**, *277*, 10346–10353. [CrossRef] [PubMed]
68. Kurata, A.; Katayama, R.; Watanabe, T.; Tsuruo, T.; Fujita, N. TUSC4/NPRL2, a novel PDK1-interacting protein, inhibits PDK1 tyrosine phosphorylation and its downstream signaling. *Cancer Sci.* **2008**, *99*, 1827–1834. [CrossRef] [PubMed]
69. Wang, C.; Liu, H.; Qiu, Q.; Zhang, Z.; Gu, Y.; He, Z. TCRP1 promotes NIH/3T3 cell transformation by over-activating PDK1 and AKT1. *Oncogenesis* **2017**, *6*, e323. [CrossRef] [PubMed]
70. Liu, X.; Wang, C.; Gu, Y.; Zhang, Z.; Zheng, G.; He, Z. TCRP1 contributes to cisplatin resistance by preventing Pol β degradation in lung cancer cells. *Mol. Cell. Biochem.* **2015**, *398*, 175–183. [CrossRef] [PubMed]
71. Gu, Y.; Fan, S.; Liu, B.; Zheng, G.; Yu, Y.; Ouyang, Y.; He, Z. TCRP1 promotes radioresistance of oral squamous cell carcinoma cells via Akt signal pathway. *Mol. Cell. Biochem.* **2011**, *357*, 107–113. [CrossRef] [PubMed]
72. Ericson, K.; Gan, C.; Cheong, I.; Rago, C.; Samuels, Y.; Velculescu, V.E.; Kinzler, K.W.; Huso, D.L.; Vogelstein, B.; Papadopoulos, N. Genetic inactivation of AKT1, AKT2, and PDPK1 in human colorectal cancer cells clarifies their roles in tumor growth regulation. *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 2598–2603. [CrossRef] [PubMed]
73. Ellwood-Yen, K.; Keilhack, H.; Kunii, K.; Dolinski, B.; Connor, Y.; Hu, K.; Nagashima, K.; O'Hare, E.; Erkul, Y.; Di Bacco, A. PDK1 attenuation fails to prevent tumor formation in PTEN-deficient transgenic mouse models. *Cancer Res.* **2011**, *71*, 3052–3065. [CrossRef] [PubMed]
74. Tan, J.; Li, Z.; Lee, P.L.; Guan, P.; Aau, M.Y.; Lee, S.T.; Feng, M.; Lim, C.Z.; Lee, E.Y.J.; Wee, Z.N. PDK1 signaling toward PLK1-MYC activation confers oncogenic transformation, tumor-initiating cell activation, and resistance to mTOR-targeted therapy. *Cancer Discov.* **2013**, *3*, 1156–1171. [CrossRef] [PubMed]
75. Wong, D.J.; Liu, H.; Ridky, T.W.; Cassarino, D.; Segal, E.; Chang, H.Y. Module map of stem cell genes guides creation of epithelial cancer stem cells. *Cell Stem Cell* **2008**, *2*, 333–344. [CrossRef] [PubMed]
76. Pan, D. The hippo signaling pathway in development and cancer. *Dev. Cell* **2010**, *19*, 491–505. [CrossRef] [PubMed]
77. Zhao, B.; Ye, X.; Yu, J.; Li, L.; Li, W.; Li, S.; Yu, J.; Lin, J.D.; Wang, C.Y.; Chinnaiyan, A.M.; et al. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev.* **2008**, *22*, 1962–1971. [CrossRef] [PubMed]
78. Fan, R.; Kim, N.G.; Gumbiner, B.M. Regulation of Hippo pathway by mitogenic growth factors via phosphoinositide 3-kinase and phosphoinositide-dependent kinase-1. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 2569–2574. [CrossRef] [PubMed]
79. Engelman, J.A. Targeting PI3K signalling in cancer: Opportunities, challenges and limitations. *Nat. Rev. Cancer* **2009**, *9*, 550–562. [CrossRef] [PubMed]
80. Fruman, D.A.; Rommel, C. PI3K and cancer: Lessons, challenges and opportunities. *Nat. Rev. Drug Discov.* **2014**, *13*, 140–156. [CrossRef] [PubMed]
81. Juric, D.; Rodon, J.; Gonzalez-Angulo, A.; Burris, H.; Bendell, J.; Berlin, J.; Middleton, M.; Bootle, D.; Boehm, M.; Schmitt, A. BYL719, a next generation PI3K alpha specific inhibitor: Preliminary safety. In Proceedings of the AACR 103rd Annual Meeting, Chicago, IL, USA, 6–10 April 2013.

82. Juric, D.; Krop, I.; Ramanathan, R.; Xiao, J.; Sanabria, S.; Wilson, T.; Choi, Y.; Parmar, H.; Hsu, J.; Baselga, J. GDC-0032, a beta isoform-sparing PI3K inhibitor: Results of a first-in-human dose escalation study. In Proceedings of the AACR 104th Annual Meeting, Washington, DC, USA, 31 March–4 April 2012.
83. Elkabets, M.; Vora, S.; Juric, D.; Morse, N.; Mino-Kenudson, M.; Muranen, T.; Tao, J.; Campos, A.B.; Rodon, J.; Ibrahim, Y.H.; et al. mTORC1 inhibition is required for sensitivity to PI3K p110alpha inhibitors in PIK3CA-mutant breast cancer. *Sci. Transl. Med.* **2013**, *5*, 196ra99. [CrossRef] [PubMed]
84. Dibble, C.C.; Cantley, L.C. Regulation of mTORC1 by PI3K signaling. *Trends Cell Biol.* **2015**, *25*, 545–555. [CrossRef] [PubMed]
85. Castel, P.; Ellis, H.; Bago, R.; Toska, E.; Razavi, P.; Carmona, F.J.; Kannan, S.; Verma, C.S.; Dickler, M.; Chandarlapaty, S. PDK1-SGK1 signaling sustains AKT-independent mTORC1 activation and confers resistance to PI3Kα inhibition. *Cancer Cell* **2016**, *30*, 229–242. [CrossRef] [PubMed]
86. Webb, A.E.; Brunet, A. FOXO transcription factors: Key regulators of cellular quality control. *Trends Biochem. Sci.* **2014**, *39*, 159–169. [CrossRef] [PubMed]
87. Murray, J.T.; Campbell, D.G.; Morrice, N.; Auld, G.C.; Shpiro, N.; Marquez, R.; Peggie, M.; Bain, J.; Bloomberg, G.B.; Grahammer, F.; et al. Exploitation of KESTREL to identify NDRG family members as physiological substrates for SGK1 and GSK3. *Biochem. J.* **2004**, *384*, 477–488. [CrossRef] [PubMed]
88. Sommer, E.M.; Dry, H.; Cross, D.; Guichard, S.; Davies, B.R.; Alessi, D.R. Elevated SGK1 predicts resistance of breast cancer cells to Akt inhibitors. *Biochem. J.* **2013**, *452*, 499–508. [CrossRef] [PubMed]
89. Zurashvili, T.; Cordón-Barris, L.; Ruiz-Babot, G.; Zhou, X.; Lizcano, J.M.; Gómez, N.; Giménez-Llort, L.; Bayascas, J.R. Interaction of PDK1 with phosphoinositides is essential for neuronal differentiation but dispensable for neuronal survival. *Mol. Cell. Biol.* **2013**, *33*, 1027–1040. [CrossRef] [PubMed]
90. Scortegagna, M.; Lau, E.; Zhang, T.; Feng, Y.; Sereduk, C.; Yin, H.; De, S.K.; Meeth, K.; Platt, J.T.; Langdon, C.G.; et al. PDK1 and SGK3 Contribute to the Growth of BRAF-Mutant Melanomas and Are Potential Therapeutic Targets. *Cancer Res.* **2015**, *75*, 1399–1412. [CrossRef] [PubMed]
91. Silva, J.M.; Bulman, C.; McMahon, M. BRAFV600E cooperates with PI3K signaling, independent of AKT, to regulate melanoma cell proliferation. *Mol. Cancer Res.* **2014**, *12*, 447–463. [CrossRef] [PubMed]
92. Bago, R.; Sommer, E.; Castel, P.; Crafter, C.; Bailey, F.P.; Shpiro, N.; Baselga, J.; Cross, D.; Eyers, P.A.; Alessi, D.R. The hVps34-SGK3 pathway alleviates sustained PI3K/Akt inhibition by stimulating mTORC1 and tumour growth. *EMBO J.* **2016**, *35*, 1902–1922. [CrossRef] [PubMed]
93. Barile, E.; De, S.K.; Pellecchia, M. PDK1 inhibitors. *Pharm. Pat. Anal.* **2012**, *1*, 145–163. [CrossRef] [PubMed]
94. Peifer, C.; Alessi, D.R. Small-Molecule Inhibitors of PDK1. *ChemMedChem* **2008**, *3*, 1810–1838. [CrossRef] [PubMed]
95. Komander, D.; Fairservice, A.; Deak, M.; Kular, G.S.; Prescott, A.R.; Downes, C.P.; Safrany, S.T.; Alessi, D.R.; van Aalten, D.M. Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates. *EMBO J.* **2004**, *23*, 3918–3928. [CrossRef] [PubMed]
96. Maffucci, T.; Piccolo, E.; Cumashi, A.; Iezzi, M.; Riley, A.M.; Saiardi, A.; Godage, H.Y.; Rossi, C.; Broggini, M.; Iacobelli, S. Inhibition of the phosphatidylinositol 3-kinase/Akt pathway by inositol pentakisphosphate results in antiangiogenic and antitumor effects. *Cancer Res.* **2005**, *65*, 8339–8349. [CrossRef] [PubMed]
97. Nesi, G.; Sestito, S.; Mey, V.; Ricciardi, S.; Falasca, M.; Danesi, R.; Lapucci, A.; Breschi, M.C.; Fogli, S.; Rapposelli, S. Synthesis of novel 3, 5-disubstituted-2-oxindole derivatives as antitumor agents against human nonsmall cell lung cancer. *ACS Med. Chem. Lett.* **2013**, *4*, 1137–1141. [CrossRef] [PubMed]
98. Sestito, S.; Nesi, G.; Daniele, S.; Martelli, A.; Digiacomo, M.; Borghini, A.; Pietra, D.; Calderone, V.; Lapucci, A.; Falasca, M. Design and synthesis of 2-oxindole based multi-targeted inhibitors of PDK1/Akt signaling pathway for the treatment of glioblastoma multiforme. *Eur. J. Med. Chem.* **2015**, *105*, 274–288. [CrossRef] [PubMed]
99. Najafov, A.; Sommer, E.M.; Axten, J.M.; Deyoung, M.P.; Alessi, D.R. Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. *Biochem. J.* **2011**, *433*, 357–369. [CrossRef] [PubMed]
100. Leroux, A.E.; Schulze, J.; Biondi, R.M. AGC kinases, mechanisms of regulation and innovative drug development. *Semin. Cancer Biol.* **2017**, in press. [CrossRef] [PubMed]
101. Biondi, R.M. Phosphoinositide-dependent protein kinase 1, a sensor of protein conformation. *Trends Biochem. Sci.* **2004**, *29*, 136–142. [CrossRef] [PubMed]

102. Arencibia, J.M.; Pastor-Flores, D.; Bauer, A.F.; Schulze, J.O.; Biondi, R.M. AGC protein kinases: From structural mechanism of regulation to allosteric drug development for the treatment of human diseases. *Biochim. Biophys. Acta* **2013**, *1834*, 1302–1321. [CrossRef] [PubMed]
103. Najafov, A.; Shpiro, N.; Alessi, D.R. Akt is efficiently activated by PIF-pocket-and PtdIns (3, 4, 5) P3-dependent mechanisms leading to resistance to PDK1 inhibitors. *Biochem. J.* **2012**, *448*, 285–295. [CrossRef] [PubMed]
104. Jansen, V.M.; Bhola, N.E.; Bauer, J.A.; Formisano, L.; Lee, K.-M.; Hutchinson, K.E.; Witkiewicz, A.K.; Moore, P.D.; Estrada, M.V.; Sánchez, V. Kinome-wide RNA interference screen reveals a role for PDK1 in acquired resistance to CDK4/6 inhibition in ER-positive breast cancer. *Cancer Res.* **2017**, *77*, 2488–2499. [CrossRef] [PubMed]
105. Yang, C.; Huang, X.; Liu, H.; Xiao, F.; Wei, J.; You, L.; Qian, W. PDK1 inhibitor GSK2334470 exerts antitumor activity in multiple myeloma and forms a novel multitargeted combination with dual mTORC1/C2 inhibitor PP242. *Oncotarget* **2017**, *8*, 39185–39197. [CrossRef] [PubMed]
106. Hansen, S.; Enquist, J.; Iwig, J.; Binnerts, M.E.; Jamieson, G.; Fox, J.A.; Craig, A.R. Abstract C198: PDK1 inhibitors SNS-229 and SNS-510 cause pathway modulation, apoptosis and tumor regression in hematologic cancer models in addition to solid tumors. *Mol. Cancer. Ther.* **2015**, *14*, C198. [CrossRef]
107. Carceller, V. AACR-NCI-EORTC—27th International Symposium—Molecular Targets and Cancer Therapeutics (November 5–9, 2015—Boston, Massachusetts, USA). *Drugs Today* **2015**, *51*, 669–675. [CrossRef] [PubMed]
108. Nagashima, K.; Shumway, S.D.; Sathyanarayanan, S.; Chen, A.H.; Dolinski, B.; Xu, Y.; Keilhack, H.; Nguyen, T.; Wiznerowicz, M.; Li, L. Genetic and pharmacological inhibition of PDK1 in cancer cells characterization of a selective allosteric kinase inhibitor. *J. Biol. Chem.* **2011**, *286*, 6433–6448. [CrossRef] [PubMed]
109. Daniele, S.; Sestito, S.; Pietrobono, D.; Giacomelli, C.; Chiellini, G.; Di Maio, D.; Marinelli, L.; Novellino, E.; Martini, C.; Rapposelli, S. Dual inhibition of PDK1 and Aurora kinase A: An effective strategy to induce differentiation and apoptosis of human glioblastoma multiforme stem cells. *ACS Chem. Neurosci.* **2016**, *8*, 100–114. [CrossRef] [PubMed]
110. Biddinger, S.B.; Kahn, C.R. From mice to men: Insights into the insulin resistance syndromes. *Annu. Rev. Physiol.* **2006**, *68*, 123–158. [CrossRef] [PubMed]
111. Vanhaesebroeck, B.; Leevers, S.J.; Ahmadi, K.; Timms, J.; Katso, R.; Driscoll, P.C.; Woscholski, R.; Parker, P.J.; Waterfield, M.D. Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **2001**, *70*, 535–602. [CrossRef] [PubMed]
112. Bayascas, J.R.; Leslie, N.R.; Parsons, R.; Fleming, S.; Alessi, D.R. Hypomorphic mutation of PDK1 suppresses tumorigenesis in PTEN(+/−) mice. *Curr. Biol.* **2005**, *15*, 1839–1846. [CrossRef] [PubMed]
113. Raimondi, C.; Calleja, V.; Ferro, R.; Fantin, A.; Riley, A.M.; Potter, B.V.L.; Brennan, C.H.; Maffucci, T.; Larijani, B.; Falasca, M. A Small Molecule Inhibitor of PDK1/PLCγ1 Interaction Blocks Breast and Melanoma Cancer Cell Invasion. *Sci. Rep.* **2016**, *6*, 26142. [CrossRef] [PubMed]
114. Ling, L.; Voskas, D.; Woodgett, J. Activation of PDK-1 maintains mouse embryonic stem cell self-renewal in a PKB-dependent manner. *Oncogene* **2013**, *32*, 5397–5408. [CrossRef] [PubMed]
115. Signore, M.; Pelacchi, F.; Di Martino, S.; Runci, D.; Biffoni, M.; Giannetti, S.; Morgante, L.; De Majo, M.; Petricoin, E.; Stancato, L. Combined PDK1 and CHK1 inhibition is required to kill glioblastoma stem-like cells in vitro and in vivo. *Cell Death Dis.* **2014**, *5*, e1223. [CrossRef] [PubMed]
116. Ferro, R.; Falasca, M. Emerging role of the KRAS-PDK1 axis in pancreatic cancer. *World J. Gastroenterol.* **2014**, *20*, 10752–10757. [CrossRef] [PubMed]
117. Eser, S.; Reiff, N.; Messer, M.; Seidler, B.; Gottschalk, K.; Dobler, M.; Hieber, M.; Arbeiter, A.; Klein, S.; Kong, B. Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer. *Cancer Cell* **2013**, *23*, 406–420. [CrossRef] [PubMed]
118. Gagliardi, P.A.; Puliafito, A.; Primo, L. PDK1: At the crossroad of cancer signaling pathways. *Semin. Cancer Biol.* **2017**. [CrossRef] [PubMed]
119. Sargeant, A.M.; Klein, R.D.; Rengel, R.C.; Clinton, S.K.; Kulp, S.K.; Kashida, Y.; Yamaguchi, M.; Wang, X.; Chen, C.-S. Chemopreventive and bioenergetic signaling effects of PDK1/Akt pathway inhibition in a transgenic mouse model of prostate cancer. *Toxicol. Pathol.* **2007**, *35*, 549–561. [CrossRef] [PubMed]

120. Lee, T.X.; Packer, M.D.; Huang, J.; Akhmametyeva, E.M.; Kulp, S.K.; Chen, C.-S.; Giovannini, M.; Jacob, A.; Welling, D.B.; Chang, L.-S. Growth inhibitory and anti-tumour activities of OSU-03012, a novel PDK-1 inhibitor, on vestibular schwannoma and malignant schwannoma cells. *Eur. J. Cancer* **2009**, *45*, 1709–1720. [CrossRef] [PubMed]
121. Pietri, M.; Dakowski, C.; Hannaoui, S.; Alleaume-Butaux, A.; Hernandez-Rapp, J.; Ragagnin, A.; Mouillet-Richard, S.; Haik, S.; Bailly, Y.; Peyrin, J.-M. PDK1 decreases TACE-mediated [alpha]-secretase activity and promotes disease progression in prion and Alzheimer's diseases. *Nat. Med.* **2013**, *19*, 1124–1131. [CrossRef] [PubMed]
122. Feldman, R.I.; Wu, J.M.; Polokoff, M.A.; Kochanny, M.J.; Dinter, H.; Zhu, D.; Biroc, S.L.; Alicke, B.; Bryant, J.; Yuan, S. Novel small molecule inhibitors of 3-phosphoinositide-dependent kinase-1. *J. Biol. Chem.* **2005**, *280*, 19867–19874. [CrossRef] [PubMed]
123. Paweletz, C.P.; Andersen, J.N.; Pollock, R.; Nagashima, K.; Hayashi, M.L.; Shangshuan, U.Y.; Guo, H.; Bobkova, E.V.; Xu, Z.; Northrup, A. Identification of direct target engagement biomarkers for kinase-targeted therapeutics. *PLoS ONE* **2011**, *6*, e26459. [CrossRef] [PubMed]
124. Gao, X.; Harris, T.K. Role of the PH domain in regulating in vitro autophosphorylation events required for reconstitution of PDK1 catalytic activity. *Bioorg. Chem.* **2006**, *34*, 200–223. [CrossRef] [PubMed]

Publication 4

Emmanouilidi et al. Journal of Experimental & Clinical Cancer Research
(2019) 38:191
https://doi.org/10.1186/s13046-019-1191-2

Journal of Experimental & Clinical Cancer Research

RESEARCH Open Access

Preclinical validation of 3-phosphoinositide-dependent protein kinase 1 inhibition in pancreatic cancer

Aikaterini Emmanouilidi[1], Chanse A. Fyffe[2], Riccardo Ferro[2], Charlotte E. Edling[2], Emily Capone[3], Simona Sestito[4], Simona Rapposelli[4], Rossano Lattanzio[3], Stefano Iacobelli[3,5], Gianluca Sala[3], Tania Maffucci[2†] and Marco Falasca[1,2*†]

Abstract

Background: The very aggressive nature and low survival rate of pancreatic ductal adenocarcinoma (PDAC) dictates the necessity to find novel efficacious therapies. Recent evidence suggests that phosphoinositide 3-kinase (PI3K) and 3-phosphoinositide-dependent protein kinase 1 (PDK1) are key effectors of oncogenic KRAS in PDAC. Herein, we report the role and mechanism of action of PDK1, a protein kinase of the AGC family, in PDAC.

Methods: PDAC cell lines were treated with selective PDK1 inhibitors or transfected with specific PDK1-targeting siRNAs. In vitro and in vivo assays were performed to investigate the functional role of PDK1 in PDAC. Specifically, anchorage-dependent and anchorage-independent growth was assessed in PDAC cells upon inhibition or downregulation of PDK1. Detailed investigation of the effect of PDK1 inhibition/downregulation on specific signalling pathways was also performed by Western blotting analysis. A xenograft tumour mouse model was used to determine the effect of pharmacological inhibition of PDK1 on PDAC cells growth in vivo.

Results: Treatment with specific inhibitors of PDK1 impaired anchorage-dependent and anchorage-independent growth of pancreatic cancer cell lines, as well as pancreatic tumour growth in a xenograft model. Mechanistically, inhibition or downregulation of PDK1 resulted in reduced activation of the serum/glucocorticoid regulated kinase family member 3 and subsequent reduced phosphorylation of its target N-Myc downstream regulated 1. Additionally, we found that combination of sub-optimal concentrations of inhibitors selective for PDK1 and the class IB PI3K isoform p110γ inhibits pancreatic cancer cell growth and colonies formation more potently than each single treatment.

Conclusions: Our data indicate that PDK1 is a suitable target for therapeutic intervention in PDAC and support the clinical development of PDK1 inhibitors for PDAC.

Keywords: Pancreatic ductal adenocarcinoma, Signal transduction, Targeted therapy, Phosphoinositide 3-kinase, 3-phosphoinositide-dependent protein kinase 1, Serum/glucocorticoid regulated kinase family member 3

Background

Pancreatic ductal adenocarcinoma (PDAC) is amongst the five most deadly human malignancies, having a 5-year relative survival rate of ~8% [1]. PDAC is associated with high-frequency somatic mutations in a subset of genes, most frequently in the gene encoding the small GTPase KRAS, which is mutated in the majority of human PDAC (> 95%) [2]. The genes encoding the tumour suppressor p53, SMAD4 and $p16^{Ink4A}$ are also frequently mutated in PDAC with many other accessory mutations also being observed at varied frequency [3].

Since their discovery, phosphoinositide 3-kinases (PI3Ks) have been established as major signalling molecules implicated in different cellular functions such as

* Correspondence: marco.falasca@curtin.edu.au
†Tania Maffucci and Marco Falasca jointly supervised this work
[1]Metabolic Signalling Group, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia 6102, Australia
[2]Queen Mary University of London, Barts and The London School of Medicine and Dentistry, Blizard Institute, Centre for Cell Biology and Cutaneous Research, E1 2AT, London, UK
Full list of author information is available at the end of the article

glucose metabolism, cellular proliferation, cellular survival and angiogenesis. Abnormal PI3K signalling has been estimated to occur in as many as 50% of all human malignancies and this pathway is a well-established target for anti-cancer therapies [4]. The PI3K family comprises of eight mammalian isoforms grouped into three classes. Class IA consists of a catalytic subunit and a regulatory subunit. The catalytic subunits include p110α, p110β, or p110δ, while the regulatory subunits consist of p85α, p85β or p55γ. Class IB consists of only one catalytic subunit, p110γ, and two regulatory subunits, p87 and p101. We previously reported that p110γ is overexpressed in human PDAC and plays a key role in pancreatic cancer cell proliferation [5]. These data were confirmed by a recent study also reporting increased levels of p110γ in human pancreatic cancer tissues as well as its role in regulation of pancreatic cancer cell growth [6]. Similarly, p110γ overexpression was detected in human hepatocellular carcinoma (HCC) where the enzyme was also shown to be a key regulator of cellular proliferation [7].

In response to receptor tyrosine kinases or G-protein coupled receptors activation, class I PI3Ks catalyse the phosphorylation of the 3′ position of the inositol ring of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5) P_2] which leads to the synthesis of the membrane bound phospholipid phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5) P_3] [8]. PtdIns(3,4,5) P_3 then acts as a second messenger and regulates both plasma membrane translocation and activation of several proteins. The tumour suppressor phosphatase and tensin homolog (PTEN) inhibits this signalling pathway by dephosphorylating PtdIns(3,4,5) P_3 back to PtdIns(4,5) P_2. The best characterized PI3K/PtdIns(3,4,5) P_3 downstream effector is the Serine/Threonine-specific protein kinase B (PKB)/Akt that binds to PtdIns(3,4,5) P_3 via its pleckstrin homology (PH) domain. Once at the plasma membrane, Akt is phosphorylated at its residue Thr308 by the 3-phosphoinositide-dependent protein kinase 1 (PDK1), which itself associates to the membrane via PH domain-dependent binding to PtdIns(3,4,5) P_3, and at its residue Ser473 by other kinases, including the complex 2 of mechanistic target of rapamycin. PDK1 belongs to the family of AGC kinases and was first discovered in 1997 for its ability to phosphorylate Akt at Thr308. Although the contribution of PI3K and PI3K-dependent pathways to cancer development and progression has been well established for many years, the first major indication that PDK1 itself might be a viable target in cancer only appeared in 2005 when Bayascas et al generated transgenic hypomorphic PDK1 mice [9]. When these mice were crossed with tumourigenic heterozygous $PTEN^{+/-}$ mice, the prevalence of tumour development was reduced in mice with deficient PDK1 levels, confirming the importance of PDK1 in tumour development driven by loss of PTEN [9]. Further evidence of a specific role for PDK1 in cancer is provided by the observation that increased copy number of *PDPK1*, the gene encoding for PDK1, is frequently observed in different cancer types [10–12]. Evidence is also emerging indicating that PDK1 inhibition can impact on several cellular functions associated with cancer progression, such as reduced invasion on Matrigel of breast, prostate and melanoma cancer cell lines [13–15]. Similarly, stable downregulation of PDK1 inhibited migration of the breast cancer cell line MDA-MB-231 and metastasis formation upon implantation of cells in immunodeficient mice [16]. PDK1 has also been shown to be implicated in ovarian cancer aggressiveness via a short form of the Ron receptor tyrosine kinase [17], and COLL11A1 [18], whereas its pharmacological inhibition had been previously found to be able to enhance the effect of chemotherapeutic drugs in the ovarian cancer cell line SKOV-3 cells [19]. Furthermore, PDK1 mRNA is correlated with poor survival rates in untreated HCC patients and is the most prominent factor in the time to recurrence prediction, post-operatively [20].

