Faculty of Health Sciences

School of Pharmacy and Biomedical Sciences

ABC transporters and G protein-coupled receptors: perspectives for novel anti-cancer drugs

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Doctor of Philosophy

of

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Declaration

I, Aleksandra Adamska, confirm that the research included in this thesis is my own work and where it was carried out in collaboration with, or supported by others, this is duly acknowledged. To the best of my knowledge this thesis contains no material previously published by any other person except where due acknowledgment has been made. This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

Animal Ethics

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal research ethics approval from the Curtin University Animal Ethics Committee, Approval Number AEC 2016 40. Xenograft mouse work was approved by the Italian Ministry of Health (N.484/2016-PR) and was performed at the University of Chieti, Chieti, Italy.

Signature: ………………………………………….

Date:

General introduction

Pancreatic ductal adenocarcinoma (PDAC) is a 4th cause of cancer-related deaths in modern world. It is characterized by marginal survival rates, with only 8% of the patients having chance to survive over 5 years, placing it among the most malignant cancers. This poor prognosis is the multifactor result of aggressive phenotype, early metastatic dissemination, lack of symptoms leading to late diagnosis, high resistance to chemotherapy and lack of effective therapeutic approach. Up to now, the reference drugs: Gemcitabine, FOLFIRINOX and ABRAXANE, only marginally improved patients' survival and their activity was coupled with several adverse effects. Attempts have been made to increase their efficiency rates by combinatorial and targeted therapies; however, no significant improvement could be achieved so far. Therefore, it is pivotal to investigate novel molecules and signalling pathways essential for PDAC progression that can be explored clinically.

One of the reasons for the marginal improvement in survival of PDAC patients is resistance of pancreatic cancer to therapies, caused in part by overexpression of ATP binding cassette (ABC) transporters. By shuffling a wide range of molecules outside of the cells, including drugs and xenobiotics, ABC transporters contribute the development of multidrug resistance in cancer cells. However, recent evidence suggest that by transporting bioactive molecules, including lipids involved in activation of pathways essential for cell proliferation and migration, ABC transporters activity might directly impact cancer progression. However, no direct contribution of ABC transporters in PDAC progression has been demonstrated so far and their pharmacological potential in PDAC remains to be explored.

It was demonstrated in our group that ABC transporters and G protein-coupled receptors could define an autocrine loop involving lysophosphatidylinositol (LPI) in cancer cells. The original research conducted in the laboratory of Prof Falasca reported that GPR55, one of G protein-coupled receptors (GPCRs), is overexpressed in many cancer types, including pancreatic cancer. Additionally, GPR55 activity has been linked with a bioactive lipid LPI, which contribution to cancer progression has been demonstrated in several cancer types. The involvement of LPI-GPR55 axis in regulation of cancer cell proliferation and the correlation between GPR55 expression

and PDAC aggressiveness and progression was shown. However, so far no studies investigated in depth the role of GPR55 in PDAC progression and addressed the potential of pharmacological inhibition of GPR55 in the therapy of pancreatic cancer. In this study, I aimed to investigate the role of ABCC3, one of ABC transporters, and GPR55 in pancreatic cancer. I also aimed to verify the existence of the ABCC3-LPI-GPR55 loop and validate the pharmacological potential of targeting the components of this loop to counteract progression of pancreatic cancer.

Using both *in vitro* and *in vivo* approaches, I could demonstrate that GPR55 and ABCC3 play critical role in PDAC progression. Functionally, I showed that GPR55 and ABCC3 are involved in control of cell growth by regulation of cell cycle, autophagy and apoptosis through activation of MAPK and STAT3 signalling. In addition, the dependence of GPR55 and ABCC3 expression on TP53 status was demonstrated. Moreover, involvement of ABCC3 in release of LPI was demonstrated, confirming the link between ABCC3 and GPR55. Importantly, potent inhibitors of both proteins were identified and their high efficacy in slowing down PDAC progression was demonstrated *in vitro* and in animal models of PDAC. In addition, the role of a cancer stem-like subpopulation of PDAC cells in PDAC chemoresistance was indicated and was proposed as a potent tool for validation of new pharmacological interventions in pancreatic cancer. Collectively, in my project I could demonstrate that ABCC3-LPI-GPR55 loop is a novel and potent pharmacological target in PDAC, which inhibition should be explored clinically in a cohort of PDAC patients with p53 mutations.