Whilst PDK1 is most commonly associated with Akt signalling, it has become increasingly evident that the role of PDK1 in cancer is not limited to Akt activation. PDK1 can phosphorylate and activate at least 23 AGC kinases including S6 Kinase, protein kinase C and serum-and glucocorticoid-induced protein kinase (SGK) [21]. The diversity of substrates that can be activated by PDK1 are of high relevance in cancer signalling. For instance, the PDK1 substrate serum/glucocorticoid regulated kinase family member 3 (SGK3) is frequently overexpressed in HCC and its downregulation reduced both colonies formation and tumour formation in nude mice [22]. In a subset of breast cancer cell lines, hyperactivation of PI3K pathways was reported to be independent from Akt activation and it was shown that tumourigenicity of the cells, as assessed by anchorage-independent growth, was dependent on PDK1 and SGK3 [23]. This provided the first evidence to suggest that PDK1 can represent an Akt-independent molecular target in human malignancies. It is now well established that PI3K/PDK1-dependent, Akt-independent signalling pathways can contribute to tumourigenesis [24, 25].

A previous study reported that deletion of *PDPK1* inhibits $KRas^{G12D}$- driven PDAC development in a transgenic mouse model [26], revealing a key role for PDK1 in PDAC initiation. Whether pharmacological inhibition of the enzyme can inhibit PDAC progression remains to be established. Here we determined the effect of selective PDK1 inhibitors on PDAC growth in vitro and in vivo. This study identified PDK1 as a novel potential target to develop new treatment strategies in pancreatic cancer.

Methods

Cell culture and transfection

HPAF-II, AsPC-1, CFPAC-1 and PANC-1 cells were obtained from ATCC and grown in complete growth media (Eagle's Minimum Essential Medium, RPMI-1640 Medium, Iscove's Modified Dulbecco's Medium and Dulbecco's Modified Eagle Medium, respectively) supplemented with 10% FBS (Bovogen Biologicals) and 1X Penicillin-Streptomycin-Glutamine (HyClone) at 37 °C in a 5% CO_2 atmosphere. HPDE cells were kindly provided by Prof H. Kocher (Queen Mary University of London) and were cultured in keratinocyte serum-free medium supplemented with epidermal growth factor (EGF) and bovine pituitary extract (Life Technologies, Inc.). hTERT-HPNE cells were obtained from ATCC and cultured in 75% DMEM without glucose supplemented with 25% Medium M3 Base (INCELL Corporation LLC), 5% FBS, 10 ng/ml human recombinant EGF, 5.5 mM D-glucose and 750 ng/ml puromycin. For serum starvation, cells were seeded in a 6-well plate at a density of 3.5×10^6 cells/well and were serum starved for 24 h. After that, cells were stimulated with media containing 10% FBS for 1 h in the presence or absence of the indicated inhibitors.

Downregulation of PDK1 was obtained using the following siRNAs from Dharmacon: Sequence 1 ON-TARGETplus Standard GACCAGAGGCCAAGAAUUUUU; Sequence 2 ON-TARGETplus Standard (A4) CAAGAGACCUCGUGGAGAAUU. Downregulation of SGK3 was obtained using the following siRNAs from Qiagen: Gene Solution siRNA SI00101003 (SGKL 3) and Gene Solution siRNA SI00287588 (SGKL 6). Cells were transfected using 75 nM of siRNAs and DharmaFECT 1 and DharmaFECT 2 transfection reagents (Dharmacon) according to manufacturer's instructions.

Cell viability assay

Effect of the drugs on anchorage-dependent growth was assessed by trypan blue exclusion assay. Briefly, cells were seeded in 12-well plates at a density of 5×10^4 cells/well and treated with different concentrations of drugs for 72 h. Cells were then trypsinized, complete media was added and 10 μl of cell suspension was mixed with trypan blue dye [1]. The mixture was loaded on a Neubauer chamber and the number of viable cells per mL was calculated as (number of viable cells / 4) $\times 10^4$, corrected for the dilution factor.

Anchorage-independent growth – soft agar assay

In order to assess the long-term effect of the drugs and the PDK1/SGK3 downregulation on the ability of cells to form 3D colonies (tumourigenicity), anchorage-independent growth assays were performed. Six-well plates were coated with a mixture of 1% noble agar: 2XRPMI [1:1(v/v)] (bottom layer). Once the first layer had solidified, a second layer was added, comprising of 0.6% noble agar: 2XRPMI [1:1(v/v)] containing 10,000 cells and supplemented with the required inhibitor or corresponding vehicle.

Alternatively, 10,000 cells that had been transfected with siRNAs were plated. After the second layer had solidified, 1x RPMI was added and plates were kept in a humidified incubator, at 37 °C in a 5% CO_2 atmosphere. After 5 weeks incubation, colonies were fixed and stained with Crystal Violet (0.05%), visualized with ChemiDoc XRS+ System (Bio-Rad) and quantified with ImageJ software.

Cell lysis and Western blotting analysis

Cells were lysed using cold radioimmunoprecipitation assay buffer (150 mM sodium chloride, 1.0% NP-40 or Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris HCl, pH 8.0) supplemented with 1X Protease/Phosphatase Inhibitor Cocktail (100X stock, Cell Signaling Technology). After sonication at 4 °C, lysates were centrifuged at 10,000 g for 10 mins at 4 °C. Supernatants were transferred to a 1.5 ml tube and protein concentrations were determined using the Direct Detect Assay-Free cards and the Direct Detect Spectrometer (Merck Millipore, Darmstadt, Germany). Samples (35 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in TBS containing Tween-20 (0.05% v/v) and supplemented with 3% bovine serum albumin (TBST -BSA) at room temperature (RT) for 1 h followed by overnight incubation with primary antibodies at 4 °C. The following day, membranes were washed with TBST at RT (3 × 10 mins), and incubated for 1 h at RT with the appropriate secondary antibody (1:20,000). After three washes in TBST and one wash in TBS, membranes were incubated with Clarity Western ECL Blotting Substrates (Bio-Rad) and images were acquired using a ChemiDoc XRS+ System (Bio-Rad). Primary antibodies used were: pFoxO1 (Thr24)/FoxO3a (Thr32) (#9464), pAkt (Thr308) (#4056), pSGK3 (Thr320) (#5642), pNDRG1 (Thr346) (#3217), Akt (#9272), SGK3 (#8156), PDK1 (#3062), NDRG1 (#5196), GAPDH (#5174). All antibodies were purchased from Cell Signaling Technology. Antibodies were diluted in TBST-BSA (1:1000, apart from pSGK3, 1:500).

In vivo experiments

Athymic CD-1 nu/nu mice (5–7 weeks old) were purchased from Charles River Laboratories (Calco, LC, Italy) and maintained under specific pathogen-free conditions with food and water provided ad libitum. The animals' health status was monitored daily. Procedures involving animals and their care were established

according to the institutional guidelines in compliance with national and international policies (Autorizzazione N 484/2016-PR Ministero della Salute). HPAF-II (3.5×10^6) cells were injected subcutaneously into the right flank of mice. When xenografts became palpable, tumour-bearing mice were divided into two groups ($n = 7$) with mice receiving MP7 (75 mg/kg) or vehicle by oral gavage (5 days/week) for 3 weeks. For this purpose, MP7 was administered as a suspension in 0.5% methylcellulose + 0.4% tween 80. Tumour volumes were monitored every week using a caliper and volumes were calculated using the following formula: tumour volume = (length* width^2)/2. The PDK1 inhibitor MP7 was synthesized as previously reported [27] and characterized as described in Additional file 1. For in vivo xenograft curves, p values were determined by Student's t-test and considered significant at $p < 0.05$. All statistical analysis was performed with GraphPad Prism 5.0 software.

Results

PDK1 inhibition reduces pancreatic cancer cell growth in vitro and in vivo

To investigate the specific role of PDK1 in pancreatic cancer proliferation, PDAC cell lines were transiently transfected with two siRNAs specifically targeting the protein, as well as a non-targeting siRNA ("siControl"). Downregulation of PDK1 expression reduced AsPC-1, HPAF-II and CFPAC-1 anchorage-dependent (Fig. 1 a, d, g), and anchorage-independent growth (Fig. 1 b, e, h). Efficient downregulation of PDK1 was confirmed by Western blotting (Fig. 1 c, f, i). We next determined the effect of PDK1 pharmacological inhibition on PDAC cells. Four malignant (HPAF-II, AsPC-1, CFPAC-1, PANC-1) and two non-malignant pancreatic epithelial cell lines (hTERT-HPNE, HPDE) were treated with increasing concentrations of selective PDK1 inhibitors. Specifically, cells were treated with the small molecules GSK2334470 [28]

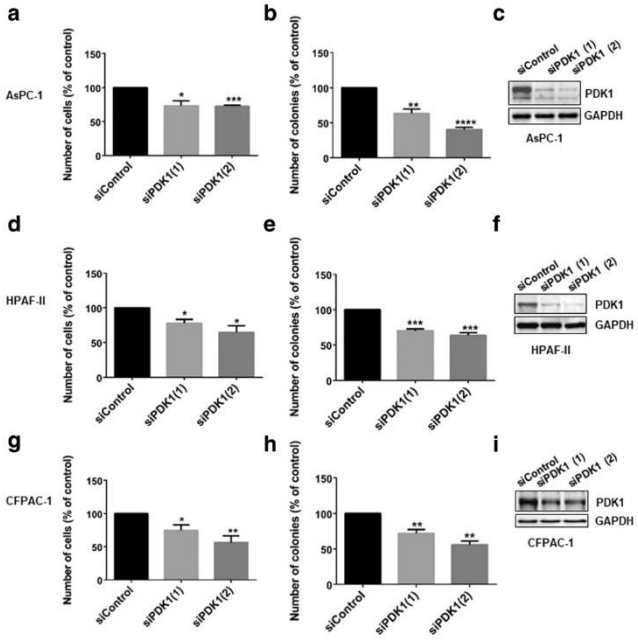


Fig. 1 siRNA-mediated PDK1 downregulation inhibits cell proliferation and anchorage-independent growth of PDAC cells. AsPC-1 (**a-c**), HPAF-II (**d-f**) and CFPAC-1 (**g-i**) cells were transfected with two siRNA sequences specifically targeting PDK1 or a non-targeting siRNA (siControl) and then manually counted with trypan blue exclusion (**a,d,g**) or plated on soft agar (**b,e,h**), as specified in the Methods. Data are expressed as percentage of cells transfected with siControl and are means ± SEM of $n \geq 3$ independent experiments performed in duplicate. T-test and GraphPad Prism version 6.0 were used for statistical analysis, and data from cells transfected with each siRNA were compared to data from cells transfected with siControl. Representative blots confirming efficient PDK1 downregulation are also shown (**c,f,i,**). GAPDH was used as loading control. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

and MP7 [27] as well as the Inositol (1,3,4,5,6) pentakisphosphate ($InsP_5$) derivative 2-*O*-benzyl-$InsP_5$ (2-*O*-Bn-$InsP_5$) that we previously reported to target PDK1 selectively [29]. We observed that treatment of all cell lines with each inhibitor significantly and dose-dependently reduced the number of AsPC-1 and HPAF-II cells compared to control cells treated with vehicle (Fig. 2 a-c). Overall, non-malignant hTERT-HPNE and HPDE cells appeared to be slightly or not sensitive to PDK1 inhibition compared to PDAC cells, with 2-*O*-Bn-$InsP_5$ not having any statistically significant effect on hTERT-HPNE and HPDE cell numbers at any tested concentrations (Fig. 2 a-c). Similar results were obtained in CFPAC-1 (Additional file 2: Figure S1a,b) and PANC-1 (Additional file 2: Figure S2a,b). Moreover, treatment of AsPC-1 cells with the three inhibitors significantly reduced their anchorage-independent growth, as

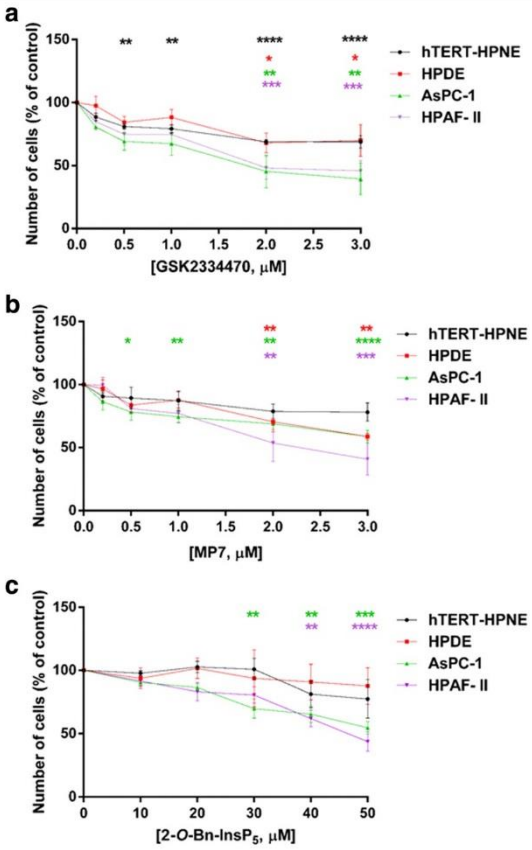


Fig. 2 Pharmacological inhibition of PDK1 reduces PDAC cells proliferation in monolayer culture. Non-malignant epithelial pancreatic cells (hTERT-HPNE, HPDE) as well as PDAC cells (AsPC-1, HPAF-II) were treated with increasing concentrations of the PDK1 inhibitors GSK2334470 (**a**), MP7 (**b**) and 2-*O*-Bn-$InsP_5$ (**c**) for 72 h, and cell viability was assessed. Data are expressed as percentage of control cells treated with vehicle alone (DMSO) and are means ± SEM of n ≥ 3 independent experiments performed in duplicate. For each cell line, one-way ANOVA with Dunnett's multiple comparisons test was used for statistical analysis between each treatment and its corresponding DMSO-treated control. Analysis was performed with GraphPad Prism version 6.0. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

assessed by soft agar assays (Fig. 3 a-c, Additional file 2: Figure S3a). PDK1 inhibition also reduced anchorage-independent growth in CFPAC-1 (Additional file 2: Figure S1c,d), PANC-1 (Additional file 2: Figure S2c,d, Additional file 2: Figure S3b-d), and HPAF-II (Additional file 2: Figure S4a,b) cells. It has been reported previously that genetic ablation of *PDPK1* reduces $KRas^{G12D}$- driven PDAC development in a transgenic mouse model and that treatment with a pan class I PI3Ks inhibitor reduces PDAC progression in KPC mice, the animal model that most accurately recapitulates the human disease [26]. Whether pharmacological inhibition of PDK1 could also affect progression of PDAC in vivo has not been previously assessed. To investigate this possibility, HPAF-II cells (3.5×10^6) were injected subcutaneously into the right flank of athymic CD-1 nu/nu mice (5–7 weeks old) and mice were treated with MP7 (75 mg/kg) or vehicle, once xenografts became palpable. No body weight loss was observed during treatments, indicating that MP7 is well tolerated at the dose used for this study. Tumour volumes were monitored every week with the use of a caliper, and volumes were calculated as described in the Methods section. Treatment of mice with MP7 significantly inhibited tumour growth in vivo (Fig. 3d), indicating that targeting PDK1 pharmacologically is able to reduce PDAC progression in vivo.

Taken together these data indicate that PDK1 regulates PDAC cell proliferation and that inhibition of the enzyme can efficiently reduce PDAC growth in vitro. At the same time, our in vivo results, together with previous evidence using genetic approaches, indicate that PDK1 represents a novel important target to counteract PDAC progression, and that MP7 might represent a valuable drug to be used for PDAC treatment.

PDK1 regulates SGK3 activation in PDAC cells

In order to gain further insight into the mechanisms involved in the PDK1-dependent regulation of PDAC cell growth, we next analysed the effect of PDK1 inhibition on activation of specific signalling pathways. First, we observed that treatment of AsPC-1 (Fig. 4 a), CFPAC-1

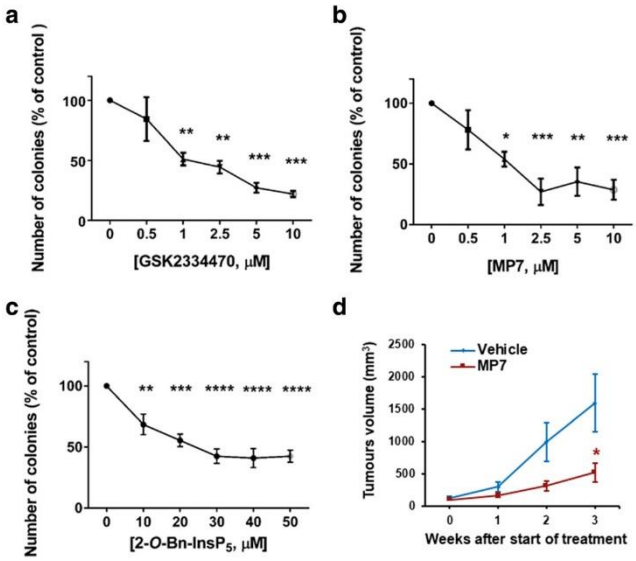


Fig. 3 Pharmacological inhibition of PDK1 reduces PDAC cells anchorage independent growth and PDAC cells growth in vivo. **(a-c)** AsPC-1 cells were treated with PDK1 inhibitors and plated on soft agar. After 5 weeks, colonies were stained and counted using ImageJ software. Data are expressed as percentage of control cells treated with DMSO and are means ± SEM of n ≥ 3 independent experiments performed in duplicate. One-way ANOVA with Dunnett's multiple comparisons test was used for statistical analysis between each treatment and its corresponding DMSO-treated control. Analysis was performed with GraphPad Prism version 6.0. *p < 0.05, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. **(d)** Mice ($n = 7$/group) were injected with HPAF-II cells and treated as described in the Methods section. Data indicate means ± SEM of tumour volumes at the indicated times after start of treatment with MP7. * $p < 0.05$ as assessed by Student's t-test

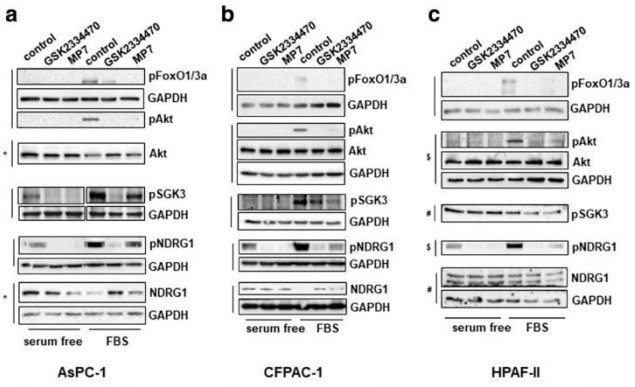


Fig. 4 Effect of PDK1 pharmacological inhibition on signalling pathways in PDAC cell lines. AsPC-1 (**a**), CFPAC-1 (**b**) and HPAF-II (**c**) cells were serum starved for 24 h and then treated with GSK2334470 (10μM) or MP7 (10μM) in the presence or absence of FBS for 1h, prior to cell lysis. Lysates were analysed by Western blotting using the indicated antibodies (details of antibodies are provided in the Methods section). In all blots GAPDH was used as loading control. Representative blots are shown. Vertical lines indicate membranes derived from the same gel. In (**b**,**c**) membranes incubated with anti-pAkt (Thr308) were stripped and re-incubated with anti-Akt. *, #, $ indicate membranes that derived from the same gel (therefore only one GAPDH is shown)

(Fig. 4 b) and HPAF-II (Fig. 4 c) cells with either GSK2334470 or MP7 efficiently blocked the FBS-induced phosphorylation of Akt at its residue Thr308, a bona fide readout of PDK1 activity, without affecting the total levels of Akt. Consistent with reduced Akt activation, inhibition of FBS-mediated FoxO1(Thr24)/FoxO3a(Thr32) phosphorylation was also detected in these cells (Fig. 4 a-c). Since PDK1 has been described to regulate SGK3 activation [30] we next investigated the effect of PDK1 inhibition on SGK3 phosphorylation at its residue Thr320 in PDAC cells. Consistent with data on Akt Thr308, inhibition of PDK1 also reduced SGK3 phosphorylation (Fig. 4 a-c). To investigate further the signalling pathways regulated by PDK1/SGK3 in PDAC cells we next analysed the effect of PDK1 inhibition on the metastasis suppressor N-Myc downstream regulated 1 (NDRG1) which has been recently identified as a downstream target of SGK3 [30]. SGK3 has been reported to regulate NDRG1 phosphorylation at its residue Thr346, which leads to degradation of the protein [30]. A clear inhibition of NDRG1 Thr346 phosphorylation (both in the absence and in the presence of FBS) was detected in AsPC-1 (Fig. 4a), CFPAC-1 (Fig. 4b) and HPAF-II (Fig. 4c) cells upon treatment with both PDK1 inhibitors. Increased protein levels of NDRG1 were observed in FBS-stimulated AsPC-1 (Fig. 4a) and CFPAC-1 (Fig. 4b) cells upon treatment with both PDK1 inhibitors compared to FBS-stimulated, untreated cells. Consistent with data obtained using the two inhibitors, siRNAs-mediated downregulation of PDK1 also clearly reduced SGK3 and NDRG1 phosphorylation in AsPC-1 (Fig. 5a), CFPAC-1 (Fig. 5b) and HPAF-II (Fig. 5c) cells.

Taken together these data indicate that PDK1 regulates both SGK3 and NDRG1 in PDAC cell lines. Interestingly, we noticed that downregulation of SGK3 efficiently reduced the number of AsPC-1 (Fig. 6a), CFPAC-1 (Fig. 6b) and HPAF-II (Fig. 6c) cells, suggesting that inhibition of PDK1 can partly affect PDAC cell proliferation through its effect on SGK3 activation.

Combination of PDK1 and p110γ inhibitors strongly reduces pancreatic cancer cell growth in vitro

Previous work in our laboratory demonstrated a key role for the PI3K isoform p110γ in PDAC cell proliferation [5]. Consistent with our previous report, we observed that treatment of HPAF-II and AsPC-1 cells with the p110δ/γ inhibitor IPI-145 (Infinity Pharmaceuticals, Inc.) significantly and dose dependently reduced cell growth (Fig. 7a). Similarly, treatment with the more potent p110δ/γ inhibitor IPI-742 (Infinity Pharmaceuticals, Inc.) reduced cell growth (Fig. 7b) and colony formation (Fig. 7c) in HPAF-II cells. Reduced cell growth was also detected in AsPC-1 cells upon treatment with IPI-742 (Additional file 2: Figure S5a). Importantly, the detected inhibition was mainly due to the effect of the compound on p110γ as in the same experimental conditions the specific p110δ inhibitor CAL-101 affected cell numbers only slightly when used at the highest concentration in HPAF-II (Fig. 7b) while it did not affect AsPC-1 cells at any of the

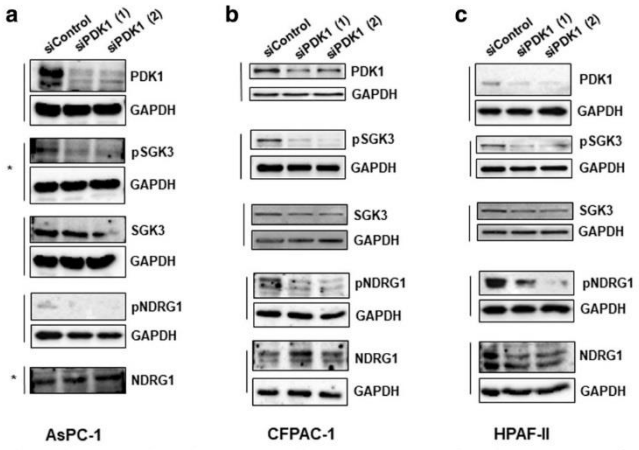


Fig. 5 Effect of PDK1 downregulation on signalling pathways in PDAC cell lines. AsPC-1 (**a**), CFPAC-1 (**b**) and HPAF-II (**c**) were transfected with the indicated siRNAs and lysed 72 h post-transfection. Lysates were then analysed by Western blotting using the indicated antibodies. Representative blots are shown. In all blots GAPDH was used as loading control. *indicates membranes that derived from the same gel (therefore only one GAPDH is shown)

concentrations used (Additional file 2: Figure S5a). Consistent with a specific role for p110γ, treatment with IPI-549 (Selleckchem), a novel and specific p110γ inhibitor, reduced anchorage-dependent growth of PDAC cells without affecting the non-malignant pancreatic epithelial cell lines (Additional file 2: Figure S5b). Reduced anchorage-independent growth was also detected in AsPC-1 (Additional file 2: Figure S5c, d) and PANC-1 (Additional file 2: Figure S5c) cells upon treatment with IPI-549. Interestingly, we observed that combination of IPI-145 with 2-*O*-Bn-InsP$_5$ (Fig. 7d) or GSK2334470 (Fig. 7e) used at sub-optimal concentrations resulted in enhanced reduction of HPAF-II cell numbers. Consistent with this, combination of GSK2334470 with IPI-742 (Additional file 2: Figure S6a) reduced numbers of AsPC-1 cells more potently than each inhibitor used at sub-optimal concentrations. A similar trend was observed in AsPC-1 cells upon combination of IPI-549 with GSK2334470 (Additional file 2: Figure S6b) and in CFPAC-1 cells upon combination of IPI-549 with either GSK2334470 or 2-*O*-Bn-InsP$_5$ (Additional file 2: Figure S6c). Finally, we analysed the effect of these combinations on anchorage-independent growth. Data indicated that treatment with IPI-742 in combination with either GSK2334470 or 2-*O*-Bn-InsP$_5$ strongly reduced the number of HPAF-II colonies in soft agar assays, when sub-optimal concentrations of each inhibitor were used (Fig. 7f). A similar trend was observed in AsPC-1 (Additional file 2: Figure S6d) and CFPAC-1 (Additional file 2: Figure S6e) cells when 2-*O*-Bn-InsP$_5$ was combined with IPI-549.

Taken together, these data indicate that simultaneous inhibition of the PI3K isoform p110γ and PDK1 affects PDAC cell growth more potently than inhibition of p110γ or PDK1 alone, indicating that combination of drugs targeting the two proteins can enhance their effect.

Discussion

In this study, we identified PDK1 as a novel potential therapeutic target in PDAC. First, our data demonstrated that downregulation of the protein using either pharmacological inhibitors or specific siRNAs reduced PDAC cell numbers and colonies formation in soft agar assays, indicating that PDK1 plays a central role in regulation of PDAC cell growth. Our results are consistent with a previous study reporting that pancreas-specific deletion of *PDPK1* reduced acinar-to-ductal metaplasia, pancreatic intraepithelial neoplasia formation and PDAC formation in a KRas^{G12D}-driven transgenic model [26]. Interestingly, ablation of *PDPK1*1 did not affect lung tumour formation in KRas^{G12D}-driven models of non small cell lung carcinoma [26], suggesting a specific role for the enzyme during PDAC development. Whether PDK1 might act specifically downstream of mutant KRas in the context of pancreatic cancer remains to be established [31]. In this respect, it is worth mentioning that all PDAC cell lines used in our study bear a KRas mutation in G12 [32], possibly providing further evidence of a specific KRas/PDK1 pathway in PDAC [31].

In an effort to define the mechanisms of PDK1-mediated regulation of PDAC cell growth, we observed

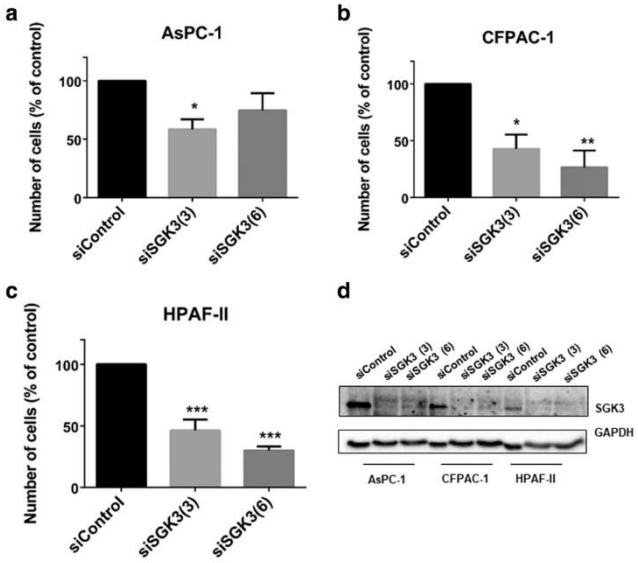


Fig. 6 siRNA-mediated SGK3 downregulation inhibits PDAC cells growth**.** AsPC-1 (**a**), CFPAC-1 (**b**) and HPAF-II (**c**) cells were transfected with two siRNAs specifically targeting SGK3 or a non-targeting siRNA (siControl) and then manually counted with trypan blue exclusion as specified in the Methods. Data are expressed as percentage of cells transfected with siControl and are means ± SEM of n ≥ 3 independent experiments performed in duplicate. T-test and GraphPad Prism version 6.0 were used for statistical analysis. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (**d**) Representative blot confirming efficient SGK3 downregulation in all cell lines. GAPDH was used as loading control

that PDK1 regulated both SGK3 and its downstream effector NDRG1 in PDAC cell lines. Furthermore, downregulation of SGK3 using selective siRNAs reduced PDAC cell numbers, strongly suggesting that PDK1 regulates PDAC cell growth through SGK3 activation, at least partly. These data are consistent with accumulating evidence indicating that PDK1 can contribute to cancer through activation of several downstream effectors. Indeed, while for many years the potential role of this enzyme in cancer was almost exclusively associated with its regulatory role on Akt activation, several recent data have revealed additional roles for PDK1, independently of Akt activation [24, 25]. For instance, it was reported that constitutively active Akt was not able to rescue the reduced anchorage-independent growth or the increased apoptosis resulting from downregulation of PDK1 in breast cancer cells MDA-MB-231 [13]. Importantly, SGK3, that can be regulated through PDK1-dependent phosphorylation of residue Thr320 within its T-loop, has been identified as a key regulator of such PI3K/PDK1-dependent, Akt-independent signalling pathways in cancer [33]. Evidence includes a study demonstrating that PDK1-mediated SGK3 activation was critical for anchorage-independent growth in a subset of *PIK3CA* (the gene encoding for p110α) mutant breast cancer cell lines with minimal Akt activation [23]. Similarly, SGK3 was implicated in Akt-independent oncogenic signalling [33]. Our data strongly suggest that SGK3 is involved in regulation of PDAC cell growth downstream of PDK1. Whether PDK1 controls PDAC growth solely through SGK3 activation, in an Akt-independent mechanism, remains to be established.

Our study further demonstrates that pharmacological inhibition of PDK1, using three distinct chemical compounds, strongly reduced both anchorage-dependent and anchorage-independent PDAC cell growth in vitro. Importantly, we also show that chemical inhibition of PDK1 reduced growth of PDAC cells in vivo.

Several lines of evidence now support the conclusion that PDK1 is an important potential therapeutic target in different cancer types and that its inhibition can prove beneficial to reduce growth of several cancer cell types. For instance, we reported that inhibition of PDK1 with 2-*O*-Bn-InsP_5 was able to reduce cell numbers in different cancer cell lines as well as growth of prostate cancer

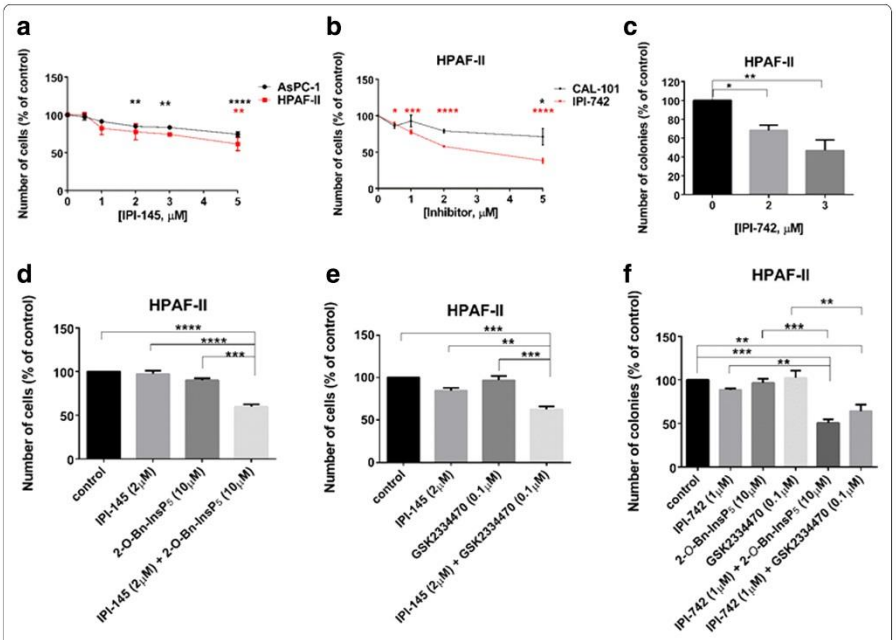


Fig. 7 PDK1 inhibitors enhance the effect of p110δ/γ inhibitors on PDAC cell growth. (**a**,**b**) HPAF-II and AsPC-1 cells were treated with the indicated concentrations of the p110δ/γ inhibitor IPI-145 (**a**). Alternatively, HPAF-II cells were treated with the indicated concentrations of the p110δ/γ inhibitor IPI-742 or the selective p110δ inhibitor CAL-101 (**b**). The number of cells was assessed after 72 h. Data are expressed as percentage of cells treated with vehicle (control) and are means ± SEM of $n = 3$ independent experiments performed in duplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs control cells. (**c**) HPAF-II cells were plated on soft agar and treated with the indicated concentrations of IPI-742. Data are expressed as percentage of colonies from cells treated with vehicle (control) and are means ± SEM of $n = 4$ independent experiments performed in duplicate. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control. (**d**, **e**) HPAF-II cells were treated with IPI-145 (2 μM), 2-O-Bn-$InsP_5$ (10 μM), GSK2334470 (0.1 μM) or the indicated combinations. The number of cells was assessed after 72 h. Data are expressed as percentage of cells treated with vehicle (control) and are means ± SEM of n = 3 independent experiments performed in duplicate. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. (**f**) HPAF-II cells were plated on soft agar and treated with IPI-742 (1 μM), 2-O-Bn-$InsP_5$ (10 μM), GSK2334470 (0.1 μM) or the indicated combinations. Data are expressed as percentage of colonies from cells treated with vehicle (control) and are means ± SEM of $n = 3$ independent experiments performed in duplicate. $^{**}p < 0.01$, $^{***}p < 0.001$

PC3 cells in a xenograft model in nude mice [29]. Similarly, MP7 reduced soft agar colony formation in a subset of cancer cell lines as well as primary tumour xenograft lines [34]. Results from our study further supports the conclusion that PDK1 represents an important molecular target to develop novel therapeutic anti-cancer strategies. More importantly, our results indicate that inhibition of PDK1 can represent a useful strategy to counteract progression of PDAC. As there are very few treatments available for pancreatic cancer patients which provide a very limited increase in survival, these results might represent an important step towards the identification of novel, much needed, therapeutic options for this deadly disease.

Finally, we report that inhibition of PDK1 potentiates the effect of other drugs in PDAC cell lines. We previously demonstrated that the class IB PI3K isoform p110γ is overexpressed in PDAC and it has a critical role in PDAC cell proliferation [5]. Here we show that combination of selective p110γ inhibitors with PDK1-targeting compounds reduced anchorage-dependent and independent-growth of PDAC cells more potently than each treatment alone, when used at sub-optimal concentrations. It remains to be established whether p110γ and PDK1 act on the same or on distinct signalling cascades to regulate PDAC growth. Indeed, the enhanced effect of the combined treatment might be the result of full inhibition of the same signalling pathway, as opposed to the effect of each compound alone that, used

at sub-optimal concentrations, would inhibit the pathway only partially. On the other hand, we cannot rule out the possibility that the combination of the two classes of inhibitors can overcome mechanisms of resistance and result in more pronounced inhibition of PDAC cell growth. In this respect, it is worth mentioning that several lines of evidence now suggest that PDK1 can have a role in cancer chemoresistance and that its inhibition can promote chemosensitization [35]. Interestingly, SGK3 has been linked to development of mechanism of resistance to PI3K and Akt inhibitors [36] therefore it is tempting to speculate that the additive effect that we detected by using combination of p110γ and PDK1 inhibitors might be due to inhibition of potential PDK1/SGK3-mediated intrinsic mechanisms of resistance to p110γ inhibitors. Additional studies are now required to ascertain whether combination of p110γ and PDK1 inhibitors would prove to be more efficient than each single agent administration in in vivo models of PDAC.

Conclusions

In summary, in this study we demonstrated that inhibition of PDK1 reduces PDAC cell growth in vitro and in vivo. These results, together with previous evidence using genetic ablation of *PDPK1*, provide a strong rationale to investigate further the use of PDK1 inhibitors in PDAC as novel therapeutic strategies for pancreatic cancer patients. Our data further suggest that combination of PDK1 inhibitors with selective PI3K inhibitors might enhance their anti-cancer activity, possibly by targeting SGK3-dependent resistance mechanisms.

Additional file

Additional file 1: Synthetic procedure followed for the synthesis of MP7 and characterization of intermediates. (DOCX 129 kb)

Additional file 2: Figure S1. Effect of pharmacological inhibition of PDK1 on CFPAC-1 cells. CFPAC-1 cells were treated with different concentrations of PDK1 inhibitors and their effects on cell viability (a, b) and anchorage independent growth (c, d) were assessed. Data are expressed as percentage of control cells treated with DMSO and are means ± SEM of $n \geq 3$ independent experiments performed in duplicate. Statistical analysis was performed using GraphPad Prism version 6.0 and one-way ANOVA with Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs control. **Figure S2.** Effect of pharmacological PDK1 inhibition on PANC-1 cells. PANC-1 cells were treated with different concentrations of PDK1 inhibitors and their effects on cell viability (a, b) and anchorage independent growth (c, d) were assessed. Data are expressed as percentage of control cells treated with DMSO and are means ± SEM of $n \geq 3$ independent experiments performed in duplicate. Statistical analysis was performed using GraphPad Prism version 6.0 and one-way ANOVA with Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs control. **Figure S3.** Representative images of 3D colonies of AsPC-1 and PANC-1 cells treated with PDK1 inhibitors. Images of AsPC-1 colonies treated with different concentrations of GSK2344470 (a) as well as PANC-1 colonies treated with MP7 (b) and GSK2344470 (c) were acquired using 4X magnification lens. (c) Images of the 6-well plates of PANC-1 colonies treated with GSK2344470 (left) and MP7 (right), as visualized by the ChemiDoc system (BioRad). **Figure S4.** Effect of pharmacologicalInhibition of PDK1 on HPAF-II cells anchorage–independent growth. HPAF-II cells were treated with the indicated concentrations of the PDK1 inhibitors GSK2334470, 2-O-Bn-InsP$_5$ (a) and MP7 (b) and their effects on anchorage-independent growth were determined. Data are expressed as percentage of control cells treated with DMSO. Data in (a) are means ± SEM of $n = 3$ independent experiments performed in duplicate. Statistical analysis was performed using GraphPad Prism version 6.0 and one-way ANOVA with Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$. Data in (b) are means of $n = 2$ independent experiments performed in duplicate. **Figure S5.** Effect of p110δ/γ and p110γ inhibition on PDAC cells. (a,b) AsPC-1 cells were treated with the indicated concentrations of the p110δ/γ inhibitor IPI-712 or the selective p110δ inhibitor CAL-101 (a). Alternatively, PDAC cells AsPC-1 and HPAF-II, together with the two non malignant epithelial pancreatic cell lines hTERT-HPNE and HPDE, were treated with increasing concentrations of the selective p110γ inhibitor IPI-549 (b). Cell viability was assessed after 72 h. Data are expressed as percentage of cells treated with vehicle alone and are means + SEM of $n \geq 3$ independent experiments performed in duplicate. Statistical analysis was performed using GraphPad Prism version 6.0 and one-way ANOVA with Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.0001$ vs control. (c) AsPC-1 and PANC-1 were plated on soft agar and treated with the indicated concentrations of IPI-549. Data are expressed as percentage of cells treated with vehicle alone and are means ± SEM of $n \geq 3$ independent experiments performed in duplicate. Statistical analysis was performed using GraphPad Prism version 6.0 and one-way ANOVA with Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ vs corresponding control. (d) Representative images of the effect of different concentrations of IPI-549 on AsPC-1 cells colony formation (4X magnification lens). **Figure S6.** PDK1 inhibitors enhance the effect of p110δ/γ inhibitors. (a-c) AsPC-1 (a,b) and CFPAC-1 (c) cells were treated with the indicated inhibitors alone or in combination. Cell viability was assessed after 72 h. Data are expressed as percentage of cells treated with vehicle alone and are means ± SEM of $n \geq 3$ independent experiments performed in duplicate. Statistical analysis was performed using GraphPad Prism version 6.0 and one-way ANOVA with Dunnett's multiple comparisons test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (d,e) AsPC-1 (d) and CFPAC-1 (e) cells were plated on soft agar and treated with the indicated inhibitors and their combination. Data are from $n = 2$ independent experiments performed in duplicate. (ZIP 2460 kb)

Abbreviations

2-O-Bn-InsP$_5$: 2-O-benzyl-Inositol (1,3,4,5,6) pentakisphosphate; EGF: Epidermal growth factor; HCC: human hepatocellular carcinoma; NDRG1: N-Myc downstream regulated 1; PDAC: pancreatic ductal adenocarcinoma; PDK1: 3-phosphoinositide-dependent protein kinase 1; PI3K: phosphoinositide 3-kinase; PKB / Akt: protein kinase B; PtdIns(3,4,5)P$_3$: phosphatidylinositol 3,4,5-trisphosphate; phosphatidylinositol 3,4,5-trisphosphate; PtdIns (4, 5) P$_2$: phosphatidylinositol 4,5-bisphosphate; PTEN: phosphatase and tensin homolog; SGK: serum and glucocorticoid-induced protein kinase.

Acknowledgements

We thank GlaxoSmithKline for GSK2334470 and Infinity Pharmaceutical for IPI-145 and IPI-712. We also thank Prof H. Kocher (Queen Mary University of London) for providing HPDE cells. The authors acknowledge the infrastructure and staff support provided by Curtin Health Innovation Research Institute, School of Pharmacy and Biomedical Sciences, Curtin University.

Funding

This work was supported by Pancreatic Cancer Research Fund (grants to M.L.) and Avner Pancreatic Cancer Foundation. C.A.L., R.T., C.E.E. were supported by Pancreatic Cancer Research Fund (grants to M.L.). A.E. is supported by AB Analitica. G.S. was supported by Ministero Sanità Finalizzata 2011/2012.

Availability of data and materials

The data supporting the conclusions of this article are included within the article.

Authors' contributions

AE carried out the bulk of the in vitro experiments and contributed to writing and editing of the manuscript. CAF performed some of the in vitro experiments. RF, CEE, RL performed additional experiments. SS and SR synthesized MP7 used in this study. SI supervised, EC and GS performed the in vivo experiment. TM and MF conceived the idea, supervised the whole project and wrote the manuscript.

Ethics approval and consent to participate
This study has been conducted in accordance with ethical standards and according to the Declaration of Helsinki and the national and international guidelines, and has been approved by the authors' institutional review board. All processes involving animals were approved by the National Animal Ethics Committee (Autorizzazione N 484/2016-PR Ministero della Salute). Animal experiments were performed according to the guidelines of the Experimental Animals Management Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Author details
[1]Metabolic Signalling Group, School of Pharmacy and Biomedical Sciences, Curtin Health Innovation Research Institute, Curtin University, Perth, Western Australia 6102, Australia. [2]Queen Mary University of London, Barts and The London School of Medicine and Dentistry, Blizard Institute, Centre for Cell Biology and Cutaneous Research, F1 2AT, London, UK. [3]Dipartimento di Scienze Mediche, Orali e Biotecnologiche, University G. d'Annunzio di Chieti-Pescara, Centro Studi sull Invecchiamento, CeSI-MeT, 66100 Chieti, Italy. [4]Department of Pharmacy, University of Pisa, Via Bonanno, 6, 56126 Pisa, Italy. [5]MediaPharma Srl, Via della Colonnetta, 50/A, 66100 Chieti, Italy.

Received: 5 November 2018 Accepted: 25 April 2019
Published online: 14 May 2019

References

1. Falasca M, Kim M, Casari I. Pancreatic cancer: current research and future directions. Biochim Biophys Acta. 2016;1865(2):123–32.
2. Adamska A, Domenichini A, Falasca M. Pancreatic ductal adenocarcinoma: current and evolving therapies. Int J Mol Sci. 2017;18(7).
3. Kleeff J, Korc M, Apte M, La Vecchia C, Johnson CD, Biankin AV, et al. Pancreatic cancer. Nat Rev Dis Primers. 2016;2:16022.
4. Falasca M. PI3K/Akt signalling pathway specific inhibitors: a novel strategy to sensitize cancer cells to anti-cancer drugs. Curr Pharm Des. 2010;16(12):1410–6.
5. Edling CE, Selvaggi F, Buus R, Maffucci T, Di Sebastiano P, Friess H, et al. Key role of phosphoinositide 3-kinase class IB in pancreatic cancer. Clinical cancer research. 2010;1078–0432. CCR-10-1210.
6. Zhang S, Liu J, Xu K, Li Z. Notch signaling via regulation of RB and p-AKT but not PIK3CG contributes to MIA PaCa-2 cell growth and migration to affect pancreatic carcinogenesis. Oncol Lett. 2018;15(2):2105–10.
7. Dituri F, Mazzocca A, Lupo L, Edling CE, Azzariti A, Antonaci S, et al. PI3K class IB controls the cell cycle checkpoint promoting cell proliferation in hepatocellular carcinoma. Int J Cancer. 2012;130(11):2505–13.
8. Wong K-K, Engelman JA, Cantley LC. Targeting the PI3K signaling pathway in cancer. Curr Opin Genet Dev. 2010;20(1):87–90.
9. Bayascas JR, Leslie NR, Parsons R, Fleming S, Alessi DR. Hypomorphic mutation of PDK1 suppresses tumorigenesis in PTEN+/− mice. Curr Biol. 2005;15(20):1839–46.
10. Choucair KA, Guérard K-P, Ejdelman J, Chevalier S, Yoshimoto M, Scarlata E, et al. The 16p13. 3 (PDPK1) genomic gain in prostate cancer: a potential role in disease progression. Transl Oncol. 2012;5(6):453–60.
11. Maurer M, Su T, Saal LH, Koujak S, Hopkins BD, Barkley CR, et al. 3-phosphoinositide–dependent kinase 1 potentiates upstream lesions on the phosphatidylinositol 3-kinase pathway in breast carcinoma. Cancer Res. 2009.
12. Raimondi C, Falasca M. Targeting PDK1 in cancer. Curr Med Chem. 2011;18(18):2763–9.
13. Gagliardi PA, di Blasio L, Orso F, Seano G, Sessa R, Taverna D, et al. 3-phosphoinositide-dependent kinase 1 controls breast tumor growth in a kinase-dependent but Akt-independent manner. Neoplasia. 2012;14(8):IN14–IN9.
14. Raimondi C, Chikh A, Wheeler AP, Maffucci T, Falasca M. A novel regulatory mechanism links PLCγ1 to PDK1. J Cell Sci. 2012;jcs. 100511.
15. Scortegagna M, Lau E, Zhang T, Feng Y, Sereduk C, Yin H, et al. PDK1 and SGK3 contribute to the growth of BRAF-mutant melanomas and are potential therapeutic targets. Cancer Res. 2015.
16. Liu Y, Wang J, Wu M, Wan W, Sun R, Yang D, et al. Down-regulation of 3-phosphoinositide–dependent protein Kinase-1 levels inhibits migration and experimental metastasis of human breast Cancer cells. Mol Cancer Res. 2009.
17. Moxley KM, Wang L, Welm AL, Bieniasz M. Short-form Ron is a novel determinant of ovarian cancer initiation and progression. Genes Cancer. 2016;7(5–6):169–81.
18. Wu YH, Chang TH, Huang YF, Chen CC, Chou CY. COL11A1 confers chemoresistance on ovarian cancer cells through the activation of Akt/c/EBPbeta pathway and PDK1 stabilization. Oncotarget. 2015;6(27):23748–63.
19. Falasca M, Chiozzotto D, Godage HY, Mazzoletti M, Riley AM, Previdi S, et al. A novel inhibitor of the PI3K/Akt pathway based on the structure of inositol 1,3,4,5,6-pentakisphosphate. Br J Cancer. 2010;102(11):104–14.
20. Wang J, Liu F, Ao P, Li X, Zheng H, Wu D, et al. Correlation of PDK1 expression with clinicopathologic features and prognosis of hepatocellular carcinoma. Onco Targets Ther. 2016;9:5597–602.
21. Mora A, Komander D, van Aalten DM, Alessi DR, editors. PDK1, the master regulator of AGC kinase signal transduction. Seminars in cell & developmental biology; 2004: Elsevier.
22. Liu M, Chen L, Chan THM, Wang J, Li Y, Zeng TT, et al. Serum and glucocorticoid kinase 3 at 8q13. 1 promotes cell proliferation and survival in hepatocellular carcinoma. Hepatology. 2012;55(6):1754–65.
23. Vasudevan KM, Barbie DA, Davies MA, Rabinovsky R, McNear CJ, Kim JJ, et al. AKT-independent signaling downstream of oncogenic PIK3CA mutations in human cancer. Cancer Cell. 2009;16(1):21–32.
24. Lien EC, Dibble CC, Toker A. PI3K signaling in cancer: beyond AKT. Curr Opin Cell Biol. 2017;45:62–71.
25. Di Blasio L, Gagliardi PA, Puliafito A, Primo L. Serine/threonine kinase 3-phosphoinositide dependent protein kinase 1 (PDK1) as a key regulator of cell migration and cancer dissemination. Cancers. 2017;9(3):25.
26. Eser S, Reiff N, Messer M, Seidler B, Gottschalk K, Dobler M, et al. Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer. Cancer Cell. 2013;23(3):406–20.
27. Erlanson DA, Arndt JW, Cancilla MT, Cao K, Elling RA, English N, et al. Discovery of a potent and highly selective PDK1 inhibitor via fragment-based drug discovery. Bioorg Med Chem Lett. 2011;21(10):3078–83.
28. Najafov A, Sommer EM, Axten JM, Deyoung MP, Alessi DR. Characterization of GSK2334470, a novel and highly specific inhibitor of PDK1. Biochem J. 2011;433(2):357–69.
29. Falasca M, Chiozzotto D, Godage H, Mazzoletti M, Riley A, Previdi S, et al. A novel inhibitor of the PI3K/Akt pathway based on the structure of inositol 1, 3, 4, 5, 6-pentakisphosphate. Br J Cancer. 2010;102(1):104.
30. Gasser JA, Inuzuka H, Lau AW, Wei W, Beroukhim R, Toker A. SGK3 mediates INPP4B-dependent PI3K signaling in breast cancer. Mol Cell. 2014;56(4):595–607.
31. Ferro R, Falasca M. Emerging role of the KRAS-PDK1 axis in pancreatic cancer. World J Gastroenterol. 2014;20(31):10752–7.
32. Deer EL, Gonzalez-Hernandez J, Coursen JD, Shea JE, Ngatia J, Scaife CL, et al. Phenotype and genotype of pancreatic cancer cell lines. Pancreas. 2010;39(4):425–35.
33. Bruhn MA, Pearson RB, Hannan RD, Sheppard KE. AKT-independent PI3-K signaling in cancer–emerging role for SGK3. Cancer Manag Res. 2013;5:281.
34. Nagashima K, Shumway SD, Sathyanarayanan S, Chen AH, Dolinski B, Xu Y, et al. Genetic and pharmacological inhibition of PDK1 in cancer cells characterization of a selective allosteric kinase inhibitor. J Biol Chem. 2011;286(8):6433–48.
35. Emmanouilidi A, Falasca M. Targeting PDK1 for Chemosensitization of Cancer cells. Cancers (Basel). 2017;9(10).
36. Bago R, Sommer E, Castel P, Crafter C, Bailey FP, Shpiro N, et al. The hVps34-SGK3 pathway alleviates sustained PI3K/Akt inhibition by stimulating mTORC1 and tumour growth. EMBO J. 2016;35(17):1902–22.