Acknowledgements

Firstly, I would like to thank my supervisor, Prof Marco Falasca for giving me this project, for his guidance, support and mentorship and for sharing his extensive knowledge and expertise. I would also like to thank my co-supervisor Prof Philip Newsholme and my associate supervisors Dr Alice Domenichini and Prof Vincenzo de Laurenzi. I really appreciate of all the effort, dedication and enthusiasm you have devoted to my studies. In particular, I want to thank Dr Alice Domenichini for her guidance, patience, help and friendship. Thank you for dragging me out of the work to enjoy the nature, hiking and coffee in the best company. In addition, I want to thank the Associate Professor Elizabeth Watkin, the Postgraduate Co-ordinator and the chairperson of my thesis committee, for your help and support.

I would like to acknowledge Curtin University for providing the scholarship that assisted me throughout my PhD. I would also like to acknowledge the School of Pharmacy and Biomedical Sciences and the Curtin Health Innovation Research Institute (CHIRI) for providing the resources that enabled me to complete my project. I would like to thank Dr Riccardo Ferro for starting the project and contributing to its development. I also want to thank our collaborators, Professor Gary Piazza from the University of South Alabama (USA) for providing and characterizing the ABCC3 inhibitor (S3), Professor Kenneth Linton from Queen Mary University of London (UK) for ABCC3 transport studies and Dr Rossano Lattanzio from University of Chieti for the analysis of Immunohistochemistry and histopathological analysis. In particular, I would like to thank Dr Gianluca Sala, for his guidance and help during my studies at the University of Chieti, Chieti Italy and his contribution to the development of my project. Additional thanks to Dr Emily Capone and Verena Damiani for their work on the xenograft mouse models in this project. I would also like to thank Dr Pierluigi di Sebastiano for providing tumour tissues for the PDX mouse model, Prof Owen Sansom for the murine cells and Dr David Tuveson for the KPC mouse model. Many special thanks to the members of Metabolic Signalling Group and to all my friends and colleagues from the School of Biomedical Sciences and CHIRI. Thank you for all of the support and care during all these years away from home. In particular, I would

like to thank Riley for bringing the joy, happiness and support, sharing multiple adventures and enduring the most challenging times during my studies.

Finally yet importantly, I want to thank my parents and my whole family for the support in my move to Australia. Without your help, encouragement and belief in me, I would never decide on the big move to another hemisphere and would not make it through the tough days away from home.

Abbreviations

ADM	adenal to ductal metaplasia
BRCA2	breast cancer type 2 susceptibility protein
CAFs	cancer-associated fibroblasts
CBD	cannabidiol
CDKN2A	cyclin dependent kinase inhibitor 2A
CMS	carboxymethylcellulose
COX	cyclooxygenase
CSCs	cancer stem cells
CTGF	connective tissue growth factor
DAB	3,3'-Diaminobenzidine
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
EMT	epithelial mesenchymal transition
ERK	extracellular signal-regulated kinase
FAS	fatty acid synthase
GPCR	G protein-coupled receptor
HCC	hepatocellular carcinoma
HH	hedgehog
IHC	immunohistochemistry
IPMN	intraductal papillary mucinous neoplasm
JAK	janus kinase
LPA	lysophospatidic acid
MAPK	mitogen-activated protein kinase
MCN	mucinous cystic neoplasm
OS	overall survival
PanIN	pancreatic intraepithelial neoplasia
PAR	protease activated receptor
PDG	platelet-derived growth factor
PFS	progression-free survival
PI3K	phosphoinositide 3-kinases
PLA	phospholipase
PSCs	pancreatic stellate cells
PV	portal vein
RT	room temperature
S1P	sphingosine-1-phosphate
SHH	sonic hedgehog
SMA	superior mesenteric artery
SMV	superior mesenteric vein
STAT3	signal transducer and activator of transcription 3
TRP	transient receptor potential cation channel
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	vascular endothelial growth factor

Published primary papers

Adamska A, Domenichini A, Falasca M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. Int J Mol Sci. 2017 Jun 22; 18(7): 1338; doi: 10.3390/ijms18071339