CHAPTER 5

Chapter 5: General discussion, limitations and future directions

Despite the advances in the field of oncology and our understanding of the molecular biology behind pancreatic cancer, this disease still has a very dismal prognosis and comprises a great challenge in regards to early diagnosis. The overwhelming majority of PDAC patients receiving standard chemotherapy with gemcitabine, gradually presents with signs of chemoresistance and regression (Oettle, Post et al. 2007, Neoptolemos, Kleeff et al. 2018). Although gemcitabine has been the first treatment option for PDAC patients for more than a decade, two new combination regimens have been shown to improve overall survival in clinical trials. More specifically FOLFIRINOX- a combination of folinic acid, fluorouracil, irinotecan and oxaliplatin, and nab-paclitaxel (Abraxane) together with gemcitabine, have shown to surpass the efficacy of gemcitabine as monotherapy. (Fernández, Salgado et al. 2018, Kim, Signorovitch et al. 2018). Factors that affect the selection of one therapy over the other include tumour burden and biology. Due to the lower toxicity of gemcitabine/Abraxane compared to FOLFIRINOX, it is the treatment of preference in case of higher CA19.9 levels and therefore higher tumour burden (Shi and Yu 2019).

Extracellular vesicles, namely exosomes, are known to be key factors in intracellular homeostasis and intercellular communication, and depending on their cargo they can exert pro- or anti-tumorigenic functions. They comprise 30-150nm late endosomes-originating vesicles which attracted the interest of the scientific community in 1983. Being secreted by the majority of cell types, they contain a variety of molecules such as DNA, RNA types, lipids and proteins, all enclosed in their lipid bilayer membrane. Their cargo and their properties have made them appealing tools for liquid biopsies development as well as therapeutic agents (Batista and Melo 2019). Two studies published in 2015 have put the cancer-derived exosomes in centre stage of a major hallmark of cancer; metastasis. The first study successfully described how PDAC-exosomes containing the macrophage migration inhibitory factor (MIF) educate liver cells towards pre-metastatic niche formation. More specifically, they instigate TGFβ production from Kupffer cells, which activates the hepatic stellate cells and leads to fibronectin production, ultimately resulting in migration of bone marrow cells to the liver and formation of a pre-metastatic niche. MIF levels in the exosomes were found to be higher in early stage PDAC patients, than those with an already established liver metastasis, underscoring the potential of exosomal MIF as a prognostic biomarker (Costa-Silva, Aiello et al. 2015). The second study proved that the choice of the metastatic site is not a random event, but is in fact governed by the exosomal integrins. For instance, ITG$\alpha_6\beta_4$ / ITG$\alpha_6\beta_1$-containing exosomes dictate lung tropism

whereas ITG$\alpha_v\beta_5$ ones show a preference towards liver cells. Moreover, the S100 proteins family overexpression was also organ specific (Hoshino, Costa-Silva et al. 2015).

The main scope of this thesis was to characterize PDAC-derived exosomes both in regards to their proteomic and lipidomic cargo, in order to detect proteins and lipids of interest, or a PDAC-specific exosomal signature. A significant challenge of this PhD was in regards to the exosome isolation and characterization procedure, as those techniques were not previously developed in the lab.

In Chapter 2 of this thesis, exosomes derived from pancreatic epithelial (HPDE, hTERT-HPNE) and pancreatic cancer (AsPC-1, BxPC-3, MiaPaCa-II) cell lines were characterised according to MISEV criteria and together with their donor cells they were submitted to mass spectrometry proteomic analysis. Overall, the cells protein content was very distinct from the exosomal one, and also the malignant exosomes cargo was very different from the pancreatic epithelial ones. Our malignant exosomal preparations were enriched in proteins known to be key players in metastasis such as integrins and members of the S100 family, previously verified PDAC biomarkers such as GPC-1 and MIF, as well as proteins involved in a number of cancer hallmarks. Selected proteins which could be of diagnostic value due to their specific enrichment in the malignant exosomes were verified with Western Blotting. The significance of this study is that it analysed and compared malignant versus healthy state, and exosomes versus donor cells at the same time, whereas most studies focus solely on extracellular vesicles and oversee the source. Of course, it has to be taken into account that although this is a solid starting point for biomarkers discovery, cell lines are not always representative of the actual tumour, as PDAC is a very heterogeneous disease. Future studies should focus on verifying those findings in *in vivo* models, such as KPC mice, which successfully recapitulate the progress of the disease in humans. Upon successful completion of those studies, verification studies should be done in human blood and / or tissue samples, from different PanIN stages, as well as IPMNs and healthy donors if possible.

In Chapter 3, exosomes and cells of pancreatic, prostate and ovarian cancer cell lines as well as their respective healthy controls were analysed with LC-ESI MS/MS in order to characterize their lipidomic cargo and detect outstanding differences. Indeed, we detected a specific enrichment of CE in PDAC-derived exosomes. Previous studies have revealed aberrant CE accumulation in tissues of prostate cancer patients compared to healthy individuals, a phenomenon attributed to the enhanced activation

of the PI3K/ Akt pathway and the inevitable impact on COH homeostasis through SREBPs and ACAT-1 regulation. In this study, CE accumulation was correlated with increased aggressiveness of the disease (Yue, Li et al. 2014). Earlier studies had revealed that $CD4^+$ T cells-derived exosomes could be internalised by cultured THP-1 cells via PS receptors present on the membrane of the latter, and induce COH accumulation (Zakharova, Svetlova et al. 2007). Following this observation downstream experiments were performed, where $CD4^+$ and $CD8^+$ T cells isolated from healthy donors blood were co-incubated with exosomes derived from avasimibe-treated PDAC cells, and their proliferation and cytokine expression was assessed. Those are lengthy assays involving many steps, and the outcome showed variation. Preliminary data were promising, however they should be validated as part of the future work, and potentially some steps could be included towards protocol optimization. For instance, a pre-enrichment could be performed before the cell sorting, with the use of a pan-T cell marker such as CD3, available in commercial kits. This is an effective way of quickly separating the T cell population from platelets, neutrophils and other blood cells, and minimizing the cell sorting time, but also exclude cell types known to secrete a number of cytokines including IL-8, IFN-γ and IL-10 (Fauriat, Long et al. 2010). In this way, the variability of the cytokines expression assay could be controlled. Another parameter to consider is that although the kit used in this experiments detects a total of 13 cytokines, there are much more of those molecules in the human body which affect one another and are kept in a balance through strict mechanisms. For instance, IL-18 was among the cytokines discussed in Chapter 3. Nevertheless, IL-37 (not included in the available kit) is able to inhibit IL-18 downstream signalling by hampering the recruitment to its receptor (Nakanishi 2018). At the same time, the IL-18BP could be quantified, in order to determine the free IL-18, as described in (Novick, Schwartsburd et al. 2001). Of course all the above apply in case it is decided that this specific cytokine requires further attention, however the approach is the same for all other cytokines detected in this study. A major factor which impacts the T cells behaviour (including both proliferation and cytokine release) is the actual donor. A common flu can cause T cell exhaustion and therefore hamper *in vitro* proliferation despite external co-stimulation with CD3 and CD28 antibodies. The metabolism of the T cells is regulated both at transcriptional and posttranscriptional level, and the different behaviour of the two T cells subsets of our experiment could possibly be explained to a certain extent by this fact (MacIver, Michalek et al. 2013). Future experiments could involve lipidomic analysis of exosomes derived from patient derived xenografts, which better reflect the tumour microenvironment (Tentler, Tan et al. 2012), and further examination of their effects

on immune system cells. In addition to this, exosomes can be isolated from the plasma of healthy mice and KPC untreated mice (Lee, Komar et al. 2016) in order to confirm a similar pattern in CE levels.

In Chapter 4 we genetically and pharmacologically downregulated PDK1 *in vitro* in a number of PDAC cell lines bearing the *Kras*^{G12D} mutation, and we observed reduced anchorage-dependent and independent growth in all cases. One of the compounds, MP7, was also tested *in vivo* and was able to reduce tumour volume without adverse effects such as weight loss. Up to date there is no PDK1 inhibitor available in the clinic, as the currently investigated molecules exhibit solubility and pharmacokinetic profile issues, as well as side effects. In our study we demonstrated that PDK1 acts through SGK3 in the context of pancreatic cancer, and that it has a unique signal that can be Akt independent. Our study is the first one to combine simultaneous inhibition of the PI3K p110γ isoform and PK1; administration of both inhibitors in suboptimal concentrations successfully hampered PDAC cells growth both in an anchorage-dependent and independent manner. This proves that PDK1 inhibition in pancreatic cancer is not redundant and that dual inhibitors could be developed and tested *in vivo*, for better outcomes.

Bibliography

Abiko, K., N. Matsumura, J. Hamanishi, N. Horikawa, R. Murakami, K. Yamaguchi, Y. Yoshioka, T. Baba, I. Konishi and M. Mandai (2015). "IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer." British journal of cancer **112**(9): 1501.

Accioly, M. T., P. Pacheco, C. M. Maya-Monteiro, N. Carrossini, B. K. Robbs, S. S. Oliveira, C. Kaufmann, J. A. Morgado-Diaz, P. T. Bozza and J. P. Viola (2008). "Lipid bodies are reservoirs of cyclooxygenase-2 and sites of prostaglandin-E2 synthesis in colon cancer cells." Cancer research **68**(6): 1732-1740.

Adam, R. M., N. K. Mukhopadhyay, J. Kim, D. Di Vizio, B. Cinar, K. Boucher, K. R. Solomon and M. R. Freeman (2007). "Cholesterol sensitivity of endogenous and myristoylated Akt." Cancer research **67**(13): 6238-6246.

AICR. (2018). "Pancreatic cancer statistics." from https://www.wcrf.org/dietandcancer/cancer-trends/pancreatic-cancer-statistics.

Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts and J. Watson (1994). "Molecular biology of the cell Garland." New York: 18-19.

Alberts, D. S., C. Marth, R. D. Alvarez, G. Johnson, M. Bidzinski, D. R. Kardatzke, W. Z. Bradford, J. Loutit, D. H. Kirn and M. C. Clouser (2008). "Randomized phase 3 trial of interferon gamma-1b plus standard carboplatin/paclitaxel versus carboplatin/paclitaxel alone for first-line treatment of advanced ovarian and primary peritoneal carcinomas: results from a prospectively designed analysis of progression-free survival." Gynecologic oncology **109**(2): 174-181.

Alvarez, M. L., M. Khosroheidari, R. K. Ravi and J. K. DiStefano (2012). "Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers." Kidney international **82**(9): 1024-1032.

Antonucci, F., E. Turola, L. Riganti, M. Caleo, M. Gabrielli, C. Perrotta, L. Novellino, E. Clementi, P. Giussani and P. Viani (2012). "Microvesicles released from microglia stimulate synaptic activity via enhanced sphingolipid metabolism." The EMBO journal **31**(5): 1231-1240.

Aparicio-Pagés, M., H. Verspaget, A. Peña, J. Jansen and C. Lamers (1991). "Natural killer cell activity in patients with neuroendocrine tumours of the gastrointestinal tract; relation with circulating gastrointestinal hormones." Neuropeptides **20**(1): 1-7.

Ardito, C. M., B. M. Grüner, K. K. Takeuchi, C. Lubeseder-Martellato, N. Teichmann, P. K. Mazur, K. E. DelGiorno, E. S. Carpenter, C. J. Halbrook and J. C. Hall (2012). "EGF receptor is required for KRAS-induced pancreatic tumorigenesis." Cancer cell **22**(3): 304-317.

Atay, S., C. Gercel-Taylor, J. Suttles, G. Mor and D. D. Taylor (2011). "Trophoblast-derived exosomes mediate monocyte recruitment and differentiation." American journal of reproductive immunology **65**(1): 65-77.

Auciello, F. R., V. Bulusu, C. Oon, J. Tait-Mulder, M. Berry, S. Bhattacharyya, S. Tumanov, B. L. Allen-Petersen, J. Link and N. D. Kendsersky (2019). "A stromal lysolipid–autotaxin signaling axis promotes pancreatic tumor progression." Cancer discovery **9**(5): 617-627.

Awasthi, N., S. Monahan, A. Stefaniak, M. A. Schwarz and R. E. Schwarz (2018). "Inhibition of the MEK/ERK pathway augments nab-paclitaxel-based chemotherapy effects in preclinical models of pancreatic cancer." Oncotarget **9**(4): 5274.

Baietti, M. F., Z. Zhang, E. Mortier, A. Melchior, G. Degeest, A. Geeraerts, Y. Ivarsson, F. Depoortere, C. Coomans and E. Vermeiren (2012). "Syndecan–syntenin–ALIX regulates the biogenesis of exosomes." Nature cell biology **14**(7): 677.

Bakhti, M., C. Winter and M. Simons (2011). "Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles." Journal of Biological Chemistry **286**(1): 787-796.

Balaj, L., R. Lessard, L. Dai, Y.-J. Cho, S. L. Pomeroy, X. O. Breakefield and J. Skog (2011). "Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences." Nature communications **2**: 180.
Barile, L., V. Lionetti, E. Cervio, M. Matteucci, M. Gherghiceanu, L. M. Popescu, T. Torre, F. Siclari, T. Moccetti and G. Vassalli (2014). "Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction." Cardiovascular research **103**(4): 530-541.
Basturk, O., I. Coban and N. V. Adsay (2010). Pathologic Classification and Biological Behavior of Pancreatic Neoplasia. Pancreatic Cancer. New York, NY, Springer New York**:** 39-70.
Batista, I. A. and S. A. Melo (2019). "Exosomes and the future of immunotherapy in pancreatic cancer." International journal of molecular sciences **20**(3): 567.
Battke, C., R. Ruiss, U. Welsch, P. Wimberger, S. Lang, S. Jochum and R. Zeidler (2011). "Tumour exosomes inhibit binding of tumour-reactive antibodies to tumour cells and reduce ADCC." Cancer immunology, immunotherapy **60**(5): 639-648.
Bishop, N. and P. Woodman (2001). "TSG101/mammalian VPS23 and mammalian VPS28 interact directly and are recruited to VPS4-induced endosomes." Journal of Biological Chemistry **276**(15): 11735-11742.
Blackford, A., G. Parmigiani, T. W. Kensler, C. Wolfgang, S. Jones, X. Zhang, D. W. Parsons, J. C.-H. Lin, R. J. Leary and J. R. Eshleman (2009). "Genetic mutations associated with cigarette smoking in pancreatic cancer." Cancer research **69**(8): 3681-3688.
Blanc, L., C. Barres, P. Bette-Bobillo and M. Vidal (2007). "Reticulocyte-secreted exosomes bind natural IgM antibodies: involvement of a ROS-activatable endosomal phospholipase iPLA2." Blood **110**(9): 3407-3416.
Blanchard, N., D. Lankar, F. Faure, A. Regnault, C. Dumont, G. Raposo and C. Hivroz (2002). "TCR activation of human T cells induces the production of exosomes bearing the TCR/CD3/ζ complex." The Journal of Immunology **168**(7): 3235-3241.
Buscail, E., C. Alix-Panabières, P. Quincy, T. Cauvin, A. Chauvet, O. Degrandi, C. Caumont, S. Verdon, I. Lamrissi and I. J. C. Moranvillier (2019). "High Clinical Value of Liquid Biopsy to Detect Circulating Tumor Cells and Tumor Exosomes in Pancreatic Ductal Adenocarcinoma Patients Eligible for Up-Front Surgery." **11**(11): 1656.
Busnardo, A. C., L. J. DiDio, R. T. Tidrick and N. R. Thomford (1983). "History of the pancreas." The American journal of surgery **146**(5): 539-550.
Caby, M. P., D. Lankar, C. Vincendeau-Scherrer, G. Raposo and C. Bonnerot (2005). "Exosomal-like vesicles are present in human blood plasma." Int Immunol **17**(7): 879-887.
Cai, S.-W., S.-Z. Yang, J. Gao, K. Pan, J.-Y. Chen, Y.-L. Wang, L.-X. Wei and J.-H. Dong (2011). "Prognostic significance of mast cell count following curative resection for pancreatic ductal adenocarcinoma." Surgery **149**(4): 576-584.
Campbell, P. J., S. Yachida, L. J. Mudie, P. J. Stephens, E. D. Pleasance, L. A. Stebbings, L. A. Morsberger, C. Latimer, S. McLaren, M. L. Lin, D. J. McBride, I. Varela, S. A. Nik-Zainal, C. Leroy, M. Jia, A. Menzies, A. P. Butler, J. W. Teague, C. A. Griffin, J. Burton, H. Swerdlow, M. A. Quail, M. R. Stratton, C. Iacobuzio-Donahue and P. A. Futreal (2010). "The patterns and dynamics of genomic instability in metastatic pancreatic cancer." Nature **467**(7319): 1109-1113.
Canon, J., K. Rex, A. Y. Saiki, C. Mohr, K. Cooke, D. Bagal, K. Gaida, T. Holt, C. G. Knutson and N. J. N. Koppada (2019). "The clinical KRAS (G12C) inhibitor AMG 510 drives anti-tumour immunity." **575**(7781): 217-223.
Capello, M., J. V. Vykoukal, H. Katayama, L. E. Bantis, H. Wang, D. L. Kundnani, C. Aguilar-Bonavides, M. Aguilar, S. C. Tripathi and D. S. Dhillon (2019). "Exosomes harbor B cell targets in pancreatic adenocarcinoma and exert decoy function against complement-mediated cytotoxicity." Nature communications **10**(1): 254.
Carbone, A., U. Rodeck, F. A. Mauri, M. Sozzi, F. Gaspari, C. Smirne, A. Prati, A. Addeo, A. Novarino and A. Robecchi (2005). "Human pancreatic carcinoma cells secrete bioactive

interleukin-18 after treatment with 5-fluorouracil: implications for anti-tumor immune response." Cancer biology & therapy **4**(2): 239-249.
Carbone, A., B. Vizio, A. Novarino, F. A. Mauri, M. Geuna, C. Robino, G. Brondino, A. Prati, A. Giacobino and D. Campra (2009). "IL-18 paradox in pancreatic carcinoma: elevated serum levels of free IL-18 are correlated with poor survival." Journal of Immunotherapy **32**(9): 920-931.
Cassatella, M. A. (1999). Neutrophil-derived proteins: selling cytokines by the pound. Advances in immunology, Elsevier. **73:** 369-509.
Castro, F., A. P. Cardoso, R. M. Gonçalves, K. Serre and M. J. Oliveira (2018). "Interferon-gamma at the crossroads of tumor immune surveillance or evasion." Frontiers in immunology **9**: 847.
Chandler, W. L., W. Yeung and J. F. Tait (2011). "A new microparticle size calibration standard for use in measuring smaller microparticles using a new flow cytometer." J Thromb Haemost **9**(6): 1216-1224.
Chari, S. T., C. L. Leibson, K. G. Rabe, J. Ransom, M. De Andrade and G. M. Petersen (2005). "Probability of pancreatic cancer following diabetes: a population-based study." Gastroenterology **129**(2): 504-511.
Chen, C., J. Skog, C.-H. Hsu, R. T. Lessard, L. Balaj, T. Wurdinger, B. S. Carter, X. O. Breakefield, M. Toner and D. Irimia (2010). "Microfluidic isolation and transcriptome analysis of serum microvesicles." Lab on a Chip **10**(4): 505-511.
Chen, G., A. C. Huang, W. Zhang, G. Zhang, M. Wu, W. Xu, Z. Yu, J. Yang, B. Wang and H. Sun (2018). "Exosomal PD-L1 contributes to immunosuppression and is associated with anti-PD-1 response." Nature **560**(7718): 382.
Chen, L., J. Fan, H. Chen, Z. Meng, Z. Chen, P. Wang and L. Liu (2014). "The IL-8/CXCR1 axis is associated with cancer stem cell-like properties and correlates with clinical prognosis in human pancreatic cancer cases." Scientific reports **4**: 5911.
Chen, T., J. Guo, M. Yang, X. Zhu and X. Cao (2011). "Chemokine-containing exosomes are released from heat-stressed tumor cells via lipid raft-dependent pathway and act as efficient tumor vaccine." The Journal of Immunology: 1002991.
Chen, W.-x., Y.-q. Cai, M.-m. Lv, L. Chen, S.-l. Zhong, T.-f. Ma, J.-h. Zhao and J.-h. Tang (2014). "Exosomes from docetaxel-resistant breast cancer cells alter chemosensitivity by delivering microRNAs." Tumor Biology **35**(10): 9649-9659.
Chen, Z.-f., Z. Rui, X. Bing and C.-s. Deng (2012). "Interleukin-18 and-12 synergistically enhance cytotoxic functions of tumor-infiltrating lymphocytes." Chinese medical journal **125**(23): 4245-4248.
Cicero, A. L., P. D. Stahl and G. Raposo (2015). "Extracellular vesicles shuffling intercellular messages: for good or for bad." Current opinion in cell biology **35**: 69-77.
Ciravolo, V., V. Huber, G. C. Ghedini, E. Venturelli, F. Bianchi, M. Campiglio, D. Morelli, A. Villa, P. D. Mina and S. Menard (2012). "Potential role of HER2-overexpressing exosomes in countering trastuzumab-based therapy." Journal of cellular physiology **227**(2): 658-667.
Clark, C. E., S. R. Hingorani, R. Mick, C. Combs, D. A. Tuveson and R. H. Vonderheide (2007). "Dynamics of the immune reaction to pancreatic cancer from inception to invasion." Cancer research **67**(19): 9518-9527.
Clayton, A., C. L. Harris, J. Court, M. D. Mason and B. P. Morgan (2003). "Antigen-presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59." Eur J Immunol **33**(2): 522-531.
Clerc, P., N. Bensaadi, P. Pradel, A. Estival, F. Clemente and N. Vaysse (1991). "Lipid-dependent proliferation of pancreatic cancer cell lines." Cancer research **51**(14): 3633-3638.
Collisson, E. A., C. L. Trejo, J. M. Silva, S. Gu, J. E. Korkola, L. M. Heiser, R.-P. Charles, B. A. Rabinovich, B. Hann and D. Dankort (2012). "A central role for RAF→ MEK→ ERK signaling in the genesis of pancreatic ductal adenocarcinoma." Cancer discovery.