Domenichini A, **Adamska A**, Falasca M. ABC transporters as cancer drivers: Potential functions in cancer development. Biochim Biophys Acta Gen Subj. 2019 Jan; 1863(1): 52-60; doi.org/10.1016/j.bbagen.2018.09.019

Ferro R, **Adamska A**, Lattanzio R, Mavrommati I, Edling CE, Arifin SA, Fyffe CA, Sala G, Sacchetto L, Chiorino G, De Laurenzi V, Piantelli M, Sansom OJ, Maffucci T, Falasca M. GPR55 signalling promotes proliferation of pancreatic cancer cells and tumour growth in mice, and its inhibition increases effects of gemcitabine. Oncogene, 2018, 37(49):6368-6382; doi: 10.1038/s41388-018-0390-1

Adamska A, Falasca M. ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward? World J Gastroenterol. 2018 Aug 7;24(29):3222-3238. doi: 10.3748/wjg.v24.i29.3222.

Adamska A, Ferro R, Lattanzio R, Capone E, Domenichini A, Damiani V, Chiorino G, Akkaya BG, Linton KJ, De Laurenzi V, Sala G, Falasca M. ABCC3 is a novel target for the treatment of pancreatic cancer. Adv Biol Regul 2019 Apr 24. pii: S2212-4926(19)30036-3. doi: 10.1016/j.jbior.2019.04.004.

Domenichini A, Edmands JS, **Adamska A**, Begicevic RR, Paternoster S, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. Adv Biol Regul. 2019 Feb; 72: 63-77; doi: 10.1016/j.jbior.2019.02.001

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Published secondary papers

Adamska A, Elaskalani O, Emmanouilidi A, Kim M, Abdol Razak NB, Metharom P, Falasca M. Molecular and cellular mechanisms of chemoresistance in pancreatic cancer. Adv Biol Regul, 2018 May; 68: 77-87; doi: 10.1016/j.jbior.2017.11.007

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Falasca M, Hamilton JR, Selvadurai M, Sundaram K, **Adamska A**, Thompson PE. Class II Phosphoinositide 3-Kinases as Novel Drug Targets. J Med Chem. 2017 Jan 12; 60(1): 47-6; doi: 10.1021/acs.jmedchem.6b00963

Attended conferences and awards

- 05.2016- Poster session at 'Science on the Swan' conference, Perth, UK
- 06.2016- Poster session at 'Pancreas 2016' International conference, Glasgow, UK
- 11.2016- Oral presentation at ' Australasian Pancreatic Club' conference, Sydney, Australia- main prize for the best oral presentation
- 02.2017- Oral presentation at 'Cell signalling' symposium, Perth, Australia
- 09.2018- Oral presentation at ' Australasian Pancreatic Club' conference, Brisbane, Australia
- 07.2019- Oral presentation at the 'International Conference on Pharmacology: Advances in Translational Sciences and Drug Discovery- the prize for the best oral presentation

Statement of Contribution by Others

The majority of the data presented in this thesis is the effect of my own original work. If the experiments were performed by collaborators or third parties, appropriate contribution is acknowledged in the figure legends. The data and information included in this thesis resulted in several publications, which are included, in parts or whole, in this thesis. All mentioned publications are attached in whole at the end of the thesis. For each publication used in this thesis, my contribution has been acknowledged by all the authors and the declarations are attached at the end.

Thesis structure

- CHAPTER 1

In chapter 1, I present the literature review that provides the background and the rationale for my studies. In addition, I present the original work for the thesis including two reviews that I published during the course of my PhD.

In the subchapter 1.6 entitled "Therapies for pancreatic cancer", I included the fragments of the following publication: "**Adamska A**, Domenichini A, Falasca M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. Int J Mol Sci. 2017 Jun 22; 18(7): 1338; doi: 10.3390/ijms18071339."

Due to the length of the publication, I combined the fragments of this publication so that they present in a cohesive way the most crucial information for the background of my project. I acknowledged the source of the publication at the start of the chapter, and the fragments adapted from the publication are highlighted in the published version of the publication attached at the end of the thesis. The review was published under creative comments CC BY license, allowing me as the author to reproduce the content without the permission of the journal.