Corcos, L. and C. Le Jossic-Corcos (2013). "Statins: perspectives in cancer therapeutics." Digestive and liver disease **45**(10): 795-802.
Coren, L. V., T. Shatzer and D. E. Ott (2008). "CD45 immunoaffinity depletion of vesicles from Jurkat T cells demonstrates that exosomes contain CD45: no evidence for a distinct exosome/HIV-1 budding pathway." Retrovirology **5**(1): 64.
Costa-Silva, B., N. M. Aiello, A. J. Ocean, S. Singh, H. Zhang, B. K. Thakur, A. Becker, A. Hoshino, M. T. Mark and H. Molina (2015). "Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver." Nature cell biology **17**(6): 816.
Cui, S. Y., R. Wang and L. B. Chen (2014). "Micro RNA-145: a potent tumour suppressor that regulates multiple cellular pathways." Journal of cellular and molecular medicine **18**(10): 1913-1926.
Curl, C. L., C. J. Bellair, T. Harris, B. E. Allman, P. J. Harris, A. G. Stewart, A. Roberts, K. A. Nugent and L. M. Delbridge (2005). "Refractive index measurement in viable cells using quantitative phase-amplitude microscopy and confocal microscopy." Cytometry A **65**(1): 88-92.
Dahl, F., J. Stenberg, S. Fredriksson, K. Welch, M. Zhang, M. Nilsson, D. Bicknell, W. F. Bodmer, R. W. Davis and H. Ji (2007). "Multigene amplification and massively parallel sequencing for cancer mutation discovery." Proc Natl Acad Sci U S A **104**(22): 9387-9392.
Davidson, S. M., K. Takov and D. M. Yellon (2017). "Exosomes and cardiovascular protection." Cardiovascular drugs and therapy **31**(1): 77-86.
de Candia, P., A. Torri, T. Gorletta, M. Fedeli, E. Bulgheroni, C. Cheroni, F. Marabita, M. Crosti, M. Moro and E. Pariani (2013). "Intracellular modulation, extracellular disposal and serum increase of MiR-150 mark lymphocyte activation." PloS one **8**(9): e75348.
de Gonzalez, A. B., S. Sweetland and E. Spencer (2003). "A meta-analysis of obesity and the risk of pancreatic cancer." British journal of cancer **89**(3): 519.
Del Conde, I., C. N. Shrimpton, P. Thiagarajan and J. A. López (2005). "Tissue-factor–bearing microvesicles arise from lipid rafts and fuse with activated platelets to initiate coagulation." Blood **106**(5): 1604-1611.
Delmas, D., C. Rebe, O. Micheau, A. Athias, P. Gambert, S. Grazide, G. Laurent, N. Latruffe and E. Solary (2004). "Redistribution of CD95, DR4 and DR5 in rafts accounts for the synergistic toxicity of resveratrol and death receptor ligands in colon carcinoma cells." Oncogene **23**(55): 8979.
Diaz-Montero, C. M., M. L. Salem, M. I. Nishimura, E. Garrett-Mayer, D. J. Cole and A. J. Montero (2009). "Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin–cyclophosphamide chemotherapy." Cancer immunology, immunotherapy **58**(1): 49-59.
Diehl, F., M. Li, D. Dressman, Y. He, D. Shen, S. Szabo, L. A. Diaz, Jr., S. N. Goodman, K. A. David, H. Juhl, K. W. Kinzler and B. Vogelstein (2005). "Detection and quantification of mutations in the plasma of patients with colorectal tumors." Proc Natl Acad Sci U S A **102**(45): 16368-16373.
Ding, G., L. Zhou, Y. Qian, M. Fu, J. Chen, J. Chen, J. Xiang, Z. Wu, G. Jiang and L. Cao (2015). "Pancreatic cancer-derived exosomes transfer miRNAs to dendritic cells and inhibit RFXAP expression via miR-212-3p." Oncotarget **6**(30): 29877.
DoH. (2014). from http://www.health.gov.au/internet/main/publishing.nsf/content/health-pubhlth-strateg-hlthwt-obesity.htm. .
Dragovic, R. A., C. Gardiner, A. S. Brooks, D. S. Tannetta, D. J. Ferguson, P. Hole, B. Carr, C. W. Redman, A. L. Harris, P. J. Dobson, P. Harrison and I. L. Sargent (2011). "Sizing and phenotyping of cellular vesicles using Nanoparticle Tracking Analysis." Nanomedicine **7**(6): 780-788.
Dressman, D., H. Yan, G. Traverso, K. W. Kinzler and B. Vogelstein (2003). "Transforming single DNA molecules into fluorescent magnetic particles for detection and enumeration of genetic variations." Proc Natl Acad Sci U S A **100**(15): 8817-8822.

Dumstrei, K., H. Chen and H. Brenner (2016). "A systematic review of serum autoantibodies as biomarkers for pancreatic cancer detection." Oncotarget **7**(10): 11151.
Dunn, G. P., A. T. Bruce, H. Ikeda, L. J. Old and R. D. Schreiber (2002). "Cancer immunoediting: from immunosurveillance to tumor escape." Nature immunology **3**(11): 991.
Dunn, G. P., L. J. Old and R. D. Schreiber (2004). "The three Es of cancer immunoediting." Annu. Rev. Immunol. **22**: 329-360.
Ealick, S. E., W. J. Cook, S. Vijay-Kumar, M. Carson, T. L. Nagabhushan, P. P. Trotta and C. E. Bugg (1991). "Three-dimensional structure of recombinant human interferon-gamma." Science **252**(5006): 698-702.
Eberlin, L. S., M. Gabay, A. C. Fan, A. M. Gouw, R. J. Tibshirani, D. W. Felsher and R. N. Zare (2014). "Alteration of the lipid profile in lymphomas induced by MYC overexpression." Proceedings of the National Academy of Sciences **111**(29): 10450-10455.
Edling, C. E., F. Selvaggi, R. Buus, T. Maffucci, P. Di Sebastiano, H. Friess, P. Innocenti, H. M. Kocher and M. Falasca (2010). "Key role of phosphoinositide 3-kinase class IB in pancreatic cancer." Clinical cancer research: 1078-0432. CCR-1010-1210.
Eitan, E., C. Suire, S. Zhang and M. P. Mattson (2016). "Impact of lysosome status on extracellular vesicle content and release." Ageing research reviews **32**: 65-74.
El Shikh, M. E. and C. Pitzalis (2012). "Follicular dendritic cells in health and disease." Front Immunol **3**: 292.
Elfeky, O., S. Longo, A. Lai, G. E. Rice and C. Salomon (2017). "Influence of maternal BMI on the exosomal profile during gestation and their role on maternal systemic inflammation." Placenta **50**: 60-69.
Ene–Obong, A., A. J. Clear, J. Watt, J. Wang, R. Fatah, J. C. Riches, J. F. Marshall, J. Chin–Aleong, C. Chelala and J. G. Gribben (2013). "Activated pancreatic stellate cells sequester CD8+ T cells to reduce their infiltration of the juxtatumoral compartment of pancreatic ductal adenocarcinoma." Gastroenterology **145**(5): 1121-1132.
Erkan, M., G. Adler, M. V. Apte, M. G. Bachem, M. Buchholz, S. Detlefsen, I. Esposito, H. Friess, T. M. Gress and H.-J. Habisch (2012). StellaTUM: current consensus and discussion on pancreatic stellate cell research, BMJ Publishing Group.
Eser, S., N. Reiff, M. Messer, B. Seidler, K. Gottschalk, M. Dobler, M. Hieber, A. Arbeiter, S. Klein and B. Kong (2013). "Selective requirement of PI3K/PDK1 signaling for Kras oncogene-driven pancreatic cell plasticity and cancer." Cancer cell **23**(3): 406-420.
Eser, S., A. Schnieke, G. Schneider and D. Saur (2014). "Oncogenic KRAS signalling in pancreatic cancer." British journal of cancer **111**(5): 817.
Fahy, E., D. Cotter, M. Sud and S. Subramaniam (2011). "Lipid classification, structures and tools." Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids **1811**(11): 637-647.
Falasca, M., D. Chiozzotto, H. Godage, M. Mazzoletti, A. Riley, S. Previdi, B. Potter, M. Broggini and T. Maffucci (2010). "A novel inhibitor of the PI3K/Akt pathway based on the structure of inositol 1, 3, 4, 5, 6-pentakisphosphate." British Journal of Cancer **102**(1): 104.
Fauriat, C., E. O. Long, H.-G. Ljunggren and Y. T. Bryceson (2010). "Regulation of human NK-cell cytokine and chemokine production by target cell recognition." Blood **115**(11): 2167-2176.
Fernández, A., M. Salgado, A. García, E. Buxò, R. Vera, J. Adeva, P. Jiménez-Fonseca, G. Quintero, C. Llorca and M. J. B. c. Cañabate (2018). "Prognostic factors for survival with nab-paclitaxel plus gemcitabine in metastatic pancreatic cancer in real-life practice: the ANICE-PaC study." **18**(1): 1185.
Fernandis, A. Z. and M. R. Wenk (2009). "Lipid-based biomarkers for cancer." Journal of Chromatography B **877**(26): 2830-2835.
Feron, O. (2009). "Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells." Radiotherapy and oncology **92**(3): 329-333.

Fisher, W. E. (2001). "Diabetes: risk factor for the development of pancreatic cancer or manifestation of the disease?" World journal of surgery **25**(4): 503-508.
Fritsche, H. A. and R. C. Bast (1998). CA 125 in ovarian cancer: advances and controversy, Clinical Chemistry.
Frühbeis, C., D. Fröhlich, W. P. Kuo, J. Amphornrat, S. Thilemann, A. S. Saab, F. Kirchhoff, W. Möbius, S. Goebbels and K.-A. Nave (2013). "Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte–neuron communication." PLoS biology **11**(7): e1001604.
Futter, C., L. Collinson, J. Backer and C. Hopkins (2001). "Human VPS34 is required for internal vesicle formation within multivesicular endosomes." J Cell Biol **155**(7): 1251-1264.
Galli, C., D. Basso and M. Plebani (2013). "CA 19-9: handle with care." Clinical chemistry and laboratory medicine **51**(7): 1369-1383.
Gansauge, F., S. Gansauge, N. Parker, M. Beger, B. Poch, K. Link, F. Safi and H. G. Beger (1996). "CAM 17.1-a new diagnostic marker in pancreatic cancer." British journal of cancer **74**(12): 1997.
Garza-Licudine, E., D. Deo, S. Yu, A. Uz-Zaman and W. B. Dunbar (2010). "Portable nanoparticle quantization using a resizable nanopore instrument - the IZON qNano." Conf Proc IEEE Eng Med Biol Soc **2010**: 5736-5739.
Gold, D. V., D. E. Modrak, Z. Ying, T. M. Cardillo, R. M. Sharkey and D. M. Goldenberg (2006). "New MUC1 serum immunoassay differentiates pancreatic cancer from pancreatitis." J Clin Oncol **24**(2): 252-258.
Gold, E. B. and S. B. Goldin (1998). "Epidemiology of and risk factors for pancreatic cancer." Surgical oncology clinics of North America **7**(1): 67-91.
Greer, J. B., D. C. Whitcomb and R. E. Brand (2007). "Genetic predisposition to pancreatic cancer: a brief review." The American journal of gastroenterology **102**(11): 2564.
Guenther, S., L. J. Muirhead, A. V. Speller, O. Golf, N. Strittmatter, R. Ramakrishnan, R. D. Goldin, E. Jones, K. Veselkov and J. Nicholson (2015). "Spatially resolved metabolic phenotyping of breast cancer by desorption electrospray ionization mass spectrometry." Cancer research **75**(9): 1828-1837.
Guescini, M., S. Genedani, V. Stocchi and L. F. Agnati (2010). "Astrocytes and Glioblastoma cells release exosomes carrying mtDNA." Journal of neural transmission **117**(1): 1.
Guillaumond, F., G. Bidaut, M. Ouaissi, S. Servais, V. Gouirand, O. Olivares, S. Lac, L. Borge, J. Roques and O. Gayet (2015). "Cholesterol uptake disruption, in association with chemotherapy, is a promising combined metabolic therapy for pancreatic adenocarcinoma." Proceedings of the National Academy of Sciences **112**(8): 2473-2478.
Gullo, L., R. Pezzilli, A. M. Morselli-Labate and I. P. C. S. Group (1994). "Diabetes and the risk of pancreatic cancer." New England Journal of Medicine **331**(2): 81-84.
Guo, B. B., S. A. Bellingham and A. F. Hill (2015). "The neutral sphingomyelinase pathway regulates packaging of the prion protein into exosomes." Journal of Biological Chemistry **290**(6): 3455-3467.
Gysin, S., S.-H. Lee, N. M. Dean and M. McMahon (2005). "Pharmacologic inhibition of RAF→ MEK→ ERK signaling elicits pancreatic cancer cell cycle arrest through induced expression of p27Kip1." Cancer research **65**(11): 4870-4880.
György, B., M. E. Hung, X. O. Breakefield and J. N. Leonard (2015). "Therapeutic applications of extracellular vesicles: clinical promise and open questions." Annual review of pharmacology and toxicology **55**: 439-464.
György, B., K. Módos, É. Pállinger, K. Pálóczi, M. Pásztói, P. Misják, M. A. Deli, Á. Sipos, A. Szalai and I. Voszka (2011). "Detection and isolation of cell-derived microparticles are compromised by protein complexes resulting from shared biophysical parameters." Blood **117**(4): e39-e48.

Haglund, C., J. Lundin, P. Kuusela and P. J. Roberts (1994). "CA 242, a new tumour marker for pancreatic cancer: a comparison with CA 19-9, CA 50 and CEA." British journal of cancer **70**(3): 487.
Hallin, J., L. D. Engstrom, L. Hargis, A. Calinisan, R. Aranda, D. M. Briere, N. Sudhakar, V. Bowcut, B. R. Baer and J. A. J. C. d. Ballard (2020). "The KRASG12C inhibitor MRTX849 provides insight toward therapeutic susceptibility of KRAS-mutant cancers in mouse models and patients." **10**(1): 54-71.
Hamada, E., T. Taniguchi, S. Baba and M. Maekawa (2012). "Investigation of unexpected serum CA19-9 elevation in Lewis-negative cancer patients." Annals of clinical biochemistry **49**(3): 266-272.
Han, S., D. H. Gonzalo, M. Feely, C. Rinaldi, S. Belsare, H. Zhai, K. Kalra, M. H. Gerber, C. E. Forsmark and S. J. Hughes (2018). "Stroma-derived extracellular vesicles deliver tumor-suppressive miRNAs to pancreatic cancer cells." Oncotarget **9**(5): 5764.
Hanahan, D. and R. A. Weinberg (2000). "The hallmarks of cancer." cell **100**(1): 57-70.
Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." cell **144**(5): 646-674.
Hao, J. (2015). "HIF-1 is a critical target of pancreatic cancer." Oncoimmunology **4**(9): e1026535.
Hao, N.-B., M.-H. Lü, Y.-H. Fan, Y.-L. Cao, Z.-R. Zhang and S.-M. Yang (2012). "Macrophages in tumor microenvironments and the progression of tumors." Clinical and Developmental Immunology **2012**.
Haraszti, R. A., M.-C. Didiot, E. Sapp, J. Leszyk, S. A. Shaffer, H. E. Rockwell, F. Gao, N. R. Narain, M. DiFiglia and M. A. Kiebish (2016). "High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources." Journal of extracellular vesicles **5**(1): 32570.
Hedlund, M., A.-C. Stenqvist, O. Nagaeva, L. Kjellberg, M. Wulff, V. Baranov and L. Mincheva-Nilsson (2009). "Human placenta expresses and secretes NKG2D ligands via exosomes that down-modulate the cognate receptor expression: evidence for immunosuppressive function." The Journal of Immunology **183**(1): 340-351.
Heusch, G., H. E. Bøtker, K. Przyklenk, A. Redington and D. Yellon (2015). "Remote ischemic conditioning." Journal of the American College of Cardiology **65**(2): 177-195.
Higginbotham, J. N., M. D. Beckler, J. D. Gephart, J. L. Franklin, G. Bogatcheva, G.-J. Kremers, D. W. Piston, G. D. Ayers, R. E. McConnell and M. J. Tyska (2011). "Amphiregulin exosomes increase cancer cell invasion." Current Biology **21**(9): 779-786.
Higginbotham, J. N., Q. Zhang, D. K. Jeppesen, A. M. Scott, H. C. Manning, J. Ochieng, J. L. Franklin and R. J. Coffey (2016). "Identification and characterization of EGF receptor in individual exosomes by fluorescence-activated vesicle sorting." Journal of extracellular vesicles **5**(1): 29254.
Hilvo, M., C. Denkert, L. Lehtinen, B. Müller, S. Brockmöller, T. Seppänen-Laakso, J. Budczies, E. Bucher, L. Yetukuri and S. Castillo (2011). "Novel theranostic opportunities offered by characterization of altered membrane lipid metabolism in breast cancer progression." Cancer research **71**(9): 3236-3245.
Hirsch, J. G., M. E. Fedorko and Z. A. Cohn (1968). "Vesicle fusion and formation at the surface of pinocytic vacuoles in macrophages." The Journal of cell biology **38**(3): 629.
Hisey, C. L., K. D. P. Dorayappan, D. E. Cohn, K. Selvendiran and D. J. Hansford (2018). "Microfluidic affinity separation chip for selective capture and release of label-free ovarian cancer exosomes." Lab on a Chip **18**(20): 3144-3153.
Hla, T. and R. Kolesnick (2014). "C16: 0-ceramide signals insulin resistance." Cell metabolism **20**(5): 703-705.
Holder, B., T. Jones, V. Sancho Shimizu, T. F. Rice, B. Donaldson, M. Bouqueau, K. Forbes and B. Kampmann (2016). "Macrophage exosomes induce placental inflammatory cytokines: a novel mode of maternal–placental messaging." Traffic **17**(2): 168-178.

Holm, P., L. Kayser, K. Sandvig and S. Hansen (1993). "Multivesicular bodies in HEp-2 cells are maturing endosomes." European journal of cell biology **61**(2): 208-224.
Hoshino, A., B. Costa-Silva, T.-L. Shen, G. Rodrigues, A. Hashimoto, M. T. Mark, H. Molina, S. Kohsaka, A. Di Giannatale and S. Ceder (2015). "Tumour exosome integrins determine organotropic metastasis." Nature **527**(7578): 329.
House, S. W., O. Warburg, D. Burk and A. L. Schade (1956). "On respiratory impairment in cancer cells." Science **124**(3215): 267-272.
Hruban, R. H., N. V. Adsay, J. Albores-Saavedra, C. Compton, E. S. Garrett, S. N. Goodman, S. E. Kern, D. S. Klimstra, G. Kloppel, D. S. Longnecker, J. Luttges and G. J. Offerhaus (2001). "Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions." Am J Surg Pathol **25**(5): 579-586.
Hruban, R. H., K. Takaori, D. S. Klimstra, N. V. Adsay, J. Albores-Saavedra, A. V. Biankin, S. A. Biankin, C. Compton, N. Fukushima, T. Furukawa, M. Goggins, Y. Kato, G. Kloppel, D. S. Longnecker, J. Luttges, A. Maitra, G. J. Offerhaus, M. Shimizu and S. Yonezawa (2004). "An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms." Am J Surg Pathol **28**(8): 977-987.
Hu, J., W. Wang, X. Lan, Z. Zeng, Y. Liang, Y. Yan, F. Song, F. Wang, X. Zhu and W. Liao (2019). "CAFs secreted exosomes promote metastasis and chemotherapy resistance by enhancing cell stemness and epithelial-mesenchymal transition in colorectal cancer." Molecular cancer **18**(1): 91.
Hu, Y., C. Yan, L. Mu, K. Huang, X. Li, D. Tao, Y. Wu and J. Qin (2015). "Fibroblast-derived exosomes contribute to chemoresistance through priming cancer stem cells in colorectal cancer." PloS one **10**(5): e0125625.
Huang, B., P.-Y. Pan, Q. Li, A. I. Sato, D. E. Levy, J. Bromberg, C. M. Divino and S.-H. Chen (2006). "Gr-1+ CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host." Cancer research **66**(2): 1123-1131.
Hurley, J. H. (2015). "ESCRTs are everywhere." The EMBO journal **34**(19): 2398-2407.
Hurwitz, S. N., M. A. Rider, J. L. Bundy, X. Liu, R. K. Singh and D. G. Meckes Jr (2016). "Proteomic profiling of NCI-60 extracellular vesicles uncovers common protein cargo and cancer type-specific biomarkers." Oncotarget **7**(52): 86999.
Ilic, M. and I. Ilic (2016). "Epidemiology of pancreatic cancer." World journal of gastroenterology **22**(44): 9694.
Inman, K. S., A. A. Francis and N. R. Murray (2014). "Complex role for the immune system in initiation and progression of pancreatic cancer." World journal of gastroenterology: WJG **20**(32): 11160.
Iraci, N., T. Leonardi, F. Gessler, B. Vega and S. Pluchino (2016). "Focus on extracellular vesicles: physiological role and signalling properties of extracellular membrane vesicles." International journal of molecular sciences **17**(2): 171.
Ismail, N., Y. Wang, D. Dakhlallah, L. Moldovan, K. Agarwal, K. Batte, P. Shah, J. Wisler, T. D. Eubank and S. Tridandapani (2013). "Macrophage microvesicles induce macrophage differentiation and miR-223 transfer." Blood **121**(6): 984-995.
Iwao, T., E. Hiyama, T. Yokoyama, A. Tsuchida, K. Hiyama, Y. Murakami, F. Shimamoto, J. W. Shay and G. Kajiyama (1997). "Telomerase activity for the preoperative diagnosis of pancreatic cancer." J Natl Cancer Inst **89**(21): 1621-1623.
Janeway, C. A., P. Travers, M. Walport and M. Shlomchik (2001). Immunobiology: the immune system in health and disease, Garland Pub. New York.
Janeway, C. A., P. Travers, M. Walport and M. J. Shlomchik (2005). "Immunobiology: the immune system in health and disease."
Jansen, M., N. Fukushima, C. Rosty, K. Walter, R. Altink, T. V. Heek, R. Hruban, J. G. Offerhaus and M. Goggins (2002). "Aberrant methylation of the 5' CpG island of TSLC1 is common in

pancreatic ductal adenocarcinoma and is first manifest in high-grade PanlNs." Cancer Biol Ther **1**(3): 293-296.
Jenkinson, C., J. Earl, P. Ghaneh, C. Halloran, A. Carrato, W. Greenhalf, J. Neoptolemos and E. Costello (2015). "Biomarkers for early diagnosis of pancreatic cancer." Expert Rev Gastroenterol Hepatol **9**(3): 305-315.
Jeon, J. H., S. K. Kim, H. J. Kim, J. Chang, C. M. Ahn and Y. S. Chang (2010). "Lipid raft modulation inhibits NSCLC cell migration through delocalization of the focal adhesion complex." Lung Cancer **69**(2): 165-171.
Jiang, Y., N. DiVittore, M. Young, Z. Jia, K. Xie, T. Ritty, M. Kester and T. Fox (2013). "Altered sphingolipid metabolism in patients with metastatic pancreatic cancer." Biomolecules **3**(3): 435-448.
Jiao, L. and D. Li (2010). Epidemiology and prospects for prevention of pancreatic cancer. Pancreatic Cancer, Springer**:** 3-25.
Jiao, L., D. T. Silverman, C. Schairer, A. C. Thiébaut, A. R. Hollenbeck, M. F. Leitzmann, A. Schatzkin and R. Z. Stolzenberg-Solomon (2009). "Alcohol use and risk of pancreatic cancer: the NIH-AARP Diet and Health Study." American journal of epidemiology **169**(9): 1043-1051.
Kalluri, R. (2016). "The biology and function of exosomes in cancer." The Journal of clinical investigation **126**(4): 1208-1215.
Karahatay, S., K. Thomas, S. Koybasi, C. E. Senkal, S. ElOjeimy, X. Liu, J. Bielawski, T. A. Day, M. B. Gillespie and D. Sinha (2007). "Clinical relevance of ceramide metabolism in the pathogenesis of human head and neck squamous cell carcinoma (HNSCC): attenuation of C18-ceramide in HNSCC tumors correlates with lymphovascular invasion and nodal metastasis." Cancer letters **256**(1): 101-111.
Kassauei, K., N. Habbe, M. E. Mullendore, C. A. Karikari, A. Maitra and G. Feldmann (2006). "Mitochondrial DNA mutations in pancreatic cancer." Int J Gastrointest Cancer **37**(2-3): 57-64.
Kato, H. and H. Nishitoh (2015). "Stress responses from the endoplasmic reticulum in cancer." Frontiers in oncology **5**: 93.
Katsiougiannis, S., D. Chia, Y. Kim, R. P. Singh and D. T. Wong (2016). "Saliva exosomes from pancreatic tumor–bearing mice modulate NK cell phenotype and antitumor cytotoxicity." The FASEB Journal **31**(3): 998-1010.
Kennedy, K. M. and M. W. Dewhirst (2010). "Tumor metabolism of lactate: the influence and therapeutic potential for MCT and CD147 regulation." Future Oncology **6**(1): 127-148.
Kent, O. A., R. R. Chivukula, M. Mullendore, E. A. Wentzel, G. Feldmann, K. H. Lee, S. Liu, S. D. Leach, A. Maitra and J. T. Mendell (2010). "Repression of the miR-143/145 cluster by oncogenic Ras initiates a tumor-promoting feed-forward pathway." Genes & development **24**(24): 2754-2759.
Kim, H.-J., M.-H. Kim, S.-J. Myung, B.-C. Lim, E.-T. Park, K.-S. Yoo, D.-W. Seo, S.-K. Lee and Y.-I. Min (1999). "A new strategy for the application of CA19-9 in the differentiation of pancreaticobiliary cancer: analysis using a receiver operating characteristic curve." The American journal of gastroenterology **94**(7): 1941-1946.
Kim, S., J. E. Signorovitch, H. Yang, O. Patterson-Lomba, C. Q. Xiang, B. Ung, M. Parisi and J. L. J. A. i. t. Marshall (2018). "Comparative effectiveness of nab-paclitaxel plus gemcitabine vs FOLFIRINOX in metastatic pancreatic cancer: a retrospective nationwide chart review in the United States." **35**(10): 1564-1577.
Kirkegård, J., D. Cronin-Fenton, U. Heide-Jørgensen and F. V. Mortensen (2018). "Acute pancreatitis and pancreatic cancer risk: a nationwide matched-cohort study in Denmark." Gastroenterology **154**(6): 1729-1736.
Klein, A. P. J. T. L. G. and Hepatology (2019). "Pancreatic cancer: a growing burden." **4**(12): 895-896.