As the subchapter 1.7 entitled "ABC transporters", I used the following publication: "Domenichini A, **Adamska A**, Falasca M. ABC transporters as cancer drivers: Potential functions in cancer development. Biochim Biophys Acta Gen Subj. 2019 Jan; 1863(1): 52-60; doi.org/10.1016/j.bbagen.2018.09.019"

The last revised version of the article prior to publication is presented in this chapter. Published review article is attached at the end of the thesis.
All the authors have acknowledged my contribution to the publication. The review was published as an open access presentation, allowing for the reproduction by the authors, granting the acknowledgement of the journal.

The rest of the subchapters of the introductory chapter 1 were produced during the preparation of the thesis.

- CHAPTER 2

In chapter 2, I present the materials and methods that were used in the study described in this thesis. Methods that were used only for the data presented in the form of published articles are shown within the attached publications and not in the chapter 2.

- CHAPTER 3

Chapter 3 includes the data that I produced for the first part of my PhD project on the pharmacological potential of GPR55 in pancreatic cancer. This project was a continuation of the work commenced by Dr Riccardo Ferro at the Queen Mary University of London (QMUL) and resulted in the publication of the following research article:

“Ferro R, **Adamska A**, Lattanzio R, Mavrommati I, Edling CE, Arifin SA, Fyffe CA, Sala G, Sacchetto L, Chiorino G, De Laurenzi V, Piantelli M, Sansom OJ, Maffucci T, Falasca M. GPR55 signalling promotes proliferation of pancreatic cancer cells and tumour growth in mice, and its inhibition increases effects of gemcitabine. Oncogene, 2018, 37(49):6368-6382; doi: 10.1038/s41388-018-0390-1”

In this chapter, I present my contribution to this publication. Original figures produced for the thesis, as well as figures adapted from the publication are presented, referring to the original figure in the publication. When data not obtained by me is mentioned, the reference to the appropriate figure in the published paper is made. Unpublished data produced by me are also presented in this chapter. Whole publication is attached at the end of the chapter.

- CHAPTER 4

Chapter 4 presents the role of ABCC3 in pancreatic cancer.

As an introduction to this chapter, I adapted fragments of the following publication: “**Adamska A**, Falasca M. ATP-binding cassette transporters in progression and clinical

outcome of pancreatic cancer: What is the way forward? World J Gastroenterol. 2018 Aug 7; 24(29):3222-3238. doi: 10.3748/wjg.v24.i29.3222."

Fragments of the publication were used to present in a short and cohesive way the rationale for the investigation of the role of ABCC3 in pancreatic cancer. The acknowledgement of the journal is made at the beginning of this subchapter. Complete, published version of the review article with highlighted fragments that were adapted in this section is attached at the end of the thesis.

The results and discussion part of this chapter is presented in the form of published research article:

"**Adamska A**, Ferro R, Lattanzio R, Capone E, Domenichini A, Damiani V, Chiorino G, Akkaya BG, Linton KJ, De Laurenzi V, Sala G, Falasca M. ABCC3 is a novel target for the treatment of pancreatic cancer. Adv Biol Regul 2019 Apr 24. pii: S2212-4926(19)30036-3. doi: 10.1016/j.jbior.2019.04.004.

Majority of the data presented in the publication are my own work. The mRNA analysis was performed by Dr Riccardo Ferro, QMUL; The IHC analysis was done by Dr Rossano Lattanzio, University of Chieti, LPI transport experiments were performed by Prof Kenneth Linton, QMUL. The last revised version prior to publication is presented. Published version is attached at the end of the thesis.

- CHAPTER 5

Chapter 5 presents the pharmacological potential of ABCC3 in pancreatic cancer.

As an introduction to this chapter, I adapted fragments of the following publication: "**Adamska A**, Falasca M. ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward? World J Gastroenterol. 2018 Aug 7; 24(29):3222-3238. doi: 10.3748/wjg.v24.i29.3222."

Fragments concerning the role of ABC transporters in cancer therapy are presented to demonstrate the background and rationale for the study.

The presented results and discussion contain my original research. When data was obtained with the help of collaborators, appropriate acknowledgement is stated in the figure legend.

Parts of the results data presented in this chapter have been accepted for publication. However, due to the date of publication release later than the thesis submission, the chapter is not presented in the form of the publication. The accepted version of the article is attached at the end of the chapter.

- CHAPTER 6

In chapter 6, I present the link between ABCC3 and GPR55 in PDAC. The introduction, results and discussion contain unpublished material. All presented experiments contain my own work. When published data is mentioned, appropriate reference is provided.