Kobayashi, T., M.-H. Beuchat, M. Lindsay, S. Frias, R. D. Palmiter, H. Sakuraba, R. G. Parton and J. Gruenberg (1999). "Late endosomal membranes rich in lysobisphosphatidic acid regulate cholesterol transport." Nature cell biology **1**(2): 113.
Komada, M. and P. Soriano (1999). "Hrs, a FYVE finger protein localized to early endosomes, is implicated in vesicular traffic and required for ventral folding morphogenesis." Genes & development **13**(11): 1475-1485.
Koopmann, J., N. S. Fedarko, A. Jain, A. Maitra, C. Iacobuzio-Donahue, A. Rahman, R. H. Hruban, C. J. Yeo and M. Goggins (2004). "Evaluation of osteopontin as biomarker for pancreatic adenocarcinoma." Cancer Epidemiology and Prevention Biomarkers **13**(3): 487-491.
Koorstra, J. B., G. Feldmann, N. Habbe and A. Maitra (2008). "Morphogenesis of pancreatic cancer: role of pancreatic intraepithelial neoplasia (PanINs)." Langenbecks Arch Surg **393**(4): 561-570.
Kourembanas, S. (2015). "Exosomes: vehicles of intercellular signaling, biomarkers, and vectors of cell therapy." Annual review of physiology **77**: 13-27.
Koybasi, S., C. E. Senkal, K. Sundararaj, S. Spassieva, J. Bielawski, W. Osta, T. A. Day, J. C. Jiang, S. M. Jazwinski and Y. A. Hannun (2004). "Defects in cell growth regulation by C18: 0-ceramide and longevity assurance gene 1 in human head and neck squamous cell carcinomas." Journal of Biological Chemistry **279**(43): 44311-44319.
Krämer-Albers, E. M., N. Bretz, S. Tenzer, C. Winterstein, W. Möbius, H. Berger, K. A. Nave, H. Schild and J. Trotter (2007). "Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons?" PROTEOMICS–Clinical Applications **1**(11): 1446-1461.
Kshirsagar, S., S. Alam, S. Jasti, H. Hodes, T. Nauser, M. Gilliam, C. Billstrand, J. Hunt and M. Petroff (2012). "Immunomodulatory molecules are released from the first trimester and term placenta via exosomes." Placenta **33**(12): 982-990.
Kubota, T., A. Kumagai, H. Ito, S. Furukawa, Y. Someya, N. Takeda, K. Ishii, T. Wakita, H. Narimatsu and H. Shirato (2012). "Structural basis for the recognition of Lewis antigens by genogroup I norovirus." Journal of virology: JVI. 00278-00212.
Laghi, L., O. Orbetegli, P. Bianchi, A. Zerbi, V. Di Carlo, C. R. Boland and A. Malesci (2002). "Common occurrence of multiple K-RAS mutations in pancreatic cancers with associated precursor lesions and in biliary cancers." Oncogene **21**(27): 4301.
Lai, R. C., F. Arslan, M. M. Lee, N. S. K. Sze, A. Choo, T. S. Chen, M. Salto-Tellez, L. Timmers, C. N. Lee and R. M. El Oakley (2010). "Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury." Stem cell research **4**(3): 214-222.
Lai, R. C., F. Arslan, S. S. Tan, B. Tan, A. Choo, M. M. Lee, T. S. Chen, B. J. Teh, J. K. L. Eng and H. Sidik (2010). "Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles." Journal of molecular and cellular cardiology **48**(6): 1215-1224.
Lambert, A., L. Schwarz, I. Borbath, A. Henry, J.-L. Van Laethem, D. Malka, M. Ducreux and T. Conroy (2019). "An update on treatment options for pancreatic adenocarcinoma." Therapeutic Advances in Medical Oncology **11**: 1758835919875568.
Langowski, J. L., X. Zhang, L. Wu, J. D. Mattson, T. Chen, K. Smith, B. Basham, T. McClanahan, R. A. Kastelein and M. Oft (2006). "IL-23 promotes tumour incidence and growth." Nature **442**(7101): 461.
Larsson, S. C., N. Orsini and A. Wolk (2007). "Body mass index and pancreatic cancer risk: a meta-analysis of prospective studies." International journal of cancer **120**(9): 1993-1998.
Lee, J. W., C. A. Komar, F. Bengsch, K. Graham and G. L. Beatty (2016). "Genetically Engineered Mouse Models of Pancreatic Cancer: The KPC Model (LSL-KrasG12D/+; LSL-Trp53R172H/+; Pdx-1-Cre), Its Variants, and Their Application in Immuno-oncology Drug Discovery." Current protocols in pharmacology **73**(1): 14.39. 11-14.39. 20.

Lemstrova, R., V. Brynychova, D. J. Hughes, V. Hlavac, P. Dvorak, J. E. Doherty, H. A. Murray, M. Crockard, M. Oliverius and J. Hlavsa (2017). "Dysregulation of KRAS signaling in pancreatic cancer is not associated with KRAS mutations and outcome." Oncology letters **14**(5): 5980-5988.
Li, D., J. E. Mullinax, T. Aiken, H. Xin, G. Wiegand, A. Anderson, S. Thorgeirsson, I. Avital and U. Rudloff (2018). "Loss of PDPK1 abrogates resistance to gemcitabine in label-retaining pancreatic cancer cells." BMC cancer **18**(1): 772.
Li, J., D. Gu, S. S. Lee, B. Song, S. Bandyopadhyay, S. Chen, S. F. Konieczny, T. L. Ratliff, X. Liu and J. Xie (2016). "Abrogating cholesterol esterification suppresses growth and metastasis of pancreatic cancer." Oncogene **35**(50): 6378.
Li, J., S. Ren, H.-l. Piao, F. Wang, P. Yin, C. Xu, X. Lu, G. Ye, Y. Shao and M. Yan (2016). "Integration of lipidomics and transcriptomics unravels aberrant lipid metabolism and defines cholesteryl oleate as potential biomarker of prostate cancer." Scientific reports **6**: 20984.
Liou, G.-Y., H. Döppler, B. Necela, M. Krishna, H. C. Crawford, M. Raimondo and P. Storz (2013). "Macrophage-secreted cytokines drive pancreatic acinar-to-ductal metaplasia through NF-κB and MMPs." J Cell Biol **202**(3): 563-577.
Liu, X., J. R. Pitarresi, M. C. Cuitiño, R. D. Kladney, S. A. Woelke, G. M. Sizemore, S. G. Nayak, O. Egriboz, P. G. Schweickert and L. Yu (2016). "Genetic ablation of Smoothened in pancreatic fibroblasts increases acinar–ductal metaplasia." Genes & development **30**(17): 1943-1955.
Liu, Y., L. S. Zuckier and N. V. Ghesani (2010). "Dominant uptake of fatty acid over glucose by prostate cells: a potential new diagnostic and therapeutic approach." Anticancer research **30**(2): 369-374.
Liyanage, U. K., T. T. Moore, H.-G. Joo, Y. Tanaka, V. Herrmann, G. Doherty, J. A. Drebin, S. M. Strasberg, T. J. Eberlein and P. S. Goedegebuure (2002). "Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma." The Journal of Immunology **169**(5): 2756-2761.
Llorente, A., T. Skotland, T. Sylvänne, D. Kauhanen, T. Róg, A. Orłowski, I. Vattulainen, K. Ekroos and K. Sandvig (2013). "Molecular lipidomics of exosomes released by PC-3 prostate cancer cells." Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids **1831**(7): 1302-1309.
Lochan, R., H. L. Reeves, A. K. Daly and R. M. Charnley (2011). "The role of tobacco-derived carcinogens in pancreas cancer." ISRN oncology **2011**.
Lynch, H., S. Lanspa, J. R. Fitzgibbons, T. Smyrk, M. Fitzsimmons and J. McClellan (1989). "Familial pancreatic cancer (Part 1): Genetic pathology review." The Nebraska medical journal **74**(5): 109-112.
Ma, Y., R. F. Hwang, C. D. Logsdon and S. E. Ullrich (2013). "Dynamic mast cell–stromal cell interactions promote growth of pancreatic cancer." Cancer research **73**(13): 3927-3937.
MacIver, N. J., R. D. Michalek and J. C. Rathmell (2013). "Metabolic regulation of T lymphocytes." Annual review of immunology **31**: 259-283.
Madonna, R., L. W. Van Laake, S. M. Davidson, F. B. Engel, D. J. Hausenloy, S. Lecour, J. Leor, C. Perrino, R. Schulz and K. Ytrehus (2016). "Position Paper of the European Society of Cardiology Working Group Cellular Biology of the Heart: cell-based therapies for myocardial repair and regeneration in ischemic heart disease and heart failure." European heart journal **37**(23): 1789-1798.
Magnani, J. L., Z. Steplewski, H. Koprowski and V. Ginsburg (1983). "Identification of the gastrointestinal and pancreatic cancer-associated antigen detected by monoclonal antibody 19-9 in the sera of patients as a mucin." Cancer research **43**(11): 5489-5492.
Mattos, L. C. d. (2016). "Structural diversity and biological importance of ABO, H, Lewis and secretor histo-blood group carbohydrates." Revista brasileira de hematologia e hemoterapia **38**(4): 331-340.

Mayor, S. and R. E. Pagano (2007). "Pathways of clathrin-independent endocytosis." Nature reviews Molecular cell biology **8**(8): 603.
Mayourian, J., T. J. Cashman, D. K. Ceholski, B. V. Johnson, D. Sachs, D. A. Kaji, S. Sahoo, J. M. Hare, R. J. Hajjar and E. A. Sobie (2017). "Experimental and computational insight into human mesenchymal stem cell paracrine signaling and heterocellular coupling effects on cardiac contractility and arrhythmogenicity." Circulation research: CIRCRESAHA. 117.310796.
McKelvey, K. J., K. L. Powell, A. W. Ashton, J. M. Morris and S. A. McCracken (2015). "Exosomes: mechanisms of uptake." Journal of circulating biomarkers **4**: 7.
Mimura, K., J. L. Teh, H. Okayama, K. Shiraishi, L. F. Kua, V. Koh, D. T. Smoot, H. Ashktorab, T. Oike and Y. Suzuki (2018). "PD-L1 expression is mainly regulated by interferon gamma associated with JAK-STAT pathway in gastric cancer." Cancer science **109**(1): 43-53.
Mishra, G., Y. Zhao, J. Sweeney, B. C. Pineau, D. Case, C. Ho, A. W. Blackstock, K. Geisinger, R. Howerton, E. Levine, P. Shen and J. Ibdah (2006). "Determination of qualitative telomerase activity as an adjunct to the diagnosis of pancreatic adenocarcinoma by EUS-guided fine-needle aspiration." Gastrointest Endosc **63**(4): 648-654.
Miyamoto, M., Y. Shimizu, K. Okada, Y. Kashii, K. Higuchi and A. Watanabe (1998). "Effect of interleukin-8 on production of tumor-associated substances and autocrine growth of human liver and pancreatic cancer cells." Cancer Immunology, Immunotherapy **47**(1): 47-57.
Miyanishi, M., K. Tada, M. Koike, Y. Uchiyama, T. Kitamura and S. Nagata (2007). "Identification of Tim4 as a phosphatidylserine receptor." Nature **450**(7168): 435.
Mollinedo, F. and C. Gajate (2015). "Lipid rafts as major platforms for signaling regulation in cancer." Advances in biological regulation **57**: 130-146.
Molnár, E., M. Swamy, M. Holzer, K. Beck-García, R. Worch, C. Thiele, G. Guigas, K. Boye, I. F. Luescher and P. Schwille (2012). "Cholesterol and sphingomyelin drive ligand-independent T-cell antigen receptor nanoclustering." Journal of Biological Chemistry **287**(51): 42664-42674.
Momen-Heravi, F., L. Balaj, S. Alian, J. Tigges, V. Toxavidis, M. Ericsson, R. J. Distel, A. R. Ivanov, J. Skog and W. P. Kuo (2012). "Alternative methods for characterization of extracellular vesicles." Front Physiol **3**: 354.
Montecalvo, A., W. J. Shufesky, D. B. Stolz, M. G. Sullivan, Z. Wang, S. J. Divito, G. D. Papworth, S. C. Watkins, P. D. Robbins and A. T. Larregina (2008). "Exosomes as a short-range mechanism to spread alloantigen between dendritic cells during T cell allorecognition." The Journal of Immunology **180**(5): 3081-3090.
Morelli, A. E., A. T. Larregina, W. J. Shufesky, M. L. Sullivan, D. B. Stolz, G. D. Papworth, A. F. Zahorchak, A. J. Logar, Z. Wang and S. C. Watkins (2004). "Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells." Blood **104**(10): 3257-3266.
Morey, P., L. Pfannkuch, E. Pang, F. Boccellato, M. Sigal, A. Imai-Matsushima, V. Dyer, M. Koch, H.-J. Mollenkopf and P. Schlaermann (2018). "Helicobacter pylori depletes cholesterol in gastric glands to prevent interferon gamma signaling and escape the inflammatory response." Gastroenterology **154**(5): 1391-1404. e1399.
Mukherjee, S., T. T. Soe and F. R. Maxfield (1999). "Endocytic sorting of lipid analogues differing solely in the chemistry of their hydrophobic tails." The Journal of cell biology **144**(6): 1271-1284.
Möbius, W., E. Van Donselaar, Y. Ohno-Iwashita, Y. Shimada, H. Heijnen, J. Slot and H. Geuze (2003). "Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway." Traffic **4**(4): 222-231.
Nair, S. and C. Salomon (2018). Extracellular vesicles and their immunomodulatory functions in pregnancy. Seminars in immunopathology, Springer.
Naito, Y., Y. Yoshioka, Y. Yamamoto and T. Ochiya (2017). "How cancer cells dictate their microenvironment: present roles of extracellular vesicles." Cellular and Molecular Life Sciences **74**(4): 697-713.

Nakamura, K., H. Okamura, M. Wada, K. Nagata and T. Tamura (1989). "Endotoxin-induced serum factor that stimulates gamma interferon production." Infection and immunity **57**(2): 590-595.
Nakanishi, K. (2018). "Unique action of interleukin-18 on T cells and other immune cells." Frontiers in immunology **9**: 763.
Navas, C., I. Hernández-Porras, A. J. Schuhmacher, M. Sibilia, C. Guerra and M. Barbacid (2012). "EGF receptor signaling is essential for k-ras oncogene-driven pancreatic ductal adenocarcinoma." Cancer cell **22**(3): 318-330.
Nebe-von-Caron, G. (2009). "Standardization in microbial cytometry." Cytometry A **75**(2): 86-89.
Neesse, A., S. Krug, T. M. Gress, D. A. Tuveson and P. Michl (2014). "Emerging concepts in pancreatic cancer medicine: targeting the tumor stroma." OncoTargets and therapy **7**: 33.
Neoptolemos, J. P., J. Kleeff, P. Michl, E. Costello, W. Greenhalf and D. H. Palmer (2018). "Therapeutic developments in pancreatic cancer: current and future perspectives." Nature reviews Gastroenterology & hepatology **15**(6): 333.
Neri, M., I. Riezzo, N. Pascale, C. Pomara and E. Turillazzi (2017). "Ischemia/reperfusion injury following acute myocardial infarction: a critical issue for clinicians and forensic pathologists." Mediators of inflammation **2017**.
Nishihara, S., T. Hiraga, Y. Ikehara, T. Kudo, H. Iwasaki, K. Morozumi, S. Akamatsu, T. Tachikawa and N. Hisashi (1999). "Molecular mechanisms of expression of Lewis b antigen and other type I Lewis antigens in human colorectal cancer." Glycobiology **9**(6): 607-616.
Nolte, E. N., S. I. Buschow, S. M. Anderton, W. Stoorvogel and M. H. Wauben (2009). "Activated T cells recruit exosomes secreted by dendritic cells via LFA-1." Blood **113**(9): 1977-1981.
Notta, F., M. Chan-Seng-Yue, M. Lemire, Y. Li, G. W. Wilson, A. A. Connor, R. E. Denroche, S.-B. Liang, A. M. Brown and J. C. J. N. Kim (2016). "A renewed model of pancreatic cancer evolution based on genomic rearrangement patterns." **538**(7625): 378-382.
Novick, D., B. Schwartsburd, R. Pinkus, D. Suissa, I. Belzer, Z. Sthoeger, W. F. Keane, Y. Chvatchko, S.-H. Kim and G. Fantuzzi (2001). "A novel IL-18BP ELISA shows elevated serum IL-18BP in sepsis and extensive decrease of free IL-18." Cytokine **14**(6): 334-342.
Nöthlings, U., S. P. Murphy, L. R. Wilkens, B. E. Henderson and L. N. Kolonel (2007). "Flavonols and pancreatic cancer risk: the multiethnic cohort study." American journal of epidemiology **166**(8): 924-931.
Nöthlings, U., L. R. Wilkens, S. P. Murphy, J. H. Hankin, B. E. Henderson and L. N. Kolonel (2005). "Meat and fat intake as risk factors for pancreatic cancer: the multiethnic cohort study." Journal of the National Cancer Institute **97**(19): 1458-1465.
Oettle, H., S. Post, P. Neuhaus, K. Gellert, J. Langrehr, K. Ridwelski, H. Schramm, J. Fahlke, C. Zuelke and C. Burkart (2007). "Adjuvant chemotherapy with gemcitabine vs observation in patients undergoing curative-intent resection of pancreatic cancer: a randomized controlled trial." Jama **297**(3): 267-277.
Orntoft, T., E. Holmes, P. Johnson, S. Hakomori and H. Clausen (1991). "Differential tissue expression of the Lewis blood group antigens: enzymatic, immunohistologic, and immunochemical evidence for Lewis a and b antigen expression in Le (ab-) individuals." Blood **77**(6): 1389-1396.
Ostenfeld, M. S., J. B. Bramsen, P. Lamy, S. B. Villadsen, N. Fristrup, K. D. Sørensen, B. Ulhøi, M. Borre, J. Kjems and L. Dyrskjøt (2010). "miR-145 induces caspase-dependent and-independent cell death in urothelial cancer cell lines with targeting of an expression signature present in Ta bladder tumors." Oncogene **29**(7): 1073.
Ozkan, H., M. Kaya and A. Cengiz (2003). "Comparison of tumor marker CA 242 with CA 19-9 and carcinoembryonic antigen (CEA) in pancreatic cancer." Hepato-gastroenterology **50**(53): 1669-1674.

Palmulli, R. and G. van Niel (2018). "To be or not to be... secreted as exosomes, a balance finely tuned by the mechanisms of biogenesis." Essays in biochemistry **62**(2): 177-191.
Pan, B.-T. and R. M. Johnstone (1983). "Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor." Cell **33**(3): 967-978.
Pannala, R., J. B. Leirness, W. R. Bamlet, A. Basu, G. M. Petersen and S. T. Chari (2008). "Prevalence and clinical profile of pancreatic cancer-associated diabetes mellitus." Gastroenterology **134**(4): 981-987.
Panousis, C. G. and S. H. Zuckerman (2000). "Regulation of cholesterol distribution in macrophage-derived foam cells by interferon-γ." Journal of lipid research **41**(1): 75-83.
Parker, N., C. Makin, C. Ching, D. Eccleston, O. Taylor, D. Milton and J. M. Rhodes (1992). "A new enzyme-linked lectin/mucin antibody sandwich assay (CAM 17.1/WGA) assessed in combination with CA 19-9 and peanut lectin binding assay for the diagnosis of pancreatic cancer." Cancer **70**(5): 1062-1068.
Parolini, I., C. Federici, C. Raggi, L. Lugini, S. Palleschi, A. De Milito, C. Coscia, E. Iessi, M. A. Logozzi and A. Molinari (2009). "Microenvironmental pH is a key factor for exosome traffic in tumor cells." Journal of Biological Chemistry: jbc. M109. 041152.
Patel, A. V., C. Rodriguez, L. Bernstein, A. Chao, M. J. Thun and E. E. Calle (2005). "Obesity, recreational physical activity, and risk of pancreatic cancer in a large US Cohort." Cancer Epidemiology and Prevention Biomarkers **14**(2): 459-466.
Patricelli, M. P., M. R. Janes, L.-S. Li, R. Hansen, U. Peters, L. V. Kessler, Y. Chen, J. M. Kucharski, J. Feng and T. J. C. d. Ely (2016). "Selective inhibition of oncogenic KRAS output with small molecules targeting the inactive state." **6**(3): 316-329.
Pearson, A. S., P. Chiao, L. Zhang, W. Zhang, L. Larry, R. L. Katz, D. B. Evans and J. L. Abbruzzese (2000). "The detection of telomerase activity in patients with adenocarcinoma of the pancreas by fine needle aspiration." Int J Oncol **17**(2): 381-385.
Perrotti, F., C. Rosa, I. Cicalini, P. Sacchetta, P. Del Boccio, D. Genovesi and D. Pieragostino (2016). "Advances in lipidomics for cancer biomarkers discovery." International Journal of Molecular Sciences **17**(12): 1992.
Peters, P. J., J. Borst, V. Oorschot, M. Fukuda, O. Krähenbühl, J. Tschopp, J. W. Slot and H. J. Geuze (1991). "Cytotoxic T lymphocyte granules are secretory lysosomes, containing both perforin and granzymes." Journal of Experimental Medicine **173**(5): 1099-1109.
Philip, B., C. L. Roland, J. Daniluk, Y. Liu, D. Chatterjee, S. B. Gomez, B. Ji, H. Huang, H. Wang and J. B. Fleming (2013). "A high-fat diet activates oncogenic Kras and COX2 to induce development of pancreatic ductal adenocarcinoma in mice." Gastroenterology **145**(6): 1449-1458.
Piper, R. C., I. Dikic and G. L. Lukacs (2014). "Ubiquitin-dependent sorting in endocytosis." Cold Spring Harbor Perspectives in Biology **6**(1): a016808.
Porembka, M. R., J. B. Mitchem, B. A. Belt, C.-S. Hsieh, H.-M. Lee, J. Herndon, W. E. Gillanders, D. C. Linehan and P. Goedegebuure (2012). "Pancreatic adenocarcinoma induces bone marrow mobilization of myeloid-derived suppressor cells which promote primary tumor growth." Cancer Immunology, Immunotherapy **61**(9): 1373-1385.
Potter, V. R. (1958). The biochemical approach to the cancer problem. Federation proceedings.
Qiu, B., D. Ackerman, D. J. Sanchez, B. Li, J. D. Ochocki, A. Grazioli, E. Bobrovnikova-Marjon, J. A. Diehl, B. Keith and M. C. Simon (2015). "HIF2α-dependent lipid storage promotes endoplasmic reticulum homeostasis in clear-cell renal cell carcinoma." Cancer discovery **5**(6): 652-667.
Rasheed, Z. A., W. Matsui and A. Maitra (2012). Pathology of pancreatic stroma in PDAC. Pancreatic cancer and tumor microenvironment, Transworld Research Network.
Record, M., M. Poirot and S. Silvente-Poirot (2014). "Emerging concepts on the role of exosomes in lipid metabolic diseases." Biochimie **96**: 67-74.