- CHAPTER 7

In chapter 7, I investigated the role of PDAC tumorspheres in PDAC chemoresistance. In this chapter, I present the data that I obtained for the bigger project that resulted in the following publication:

“Domenichini A, Edmands JS, **Adamska A**, Begicevic RR, Paternoster S, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. Adv Biol Regul. 2019 Feb; 72: 63-77; doi: 10.1016/j.jbior.2019.02.001”

The experiments that were performed by me that were included in the publication (isolation of tumorspheres and characterization of chemoresistance of PDAC thumorspheres) are presented in this chapter. Figures published in this article that were based on my work are presented and the reference to the appropriate figure in the publication is made under the figure legends. Whole publication is attached at the end of the chapter.

- CHAPTER 8

In chapter 8, I discuss the findings and importance of the data presented in the thesis combining all the chapters together in a cohesive story. I also propose future studies that should be undertaken in order to add the value to presented study.

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1. Literature review

1.1 Pancreas

The pancreas is an organ of the digestive system that participates in the digestive processes and regulates glucose homeostasis. Structurally, pancreas can be divided in exocrine and endocrine lobules. Exocrine part of the pancreas, composed of acinar cells (82% of pancreatic mass), responsible for the production of digestive enzymes (proteases, lipases, amylases) and ductal structures (4%), transporting enzymes to intestines comprises the majority of pancreatic mass (more than 95%) (1).

Endocrine part, composed by islet cells, is scattered across the tissues. It comprises only 2-3 % of pancreatic mass and is responsible for regulating glucose levels (2). Each islet is formed by circa 5000 endocrine cells classified in four main groups: α cells, synthesizing and secreting glucagon (20% of all endocrine cells), β cells synthesizing and secreting insulin and amylin (68%), δ cells secreting somatostatin (1%) and small fraction of cells (2%) secreting pancreatic polypeptide and adrenomedullin (PP/F cells) (3). The cellular architecture of the pancreas is maintained by a variety of stromal and supporting cells that communicate with each other and with the other cellular compartments in the pancreas in a complex way. The stromal factors include fibroblasts, pancreatic stellate cells (PSC), inflammatory cells and nerves. Regarding its anatomy, the pancreas can be divided in three parts- head, the biggest part of the pancreas contacting intestines, body and tail, adjoining the spleen (4) (Figure 1.1). Two ducts run through the body of the pancreas: main pancreatic duct and accessory pancreatic duct, joining with the common bile duct, allowing for the transport of enzymes secreted form the acinar pancreatic cells. The integrity of the duct system is crucial for proper functioning of the pancreas, preventing early activation of transported enzymes, which might lead to pancreatitis. Typically, pancreas is also drained by lymph nodes that differ in localization.