Renehan, A. G., M. Tyson, M. Egger, R. F. Heller and M. Zwahlen (2008). "Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies." The Lancet **371**(9612): 569-578.
Rhim, A. D., P. E. Oberstein, D. H. Thomas, E. T. Mirek, C. F. Palermo, S. A. Sastra, E. N. Dekleva, T. Saunders, C. P. Becerra and I. W. Tattersall (2014). "Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma." Cancer cell **25**(6): 735-747.
Richards, K. E., A. E. Zeleniak, M. L. Fishel, J. Wu, L. E. Littlepage and R. Hill (2017). "Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells." Oncogene **36**(13): 1770.
Ríos-Marco, P., M. Martín-Fernández, I. Soria-Bretones, A. Ríos, M. P. Carrasco and C. Marco (2013). "Alkylphospholipids deregulate cholesterol metabolism and induce cell-cycle arrest and autophagy in U-87 MG glioblastoma cells." Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids **1831**(8): 1322-1334.
Robbins, P. D. and A. E. Morelli (2014). "Regulation of immune responses by extracellular vesicles." Nature Reviews Immunology **14**(3): 195.
Rohrmann, S., J. Linseisen, A. Vrieling, P. Boffetta, R. Z. Stolzenberg-Solomon, A. B. Lowenfels, M. K. Jensen, K. Overvad, A. Olsen and A. Tjonneland (2009). "Ethanol intake and the risk of pancreatic cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC)." Cancer Causes & Control **20**(5): 785-794.
Rysman, E., K. Brusselmans, K. Scheys, L. Timmermans, R. Derua, S. Munck, P. P. Van Veldhoven, D. Waltregny, V. W. Daniëls and J. Machiels (2010). "De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation." Cancer research **70**(20): 8117-8126.
Safaei, R., B. J. Larson, T. C. Cheng, M. A. Gibson, S. Otani, W. Naerdemann and S. B. Howell (2005). "Abnormal lysosomal trafficking and enhanced exosomal export of cisplatin in drug-resistant human ovarian carcinoma cells." Molecular cancer therapeutics **4**(10): 1595-1604.
Saio, M., S. Radoja, M. Marino and A. B. Frey (2001). "Tumor-infiltrating macrophages induce apoptosis in activated CD8+ T cells by a mechanism requiring cell contact and mediated by both the cell-associated form of TNF and nitric oxide." The Journal of Immunology **167**(10): 5583-5593.
Salazar, M., A. Carracedo, Í. J. Salanueva, S. Hernández-Tiedra, M. Lorente, A. Egia, P. Vázquez, C. Blázquez, S. Torres and S. García (2009). "Cannabinoid action induces autophagy-mediated cell death through stimulation of ER stress in human glioma cells." The Journal of clinical investigation **119**(5): 1359-1372.
Sánchez, C. A., E. I. Andahur, R. Valenzuela, E. A. Castellon, J. A. Fulla, C. G. Ramos and J. C. Trivino (2016). "Exosomes from bulk and stem cells from human prostate cancer have a differential microRNA content that contributes cooperatively over local and pre-metastatic niche." Oncotarget **7**(4): 3993.
Sansone, P., C. Savini, I. Kurelac, Q. Chang, L. B. Amato, A. Strillacci, A. Stepanova, L. Iommarini, C. Mastroleo and L. Daly (2017). "Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer." Proceedings of the National Academy of Sciences **114**(43): E9066-E9075.
Sato, N., N. Fukushima, A. Maitra, H. Matsubayashi, C. J. Yeo, J. L. Cameron, R. H. Hruban and M. Goggins (2003). "Discovery of novel targets for aberrant methylation in pancreatic carcinoma using high-throughput microarrays." Cancer Res **63**(13): 3735-3742.
Sato, N., T. Ueki, N. Fukushima, C. A. Iacobuzio-Donahue, C. J. Yeo, J. L. Cameron, R. H. Hruban and M. Goggins (2002). "Aberrant methylation of CpG islands in intraductal papillary mucinous neoplasms of the pancreas." Gastroenterology **123**(1): 365-372.
Saunderson, S. C., A. C. Dunn, P. R. Crocker and A. D. McLellan (2014). "CD169 mediates the capture of exosomes in spleen and lymph node." Blood **123**(2): 208-216.
Scott, C. C., F. Vacca and J. Gruenberg (2014). Endosome maturation, transport and functions. Seminars in cell & developmental biology, Elsevier.

Segura, E., C. Guérin, N. Hogg, S. Amigorena and C. Théry (2007). "CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo." The Journal of Immunology **179**(3): 1489-1496.
Segura, E., C. Nicco, B. Lombard, P. Véron, G. Raposo, F. Batteux, S. Amigorena and C. Théry (2005). "ICAM-1 on exosomes from mature dendritic cells is critical for efficient naive T-cell priming." Blood **106**(1): 216-223.
Semenza, G. L. (2008). "Tumor metabolism: cancer cells give and take lactate." The Journal of clinical investigation **118**(12): 3835-3837.
Sener, S. F., A. Fremgen, H. R. Menck and D. P. Winchester (1999). "Pancreatic cancer: a report of treatment and survival trends for 100,313 patients diagnosed from 1985-1995, using the National Cancer Database." J Am Coll Surg **189**(1): 1-7.
Shankaran, V., H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old and R. D. Schreiber (2001). "IFNγ and lymphocytes prevent primary tumour development and shape tumour immunogenicity." Nature **410**(6832): 1107.
Shi, C., S. H. Eshleman, D. Jones, N. Fukushima, L. Hua, A. R. Parker, C. J. Yeo, R. H. Hruban, M. G. Goggins and J. R. Eshleman (2004). "LigAmp for sensitive detection of single-nucleotide differences." Nat Methods **1**(2): 141-147.
Shi, S. and X. Yu (2019). Selecting chemotherapy for pancreatic cancer: Far away or so close? Seminars in oncology, Elsevier.
Simeone, D. M., B. Ji, M. Banerjee, T. Arumugam, D. Li, M. A. Anderson, A. M. Bamberger, J. Greenson, R. E. Brand and V. Ramachandran (2007). "CEACAM1, a novel serum biomarker for pancreatic cancer." Pancreas **34**(4): 436-443.
Simons, K. (2018). "How can omic science be improved?" Proteomics **18**(5-6): 1800039.
Skog, J., T. Würdinger, S. Van Rijn, D. H. Meijer, L. Gainche, W. T. Curry Jr, B. S. Carter, A. M. Krichevsky and X. O. Breakefield (2008). "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers." Nature cell biology **10**(12): 1470.
Smyth, L. A., B. Afzali, J. Tsang, G. Lombardi and R. Lechler (2007). "Intercellular transfer of MHC and immunological molecules: molecular mechanisms and biological significance." American journal of transplantation **7**(6): 1442-1449.
Smyth, M. J., Y. Hayakawa, K. Takeda and H. Yagita (2002). "New aspects of natural-killer-cell surveillance and therapy of cancer." Nature Reviews Cancer **2**(11): 850.
Sokolova, V., A. K. Ludwig, S. Hornung, O. Rotan, P. A. Horn, M. Epple and B. Giebel (2011). "Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy." Colloids Surf B Biointerfaces **87**(1): 146-150.
Sousa, C. M., D. E. Biancur, X. Wang, C. J. Halbrook, M. H. Sherman, L. Zhang, D. Kremer, R. F. Hwang, A. K. Witkiewicz and H. Ying (2016). "Pancreatic stellate cells support tumour metabolism through autophagic alanine secretion." Nature **536**(7617): 479.
Steinberg, W. (1990). "The clinical utility of the CA 19-9 tumor-associated antigen." American Journal of Gastroenterology **85**(4).
Stephens, P. J., C. D. Greenman, B. Fu, F. Yang, G. R. Bignell, L. J. Mudie, E. D. Pleasance, K. W. Lau, D. Beare and L. A. J. c. Stebbings (2011). "Massive genomic rearrangement acquired in a single catastrophic event during cancer development." **144**(1): 27-40.
Stoorvogel, W., M. J. Kleijmeer, H. J. Geuze and G. Raposo (2002). "The biogenesis and functions of exosomes." Traffic **3**(5): 321-330.
Sturm, P. D., R. H. Hruban, T. B. Ramsoekh, L. A. Noorduyn, G. N. Tytgat, D. J. Gouma and G. J. Offerhaus (1998). "The potential diagnostic use of K-ras codon 12 and p53 alterations in brush cytology from the pancreatic head region." J Pathol **186**(3): 247-253.
Subra, C., D. Grand, K. Laulagnier, A. Stella, G. Lambeau, M. Paillasse, P. De Medina, B. Monsarrat, B. Perret and S. Silvente-Poirot (2010). "Exosomes account for vesicle-mediated transcellular transport of activatable phospholipases and prostaglandins." Journal of lipid research **51**(8): 2105-2120.

Sun, Y., W. He, M. Luo, Y. Zhou, G. Chang, W. Ren, K. Wu, X. Li, J. Shen and X. Zhao (2015). "SREBP1 regulates tumorigenesis and prognosis of pancreatic cancer through targeting lipid metabolism." Tumor Biology **36**(6): 4133-4141.
Sunami, Y., A. Rebelo and J. Kleeff (2017). "Lipid metabolism and lipid droplets in pancreatic cancer and stellate cells." Cancers **10**(1): 3.
Suriyaphol, P., D. Fenske, U. Zähringer, S.-R. Han, S. Bhakdi and M. Husmann (2002). "Enzymatically modified nonoxidized low-density lipoprotein induces interleukin-8 in human endothelial cells: role of free fatty acids." Circulation **106**(20): 2581-2587.
Szafranska, A. E., T. S. Davison, J. John, T. Cannon, B. Sipos, A. Maghnouj, E. Labourier and S. A. Hahn (2007). "MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma." Oncogene **26**(30): 4442-4452.
Szatanek, R., M. Baj-Krzyworzeka, J. Zimoch, M. Lekka, M. Siedlar and J. Baran (2017). "The Methods of Choice for Extracellular Vesicles (EVs) Characterization." Int J Mol Sci **18**(6).
Tadros, S., S. K. Shukla, R. J. King, V. Gunda, E. Vernucci, J. Abrego, N. V. Chaika, F. Yu, A. J. Lazenby and L. Berim (2017). "De novo lipid synthesis facilitates gemcitabine resistance through endoplasmic reticulum stress in pancreatic cancer." Cancer research **77**(20): 5503-5517.
Takaoka, A., Y. Mitani, H. Suemori, M. Sato, T. Yokochi, S. Noguchi, N. Tanaka and T. Taniguchi (2000). "Cross talk between interferon-γ and-α/β signaling components in caveolar membrane domains." Science **288**(5475): 2357-2360.
Takikawa, T., A. Masamune, N. Yoshida, S. Hamada, T. Kogure and T. Shimosegawa (2017). "Exosomes derived from pancreatic stellate cells: MicroRNA signature and effects on pancreatic cancer cells." Pancreas **46**(1): 19-27.
Talbert, E. E., H. L. Lewis, M. R. Farren, M. L. Ramsey, J. M. Chakedis, P. Rajasekera, E. Haverick, A. Sarna, M. Bloomston and T. M. Pawlik (2018). "Circulating monocyte chemoattractant protein-1 (MCP-1) is associated with cachexia in treatment-naive pancreatic cancer patients." Journal of cachexia, sarcopenia and muscle **9**(2): 358-368.
Tan, A. S., J. W. Baty, L.-F. Dong, A. Bezawork-Geleta, B. Endaya, J. Goodwin, M. Bajzikova, J. Kovarova, M. Peterka and B. Yan (2015). "Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA." Cell metabolism **21**(1): 81-94.
Tan, H. T., J. Low, S. G. Lim and M. C. Chung (2009). "Serum autoantibodies as biomarkers for early cancer detection." The FEBS journal **276**(23): 6880-6904.
Tan, M. C., P. S. Goedegebuure, B. A. Belt, B. Flaherty, N. Sankpal, W. E. Gillanders, T. J. Eberlein, C.-S. Hsieh and D. C. Linehan (2009). "Disruption of CCR5-dependent homing of regulatory T cells inhibits tumor growth in a murine model of pancreatic cancer." The Journal of Immunology **182**(3): 1746-1755.
Tannetta, D. S., R. A. Dragovic, C. Gardiner, C. W. Redman and I. L. Sargent (2013). "Characterisation of syncytiotrophoblast vesicles in normal pregnancy and pre-eclampsia: expression of Flt-1 and endoglin." PloS one **8**(2): e56754.
Taylor, D. D., Ç. Gerçel-Taylor, K. S. Lyons, J. Stanson and T. L. Whiteside (2003). "T-cell apoptosis and suppression of T-cell receptor/CD3-ζ by Fas ligand-containing membrane vesicles shed from ovarian tumors." Clinical cancer research **9**(14): 5113-5119.
Teng, M. W., D. M. Andrews, N. McLaughlin, B. von Scheidt, S. F. Ngiow, A. Möller, G. R. Hill, Y. Iwakura, M. Oft and M. J. Smyth (2010). "IL-23 suppresses innate immune response independently of IL-17A during carcinogenesis and metastasis." Proceedings of the National Academy of Sciences **107**(18): 8328-8333.
Teng, Y., Y. Ren, X. Hu, J. Mu, A. Samykutty, X. Zhuang, Z. Deng, A. Kumar, L. Zhang and M. L. Merchant (2017). "MVP-mediated exosomal sorting of miR-193a promotes colon cancer progression." Nature communications **8**: 14448.

Tentler, J. J., A. C. Tan, C. D. Weekes, A. Jimeno, S. Leong, T. M. Pitts, J. J. Arcaroli, W. A. Messersmith and S. G. Eckhardt (2012). "Patient-derived tumour xenografts as models for oncology drug development." Nature reviews Clinical oncology **9**(6): 338.
Thakur, B. K., H. Zhang, A. Becker, I. Matei, Y. Huang, B. Costa-Silva, Y. Zheng, A. Hoshino, H. Brazier and J. Xiang (2014). "Double-stranded DNA in exosomes: a novel biomarker in cancer detection." Cell research **24**(6): 766.
Thery, C., S. Amigorena, G. Raposo and A. Clayton (2006). "Isolation and characterization of exosomes from cell culture supernatants and biological fluids." Curr Protoc Cell Biol **Chapter 3**: Unit 3 22.
Théry, C., K. W. Witwer, E. Aikawa, M. J. Alcaraz, J. D. Anderson, R. Andriantsitohaina, A. Antoniou, T. Arab, F. Archer and G. K. Atkin-Smith (2018). "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." Journal of extracellular vesicles **7**(1): 1535750.
Tian, T., Y.-L. Zhu, Y.-Y. Zhou, G.-F. Liang, Y.-Y. Wang, F.-H. Hu and Z.-D. Xiao (2014). "Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery." Journal of Biological Chemistry: jbc. M114. 588046.
Tonack, S., C. Jenkinson, T. Cox, V. Elliott, R. E. Jenkins, N. R. Kitteringham, W. Greenhalf, V. Shaw, C. W. Michalski, H. Friess, J. P. Neoptolemos and E. Costello (2013). "iTRAQ reveals candidate pancreatic cancer serum biomarkers: influence of obstructive jaundice on their performance." Br J Cancer **108**(9): 1846-1853.
Trajkovic, K., C. Hsu, S. Chiantia, L. Rajendran, D. Wenzel, F. Wieland, P. Schwille, B. Brügger and M. Simons (2008). "Ceramide triggers budding of exosome vesicles into multivesicular endosomes." Science **319**(5867): 1244-1247.
Tsou, P., H. Katayama, E. J. Ostrin and S. M. Hanash (2016). "The emerging role of B cells in tumor immunity." Cancer research **76**(19): 5597-5601.
Tsuchiya, R. and N. Fujisawa (1997). "On the etymology of "pancreas"." Int J Pancreatol **21**(3): 269-272.
Tsuchiya, R., T. Kuroki and S. Eguchi (2015). "The pancreas from Aristotle to Galen." Pancreatology **15**(1): 2-7.
Tsutsumi, M., T. Tsujiuchi, O. Ishikawa, T. Majima, M. Yoshimoto, Y. Sasaki, T. Fukuda, H. Oohigashi and Y. Konishi (1997). "Increased telomerase activities in human pancreatic duct adenocarcinomas." Jpn J Cancer Res **88**(10): 971-976.
Tsuzuki, Y., C. M. Carreira, M. Bockhorn, L. Xu, R. K. Jain and D. Fukumura (2001). "Pancreas microenvironment promotes VEGF expression and tumor growth: novel window models for pancreatic tumor angiogenesis and microcirculation." Laboratory investigation **81**(10): 1439.
Waghray, M., M. Yalamanchili, M. P. di Magliano and D. M. Simeone (2013). "Deciphering the role of stroma in pancreatic cancer." Current opinion in gastroenterology **29**(5): 537.
Valadi, H., K. Ekström, A. Bossios, M. Sjöstrand, J. J. Lee and J. O. Lötvall (2007). "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells." Nature cell biology **9**(6): 654.
Walseng, E., O. Bakke and P. A. Roche (2008). "Major histocompatibility complex class II-peptide complexes internalize using a clathrin-and dynamin-independent endocytosis pathway." Journal of Biological Chemistry **283**(21): 14717-14727.
Walter, K., S.-M. Hong, S. Nyhan, M. Canto, N. Fedarko, A. Klein, M. Griffith, N. Omura, S. Medghalchi and F. Kuhajda (2009). "Serum fatty acid synthase as a marker of pancreatic neoplasia." Cancer Epidemiology and Prevention Biomarkers **18**(9): 2380-2385.
van Balkom, B. W., A. S. Eisele, D. M. Pegtel, S. Bervoets and M. C. Verhaar (2015). "Quantitative and qualitative analysis of small RNAs in human endothelial cells and exosomes provides insights into localized RNA processing, degradation and sorting." Journal of extracellular vesicles **4**(1): 26760.

van der Pol, E., F. A. Coumans, A. E. Grootemaat, C. Gardiner, I. L. Sargent, P. Harrison, A. Sturk, T. G. van Leeuwen and R. Nieuwland (2014). "Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing." J Thromb Haemost **12**(7): 1182-1192.
van der Pol, E., M. J. van Gemert, A. Sturk, R. Nieuwland and T. G. van Leeuwen (2012). "Single vs. swarm detection of microparticles and exosomes by flow cytometry." J Thromb Haemost **10**(5): 919-930.
Van Meer, G., D. R. Voelker and G. W. Feigenson (2008). "Membrane lipids: where they are and how they behave." Nature reviews Molecular cell biology **9**(2): 112.
Vander Heiden, M. G., L. C. Cantley and C. B. Thompson (2009). "Understanding the Warburg effect: the metabolic requirements of cell proliferation." science **324**(5930): 1029-1033.
Wang, R., J. Bi, K. K. Ampah, C. Zhang, Z. Li, Y. Jiao, X. Wang, X. Ba and X. Zeng (2013). "Lipid raft regulates the initial spreading of melanoma A375 cells by modulating β1 integrin clustering." The international journal of biochemistry & cell biology **45**(8): 1679-1689.
Warburg, O. (1956). "On the origin of cancer cells." Science **123**(3191): 309-314.
Warburg, O. H. (1930). The metabolism of tumours: investigations from the Kaiser Wilhelm Institute for Biology, Berlin-Dahlem, Constable & Company Limited.
Webber, J., R. Steadman, M. D. Mason, Z. Tabi and A. Clayton (2010). "Cancer exosomes trigger fibroblast to myofibroblast differentiation." Cancer research **70**(23): 9621-9630.
Wei, X., C. Liu, H. Wang, L. Wang, F. Xiao, Z. Guo and H. Zhang (2016). "Surface phosphatidylserine is responsible for the internalization on microvesicles derived from hypoxia-induced human bone marrow mesenchymal stem cells into human endothelial cells." PLoS One **11**(1): e0147360.
Weir, J. M., G. Wong, C. K. Barlow, M. A. Greeve, A. Kowalczyk, L. Almasy, A. G. Comuzzie, M. C. Mahaney, J. B. Jowett and J. Shaw (2013). "Plasma lipid profiling in a large population-based cohort." Journal of lipid research **54**(10): 2898-2908.
Wenk, M. R. (2005). "The emerging field of lipidomics." Nature reviews Drug discovery **4**(7): 594.
Ventrucci, M., G. M. Ubalducci, A. Cipolla, M. A. Panella and A. Ligabue (1998). "Serum CA 242: the search for a valid marker of pancreatic cancer." Clinical chemistry and laboratory medicine **36**(3): 179-184.
Véron, P., E. Segura, G. Sugano, S. Amigorena and C. Théry (2005). "Accumulation of MFG-E8/lactadherin on exosomes from immature dendritic cells." Blood Cells, Molecules, and Diseases **35**(2): 81-88.
Vesely, M. D., M. H. Kershaw, R. D. Schreiber and M. J. Smyth (2011). "Natural innate and adaptive immunity to cancer." Annual review of immunology **29**: 235-271.
Vestad, B., A. Llorente, A. Neurauter, S. Phuyal, B. Kierulf, P. Kierulf, T. Skotland, K. Sandvig, K. B. F. Haug and R. Øvstebø (2017). "Size and concentration analyses of extracellular vesicles by nanoparticle tracking analysis: a variation study." Journal of extracellular vesicles **6**(1): 1344087.
WHO. (2017). "Cardiovascular diseases (CVDs)."
Vicencio, J. M., D. M. Yellon, V. Sivaraman, D. Das, C. Boi-Doku, S. Arjun, Y. Zheng, J. A. Riquelme, J. Kearney and V. Sharma (2015). "Plasma exosomes protect the myocardium from ischemia-reperfusion injury." Journal of the American College of Cardiology **65**(15): 1525-1536.
Vidal-Vanaclocha, F., L. Mendoza, N. Telleria, C. Salado, M. Valcárcel, N. Gallot, T. Carrascal, E. Egilegor, J. Beaskoetxea and C. A. Dinarello (2006). "Clinical and experimental approaches to the pathophysiology of interleukin-18 in cancer progression." Cancer and Metastasis Reviews **25**(3): 417-434.
Vidal, M., J. Sainte-Marie, J. R. Philippot and A. Bienvenue (1989). "Asymmetric distribution of phospholipids in the membrane of vesicles released during in vitro maturation of guinea

pig reticulocytes: evidence precluding a role for “aminophospholipid translocase”." Journal of cellular physiology **140**(3): 455-462.
Wieckowski, E. U., C. Visus, M. Szajnik, M. J. Szczepanski, W. J. Storkus and T. L. Whiteside (2009). "Tumor-derived microvesicles promote regulatory T cell expansion and induce apoptosis in tumor-reactive activated CD8+ T lymphocytes." The Journal of Immunology: jimmunol. 0900970.
Wikman, F. P., M. L. Lu, T. Thykjaer, S. H. Olesen, L. D. Andersen, C. Cordon-Cardo and T. F. Orntoft (2000). "Evaluation of the performance of a p53 sequencing microarray chip using 140 previously sequenced bladder tumor samples." Clin Chem **46**(10): 1555-1561.
Wilbur, J. (2008). "Prostate cancer screening: the continuing controversy." Am Fam Physician **78**(12): 1377-1384.
Vincent-Schneider, H., P. Stumptner-Cuvelette, D. Lankar, S. Pain, G. Raposo, P. Benaroch and C. Bonnerot (2002). "Exosomes bearing HLA-DR1 molecules need dendritic cells to efficiently stimulate specific T cells." International immunology **14**(7): 713-722.
Wolfers, J., A. Lozier, G. Raposo, A. Regnault, C. Théry, C. Masurier, C. Flament, S. Pouzieux, F. Faure and T. Tursz (2001). "Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming." Nature medicine **7**(3): 297.
Wortzel, I., S. Dror, C. M. Kenific and D. Lyden (2019). "Exosome-mediated metastasis: communication from a distance." Developmental cell **49**(3): 347-360.
Vrijsen, K., J. Sluijter, M. Schuchardt, B. Van Balkom, W. Noort, S. Chamuleau and P. Doevendans (2010). "Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells." Journal of cellular and molecular medicine **14**(5): 1064-1070.
Wu, Y., W. Deng and D. J. Klinke, 2nd (2015). "Exosomes: improved methods to characterize their morphology, RNA content, and surface protein biomarkers." Analyst **140**(19): 6631-6642.
Wubbolts, R., R. S. Leckie, P. T. Veenhuizen, G. Schwarzmann, W. Möbius, J. Hoernschemeyer, J.-W. Slot, H. J. Geuze and W. Stoorvogel (2003). "Proteomic and biochemical analyses of human B cell-derived exosomes Potential implications for their function and multivesicular body formation." Journal of Biological Chemistry **278**(13): 10963-10972.
Xie, F., Q. Huang, C. Liu, X. Lin, Z. Liu, L. Liu, D. Huang and H. Zhou (2018). "MiR-1271 negatively regulates AKT/MTOR signaling and promotes apoptosis via targeting PDK1 in pancreatic cancer." European review for medical and pharmacological sciences **22**: 678-686.
Yachida, S., S. Jones, I. Bozic, T. Antal, R. Leary, B. Fu, M. Kamiyama, R. H. Hruban, J. R. Eshleman, M. A. Nowak, V. E. Velculescu, K. W. Kinzler, B. Vogelstein and C. A. Iacobuzio-Donahue (2010). "Distant metastasis occurs late during the genetic evolution of pancreatic cancer." Nature **467**(7319): 1114-1117.
Yamada, T., Y. Inoshima, T. Matsuda and N. Ishiguro (2012). "Comparison of methods for isolating exosomes from bovine milk." Journal of Veterinary Medical Science: 12-0032.
Yamazaki, K., T. Nagao, T. Yamaguchi, H. Saisho and Y. Kondo (1997). "Expression of basic fibroblast growth factor (FGF-2)-associated with tumour proliferation in human pancreatic carcinoma." Virchows Archiv **431**(2): 95-101.
Yáñez-Mó, M., P. R.-M. Siljander, Z. Andreu, A. Bedina Zavec, F. E. Borràs, E. I. Buzas, K. Buzas, E. Casal, F. Cappello and J. Carvalho (2015). "Biological properties of extracellular vesicles and their physiological functions." Journal of extracellular vesicles **4**(1): 27066.
Yang, J.-B., Z.-J. Duan, W. Yao, O. Lee, L. Yang, X.-Y. Yang, X. Sun, C. C. Chang, T.-Y. Chang and B.-L. Li (2001). "Synergistic Transcriptional Activation of HumanAcyl-coenzyme A: Cholesterol Acyltransterase-1 Gene by Interferon-γ and All-trans-Retinoic Acid THP-1 Cells." Journal of Biological Chemistry **276**(24): 20989-20998.
Yang, W., Y. Bai, Y. Xiong, J. Zhang, S. Chen, X. Zheng, X. Meng, L. Li, J. Wang and C. Xu (2016). "Potentiating the antitumour response of CD8+ T cells by modulating cholesterol metabolism." Nature **531**(7596): 651.

Yu, S., N. Parameswaran, M. Li, Y. Wang, M. W. Jackson, H. Liu, W. Xin and L. Zhou (2017). "CRABP-II enhances pancreatic cancer cell migration and invasion by stabilizing interleukin 8 expression." Oncotarget **8**(32): 52432.
Yue, S., J. Li, S.-Y. Lee, H. J. Lee, T. Shao, B. Song, L. Cheng, T. A. Masterson, X. Liu and T. L. Ratliff (2014). "Cholesteryl ester accumulation induced by PTEN loss and PI3K/AKT activation underlies human prostate cancer aggressiveness." Cell metabolism **19**(3): 393-406.
Zakharova, L., M. Svetlova and A. F. Fomina (2007). "T cell exosomes induce cholesterol accumulation in human monocytes via phosphatidylserine receptor." Journal of cellular physiology **212**(1): 174-181.
Zech, D., S. Rana, M. W. Büchler and M. Zöller (2012). "Tumor-exosomes and leukocyte activation: an ambivalent crosstalk." Cell Communication and Signaling **10**(1): 37.
Zhang, H., T. Deng, R. Liu, M. Bai, L. Zhou, X. Wang, S. Li, X. Wang, H. Yang and J. Li (2017). "Exosome-delivered EGFR regulates liver microenvironment to promote gastric cancer liver metastasis." Nature communications **8**: 15016.
Zhao, L., J. Li, Y. Liu, L. Kang, H. Chen, Y. Jin, F. Zhao, J. Feng, C. Fang and B. Zhu (2018). "Cholesterol esterification enzyme inhibition enhances antitumor effects of human chimeric antigen receptors modified T cells." Journal of Immunotherapy **41**(2): 45-52.
Zhao, Y., M. Shen, Y. Feng, R. He, X. Xu, Y. Xie, X. Shi, M. Zhou, S. Pan and M. Wang (2018). "Regulatory B cells induced by pancreatic cancer cell-derived interleukin-18 promote immune tolerance via the PD-1/PD-L1 pathway." Oncotarget **9**(19): 14803.
Zhou, J., S. Song, S. He, X. Zhu, Y. Zhang, B. Yi, B. Zhang, G. Qin and D. Li (2014). "MicroRNA-375 targets PDK1 in pancreatic carcinoma and suppresses cell growth through the Akt signaling pathway." International journal of molecular medicine **33**(4): 950-956.
Zhou, M., J. Chen, L. Zhou, W. Chen, G. Ding and L. Cao (2014). "Pancreatic cancer derived exosomes regulate the expression of TLR4 in dendritic cells via miR-203." Cellular immunology **292**(1-2): 65-69.
Zhuang, L., J. Kim, R. M. Adam, K. R. Solomon and M. R. Freeman (2005). "Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts." The Journal of clinical investigation **115**(4): 959-968.
Özdemir, B. C., T. Pentcheva-Hoang, J. L. Carstens, X. Zheng, C.-C. Wu, T. R. Simpson, H. Laklai, H. Sugimoto, C. Kahlert and S. V. Novitskiy (2014). "Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival." Cancer cell **25**(6): 719-734.

Appendix

State of contribution by others and copyright authorisation

This thesis presents 4 first author peer-reviewed journal articles. I warrant that I have obtained, where necessary, permission from the copyright owners to use any third-party copyright material reproduced in the thesis or to use any of my own published work in which the copyright is held by another party.

Author and co-author contributions for each paper have been stated accordingly and indicated in the section below. All authors read and approved the final version of each manuscript prior to submission and publication.

`

Publication 1:

Emmanouilidi, A., Paladin, D., Greening, D. W., & Falasca, M. (2019). Oncogenic and Non-Malignant Pancreatic Exosome Cargo Reveal Distinct Expression of Oncogenic and Prognostic Factors Involved in Tumor Invasion and Metastasis. *Proteomics*, *19*(8), 1800158.

JOHN WILEY AND SONS LICENSE TERMS AND CONDITIONS

Jun 25, 2019

This Agreement between Ms. Aikaterini Emmanouilidi ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number	4615920547558
License date	Jun 25, 2019
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Proteomics
Licensed Content Title	Oncogenic and Non-Malignant Pancreatic Exosome Cargo Reveal Distinct Expression of Oncogenic and Prognostic Factors Involved in Tumor Invasion and Metastasis
Licensed Content Author	Aikaterini Emmanouilidi, Dino Paladin, David W. Greening, et al
Licensed Content Date	Apr 9, 2019
Licensed Content Volume	19
Licensed Content Issue	8
Licensed Content Pages	12
Type of use	Dissertation/Thesis
Requestor type	Author of this Wiley article
Format	Print and electronic
Portion	Full article
Will you be translating?	No
Title of your thesis / dissertation	Investigation of novel biomarkers for pancreatic cancer
Expected completion date	Oct 2019
Expected size (number of pages)	150
Requestor Location	Ms. Aikaterini Emmanouilidi Curtin University Building 305 Kent Street Perth, WA 6102 Australia Attn: Ms. Aikaterini Emmanouilidi
Publisher Tax ID	EU826007151
Total	0.00 AUD
Terms and Conditions	

TERMS AND CONDITIONS

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a"Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright Clearance Center Inc., ("CCC's Billing and Payment terms and conditions"), at the time that you opened your RightsLink account (these are available at any time at http://myaccount.copyright.com).

Terms and Conditions

- The materials you have requested permission to reproduce or reuse (the "Wiley Materials") are protected by copyright.

- You are hereby granted a personal, non-exclusive, non-sub licensable (on a stand-alone basis), non-transferable, worldwide, limited license to reproduce the Wiley Materials for the purpose specified in the licensing process. This license, **and any CONTENT (PDF or image file) purchased as part of your order,** is for a one-time use only and limited to any maximum distribution number specified in the license. The first instance of republication or reuse granted by this license must be completed within two years of the date of the grant of this license (although copies prepared before the end date may be distributed thereafter). The Wiley Materials shall not be used in any other manner or for any other purpose, beyond what is granted in the license. Permission is granted subject to an appropriate acknowledgement given to the author, title of the material/book/journal and the publisher. You shall also duplicate the copyright notice that appears in the Wiley publication in your use of the Wiley Material. Permission is also granted on the understanding that nowhere in the text is a previously published source acknowledged for all or part of this Wiley Material. Any third party content is expressly excluded from this permission.

- With respect to the Wiley Materials, all rights are reserved. Except as expressly granted by the terms of the license, no part of the Wiley Materials may be copied, modified, adapted (except for minor reformatting required by the new Publication), translated, reproduced, transferred or distributed, in any form or by any means, and no derivative works may be made based on the Wiley Materials without the prior permission of the respective copyright owner.**For STM Signatory Publishers clearing permission under the terms of the STM Permissions Guidelines only, the terms of the license are extended to include subsequent editions and for editions in other languages, provided such editions are for the work as a whole in situ and does not involve the separate exploitation of the permitted figures or extracts,** You may not alter, remove or suppress in any manner any copyright, trademark or other notices displayed by the Wiley Materials. You may not license, rent, sell, loan, lease, pledge, offer as security, transfer or assign the Wiley Materials on a stand-alone basis, or any of the rights granted to you hereunder to any other person.

- The Wiley Materials and all of the intellectual property rights therein shall at all times remain the exclusive property of John Wiley & Sons Inc, the Wiley Companies, or their respective licensors, and your interest therein is only that of having possession of and the right to reproduce the Wiley Materials pursuant to Section 2 herein during the continuance of this Agreement. You agree that you own no right, title or interest in or to the Wiley Materials or any of the intellectual property rights therein. You shall have no rights hereunder other than the license as provided for above in Section 2. No right, license or interest to any trademark, trade name, service mark or other branding ("Marks") of WILEY or its licensors is granted hereunder, and you agree that you shall not assert any such right, license or interest with respect thereto

- NEITHER WILEY NOR ITS LICENSORS MAKES ANY WARRANTY OR REPRESENTATION OF ANY KIND TO YOU OR ANY THIRD PARTY, EXPRESS, IMPLIED OR STATUTORY, WITH RESPECT TO THE MATERIALS OR THE ACCURACY OF ANY INFORMATION CONTAINED IN THE MATERIALS, INCLUDING, WITHOUT LIMITATION, ANY IMPLIED WARRANTY OF MERCHANTABILITY, ACCURACY, SATISFACTORY QUALITY, FITNESS FOR A PARTICULAR PURPOSE, USABILITY, INTEGRATION OR NON-INFRINGEMENT AND ALL SUCH WARRANTIES ARE HEREBY EXCLUDED BY WILEY AND ITS LICENSORS AND WAIVED

BY YOU.

- WILEY shall have the right to terminate this Agreement immediately upon breach of this Agreement by you.
- You shall indemnify, defend and hold harmless WILEY, its Licensors and their respective directors, officers, agents and employees, from and against any actual or threatened claims, demands, causes of action or proceedings arising from any breach of this Agreement by you.
- IN NO EVENT SHALL WILEY OR ITS LICENSORS BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR ENTITY FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL, INDIRECT, EXEMPLARY OR PUNITIVE DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, PROVISIONING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.
- Should any provision of this Agreement be held by a court of competent jurisdiction to be illegal, invalid, or unenforceable, that provision shall be deemed amended to achieve as nearly as possible the same economic effect as the original provision, and the legality, validity and enforceability of the remaining provisions of this Agreement shall not be affected or impaired thereby.
- The failure of either party to enforce any term or condition of this Agreement shall not constitute a waiver of either party's right to enforce each and every term and condition of this Agreement. No breach under this agreement shall be deemed waived or excused by either party unless such waiver or consent is in writing signed by the party granting such waiver or consent. The waiver by or consent of a party to a breach of any provision of this Agreement shall not operate or be construed as a waiver of or consent to any other or subsequent breach by such other party.
- This Agreement may not be assigned (including by operation of law or otherwise) by you without WILEY's prior written consent.
- Any fee required for this permission shall be non-refundable after thirty (30) days from receipt by the CCC.
- These terms and conditions together with CCC's Billing and Payment terms and conditions (which are incorporated herein) form the entire agreement between you and WILEY concerning this licensing transaction and (in the absence of fraud) supersedes all prior agreements and representations of the parties, oral or written. This Agreement may not be amended except in writing signed by both parties. This Agreement shall be binding upon and inure to the benefit of the parties' successors, legal representatives, and authorized assigns.
- In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall prevail.

- WILEY expressly reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

- This Agreement will be void if the Type of Use, Format, Circulation, or Requestor Type was misrepresented during the licensing process.

- This Agreement shall be governed by and construed in accordance with the laws of the State of New York, USA, without regards to such state's conflict of law rules. Any legal action, suit or proceeding arising out of or relating to these Terms and Conditions or the breach thereof shall be instituted in a court of competent jurisdiction in New York County in the State of New York in the United States of America and each party hereby consents and submits to the personal jurisdiction of such court, waives any objection to venue in such court and consents to service of process by registered or certified mail, return receipt requested, at the last known address of such party.

WILEY OPEN ACCESS TERMS AND CONDITIONS

Wiley Publishes Open Access Articles in fully Open Access Journals and in Subscription journals offering Online Open. Although most of the fully Open Access journals publish open access articles under the terms of the Creative Commons Attribution (CC BY) License only, the subscription journals and a few of the Open Access Journals offer a choice of Creative Commons Licenses. The license type is clearly identified on the article.

The Creative Commons Attribution License

The Creative Commons Attribution License (CC-BY) allows users to copy, distribute and transmit an article, adapt the article and make commercial use of the article. The CC-BY license permits commercial and non-

Creative Commons Attribution Non-Commercial License

The Creative Commons Attribution Non-Commercial (CC-BY-NC)License permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.(see below)

Creative Commons Attribution-Non-Commercial-NoDerivs License

The Creative Commons Attribution Non-Commercial-NoDerivs License (CC-BY-NC-ND) permits use, distribution and reproduction in any medium, provided the original work is properly cited, is not used for commercial purposes and no modifications or adaptations are made. (see below)

Use by commercial "for-profit" organizations

Use of Wiley Open Access articles for commercial, promotional, or marketing purposes requires further explicit permission from Wiley and will be subject to a fee.

Further details can be found on Wiley Online Library

http://olabout.wiley.com/WileyCDA/Section/id-410895.html

Other Terms and Conditions:

v1.10 Last updated September 2015

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

To Whom It May Concern:

I, Aikaterini Emmanouilidi, contributed as a first author to the conception, experimental design and procedures, data analysis, interpretation and manuscript preparation for the publication entitled "Oncogenic and Non-Malignant Pancreatic Exosome Cargo Reveal Distinct Expression of Oncogenic and Prognostic Factors Involved in Tumor Invasion and Metastasis"

Emmanouilidi, A., Paladin, D., Greening, D. W., & Falasca, M. (2019). Oncogenic and Non-Malignant Pancreatic Exosome Cargo Reveal Distinct Expression of Oncogenic and Prognostic Factors Involved in Tumour Invasion and Metastasis. *Proteomics*, 1800158.

I, Dr Dino Paladin, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Dino Paladin (Jul 25, 2019) Date _____

I, Dr David W Greening, as a corresponding author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature _____ Date _____

I, Professor Marco Falasca, as a corresponding author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature MARCO FALASCA (Jul 25, 2019) Date 25.7.2019

Publication 2:

Emmanouilidi, A., & Falasca, M. (2017). 3-Phosphoinositide-Dependent Kinase 1 (PDK1). *Encyclopedia of Signaling Molecules*, 1-4.

SPRINGER NATURE LICENSE TERMS AND CONDITIONS

Jun 26, 2019

This Agreement between Ms. Aikaterini Emmanouilidi ("You") and Springer Nature ("Springer Nature") consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.

License Number	4616401503676
License date	Jun 26, 2019
Licensed Content Publisher	Springer Nature
Licensed Content Publication	Springer eBook
Licensed Content Title	3-Phosphoinositide-Dependent Kinase 1 (PDK1)
Licensed Content Author	Aikaterini Emmanouilidi, Marco Falasca
Licensed Content Date	Jan 1, 2016
Type of Use	Thesis/Dissertation
Requestor type	academic/university or research institute
Format	print and electronic
Portion	full article/chapter
Will you be translating?	no
Circulation/distribution	<501
Author of this Springer Nature content	yes
Title	Investigation of novel biomarkers for pancreatic cancer
Institution name	n/a
Expected presentation date	Oct 2019
Order reference number	4615920547558
Requestor Location	Ms. Aikaterini Emmanouilidi Curtin University Building 305 Kent Street Perth, WA 6102 Australia Attn: Ms. Aikaterini Emmanouilidi
Total	0.00 USD
Terms and Conditions	

Springer Nature Customer Service Centre GmbH Terms and Conditions

This agreement sets out the terms and conditions of the licence (the **Licence**) between you and **Springer Nature Customer Service Centre GmbH** (the **Licensor**). By clicking 'accept' and completing the transaction for the material (**Licensed Material**), you also confirm your acceptance of these terms and conditions.

1. Grant of License

1. 1. The Licensor grants you a personal, non-exclusive, non-transferable, world-wide licence to reproduce the Licensed Material for the purpose specified in your order only. Licences are granted for the specific use requested in the order and for no other use,

subject to the conditions below.

1. 2. The Licensor warrants that it has, to the best of its knowledge, the rights to license reuse of the Licensed Material. However, you should ensure that the material you are requesting is original to the Licensor and does not carry the copyright of another entity (as credited in the published version).

1. 3. If the credit line on any part of the material you have requested indicates that it was reprinted or adapted with permission from another source, then you should also seek permission from that source to reuse the material.

2. Scope of Licence

2. 1. You may only use the Licensed Content in the manner and to the extent permitted by these Ts&Cs and any applicable laws.

2. 2. A separate licence may be required for any additional use of the Licensed Material, e.g. where a licence has been purchased for print only use, separate permission must be obtained for electronic re-use. Similarly, a licence is only valid in the language selected and does not apply for editions in other languages unless additional translation rights have been granted separately in the licence. Any content owned by third parties are expressly excluded from the licence.

2. 3. Similarly, rights for additional components such as custom editions and derivatives require additional permission and may be subject to an additional fee. Please apply to Journalpermissions@springernature.com/bookpermissions@springernature.com for these rights.

2. 4. Where permission has been granted **free of charge** for material in print, permission may also be granted for any electronic version of that work, provided that the material is incidental to your work as a whole and that the electronic version is essentially equivalent to, or substitutes for, the print version.

2. 5. An alternative scope of licence may apply to signatories of the STM Permissions Guidelines, as amended from time to time.

3. Duration of Licence

3. 1. A licence for is valid from the date of purchase ('Licence Date') at the end of the relevant period in the below table:

Scope of Licence	Duration of Licence
Post on a website	12 months
Presentations	12 months
Books and journals	Lifetime of the edition in the language purchased

4. Acknowledgement

4. 1. The Licensor's permission must be acknowledged next to the Licenced Material in print. In electronic form, this acknowledgement must be visible at the same time as the figures/tables/illustrations or abstract, and must be hyperlinked to the journal/book's homepage. Our required acknowledgement format is in the Appendix below.

5. Restrictions on use

5. 1. Use of the Licensed Material may be permitted for incidental promotional use and minor editing privileges e.g. minor adaptations of single figures, changes of format, colour and/or style where the adaptation is credited as set out in Appendix 1 below. Any other changes including but not limited to, cropping, adapting, omitting material that affect the meaning,

intention or moral rights of the author are strictly prohibited.

5. 2. You must not use any Licensed Material as part of any design or trademark.

5. 3. Licensed Material may be used in Open Access Publications (OAP) before publication by Springer Nature, but any Licensed Material must be removed from OAP sites prior to final publication.

6. Ownership of Rights

6. 1. Licensed Material remains the property of either Licensor or the relevant third party and any rights not explicitly granted herein are expressly reserved.

7. Warranty

IN NO EVENT SHALL LICENSOR BE LIABLE TO YOU OR ANY OTHER PARTY OR ANY OTHER PERSON OR FOR ANY SPECIAL, CONSEQUENTIAL, INCIDENTAL OR INDIRECT DAMAGES, HOWEVER CAUSED, ARISING OUT OF OR IN CONNECTION WITH THE DOWNLOADING, VIEWING OR USE OF THE MATERIALS REGARDLESS OF THE FORM OF ACTION, WHETHER FOR BREACH OF CONTRACT, BREACH OF WARRANTY, TORT, NEGLIGENCE, INFRINGEMENT OR OTHERWISE (INCLUDING, WITHOUT LIMITATION, DAMAGES BASED ON LOSS OF PROFITS, DATA, FILES, USE, BUSINESS OPPORTUNITY OR CLAIMS OF THIRD PARTIES), AND
WHETHER OR NOT THE PARTY HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. THIS LIMITATION SHALL APPLY NOTWITHSTANDING ANY FAILURE OF ESSENTIAL PURPOSE OF ANY LIMITED REMEDY PROVIDED HEREIN.

8. Limitations

8. 1. ***BOOKS ONLY:***Where **'reuse in a dissertation/thesis'** has been selected the following terms apply: Print rights of the final author's accepted manuscript (for clarity, NOT the published version) for up to 100 copies, electronic rights for use only on a personal website or institutional repository as defined by the Sherpa guideline (www.sherpa.ac.uk/romeo/).

9. Termination and Cancellation

9. 1. Licences will expire after the period shown in Clause 3 (above).

9. 2. Licensee reserves the right to terminate the Licence in the event that payment is not received in full or if there has been a breach of this agreement by you.

Appendix 1 — Acknowledgements:

For Journal Content:
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication papers:**
Reprinted by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION**

(Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM].)

For Adaptations/Translations:
Adapted/Translated by permission from [**the Licensor**]: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

Note: For any republication from the British Journal of Cancer, the following credit line style applies:

Reprinted/adapted/translated by permission from [**the Licensor**]: on behalf of Cancer Research UK: : [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication)

For **Advance Online Publication** papers:
Reprinted by permission from The [**the Licensor**]: on behalf of Cancer Research UK: [**Journal Publisher** (e.g. Nature/Springer/Palgrave)] [**JOURNAL NAME**] [**REFERENCE CITATION** (Article name, Author(s) Name), [**COPYRIGHT**] (year of publication), advance online publication, day month year (doi: 10.1038/sj.[JOURNAL ACRONYM])

For Book content:
Reprinted/adapted by permission from [**the Licensor**]: [**Book Publisher** (e.g. Palgrave Macmillan, Springer etc) [**Book Title**] by [**Book author**(s)] [**COPYRIGHT**] (year of publication)

Other Conditions:

Version 1.2

To Whom It May Concern:

I, Aikaterini Emmanouilidi, as the first author, contributed to the conception, wrote the manuscript and contributed to editing for the publication entitled "3-Phosphoinositide-Dependent Kinase 1 (PDK1)"

Emmanouilidi, A., & Falasca, M. (2017). 3-Phosphoinositide-Dependent Kinase 1 (PDK1). *Encyclopedia of Signaling Molecules*, 1-4.

I, Professor Marco Falasca, as the corresponding author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Marco Falasca (Jul 5, 2019) Date 5/7/2019

Publication 3:

Emmanouilidi, A., & Falasca, M. (2017). Targeting PDK1 for chemosensitization of cancer cells. *Cancers*, *9*(10), 140.

To Whom It May Concern:

I, Aikaterini Emmanouilidi, as the first author, contributed to the conception, wrote the manuscript and contributed to editing for the publication entitled "Targeting PDK1 for chemosensitization of cancer cells"

Emmanouilidi, A., & Falasca, M. (2017). Targeting PDK1 for chemosensitization of cancer cells. *Cancers*, *9*(10), 140.

I, Professor Marco Falasca, as the corresponding author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Marco Falasca Marco Falasca (Sep 17, 2019) Date 17/9/19

Publication 4:

Emmanouilidi, A., Fyffe, C. A., Ferro, R., Edling, C. E., Capone, E., Sestito, S., ... & Maffucci, T. (2019). Preclinical validation of 3-phosphoinositide-dependent protein kinase 1 inhibition in pancreatic cancer. *Journal of Experimental & Clinical Cancer Research*, *38*(1), 191.

To Whom It May Concern:

I, Aikaterini Emmanouilidi, contributed as the first author to the conception, experimental design and execution, data interpretation and manuscript preparation for the publication entitled "Preclinical validation of 3-phosphoinositide-dependent protein kinase 1 inhibition in pancreatic cancer"

Aikaterini Emmanouilidi, Chanse A. Fyffe, Riccardo Ferro, Charlotte E. Edling, Emily Capone, Simona Sestito, Simona Rapposelli, Rossano Lattanzio, Stefano Iacobelli, Gianluca Sala, Tania Maffucci and Marco Falasca (2019). Preclinical validation of 3-phosphoinositide-dependent protein kinase 1 inhibition in pancreatic cancer. *Journal of Experimental & Clinical Cancer Research*, *38*(1), 191.

I, Dr Chanse A. Fyffe, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature chanse Fyffe (Sep 17, 2019) Date Sep 17, 2019

I, Dr Riccardo Ferro, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Riccardo Ferro (Sep 19, 2019) Date Sep 19, 2019

I, Dr Charlotte E. Edling, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Charlotte Edling (Sep 17, 2019) Date Sep 17, 2019

I, Dr Emily Capone, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Emily Capone Date Sep 17, 2019

I, Dr Simona Sestito, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Simona Sestito Date Sep 17, 2019

I, Dr Simona Rapposelli, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Simona Rapposelli Date Sep 17, 2019

I, Dr Rossano Lattanzio, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Rossano Lattanzio (Sep 21, 2019) Date Sep 21, 2019

I, Professor Stefano Iacobelli, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Date Oct. 8, 2019

I, Dr Gianluca Sala, as a Co-Author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature _____ Date Sep 17, 2019

I, Dr Tania Maffucci, as corresponding author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Tania Maffucci Tania Maffucci (Sep 18, 2019) Date Sep 18, 2019

I, Marco Falasca, as corresponding author, confirm that this level of contribution by the candidate indicated above is appropriate. I agree to give permission to use this publication as part of Aikaterini Emmanouilidi's thesis.

Signature Marco Falasca Date October 9,2019