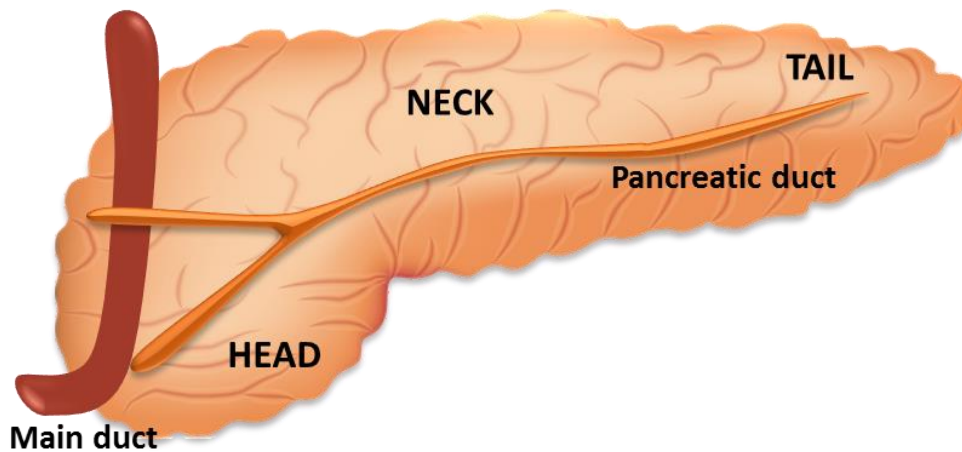

Figure 1.1 Anatomy of pancreas

Clinically, dysregulation of proper functioning of pancreas may lead to several malignancies, including pancreatitis, diabetes or cancer. Pancreatitis is an inflammation of the pancreas, acute or chronic, which results in considerable morbidity. In healthy organism, the proteases produced by pancreatic cells remain in the inactive state during their synthesis, secretion and transport through the pancreatic duct. During pancreatitis, enzymes produced by the exocrine pancreas, such as lipases or amylases, are activated in the acinar cells, causing "autodigestion" and damage the organ (5). Type 1 and Type 2 Diabetes is also associated with pancreatic functions, caused by the dysregulation of insulin homeostasis. Insulin resistance or impaired insulin secretion from pancreatic islets are main causes of type 2 diabetes, while impairment of insulin-secreting cells of the pancreas by immune cells is considered as one of the mechanisms of type 1 diabetes (6).

1.2 Pancreatic Cancer

Pancreatic cancer, in particular its most common form, Pancreatic Ductal Adenocarcinoma (PDAC), is a very aggressive disease that represents one of the major challenges in cancer research. The overall 5-year survival for PDAC patients has not changed significantly over last few decades, leaving PDAC sufferers with grim prognosis of only 8% compared to 5% in 1970s (7). Characterized by a silent nature,

which results in the lack of distinctive symptoms, PDAC is usually diagnosed at the late stage. At that point, the tumour has already spread out from the primary site to the surrounding organs (locally advanced disease) or distant organs (metastatic disease). Therefore, only ~20% of diagnosed patients are eligible for surgical resection (resectable and borderline resectable tumours). However, prognosis is grim and most of the patients will eventually relapse, reaching 5-year survival of 15-25% (8). The majority of the patients are diagnosed with a stage IV metastatic disease, which leaves them with a mere 3% of 5-year survival rate (9). With the lack of effective therapies, aging and obese population, PDAC is predicted to become the leading cause of cancer-related death in the near future. With around 380.000 patients diagnosed worldwide with pancreatic cancer, 331.000 died making PDAC the 7th cause of cancer-related deaths in 2012 (10). Nevertheless, it has been reported that until 2030 its worldwide incidence is predicted to increase up to around 420.000 cases per year, with mortality reaching almost 410.000 cases, making PDAC second most mortal cancer type (11).

Although typically considered as one disease, pancreatic cancer can be classified into different subtypes, depending on the site of origin. Endocrine pancreatic tumours account for 1-2% of all pancreatic malignancies and are typically characterized by a benign course and a more favourable prognosis (12). Derived from cells involved in hormone production, the names and the malignant consequences (the excessive production) of endocrine pancreatic tumours are reflected by the hormone that they produce (insulinomas, glucagonomas, gastrinomas) (13).

Exocrine tumours are the most common form of pancreatic cancer and can be sub-classified into sarcomas, cystic tumours or acinar cell carcinomas. The vast majority (around 85-90%) of cases originating from exocrine ducts gives rise to pancreatic ductal adenocarcinoma (PDAC). The remaining percentage accounts for the intraductal papillary mucinous neoplasms (IPMN) or mucinous cystic neoplasms (MCN) (14, 15). Considering the anatomy of the pancreas, each part is susceptible, to different extent, to cancer initiation. The vast majority of PDAC cases is localized in the head of the pancreas (~80%), with lower occurrence in the body (15%) and the tail of the pancreas (5%).

There are several factors adding to the increased risk of developing pancreatic cancer (16-19), amongst them the most distinctive are:

- Age (the risk increases in patients over 65 years of age)
- Smoking
- Obesity
- Genetic factors
- Chronic pancreatitis
- Heavy consumption of alcohol
- Diabetes mellitus
- Stomach ulcer (*Helicobacter pylori* infection)

1.3 Stages of PDAC

For clinical management, PDAC is classified into different groups, based on the tumour node metastasis (TNM) system, in which the primary tumour size (TX, T0-T4), local metastasis to the regional lymph nodes (NX, N0-1) and the distant metastasis (M0-1) are assessed (20, 21). Determination of the TNM values allows for the division of diagnosed tumours into different prognostic groups (0-II resectable or borderline resectable, III locally advanced, unresectable, IV metastatic unresectable), which determines susceptibility of the tumours to the surgical removal (22).

Stage 0- is characterized by localized tumour without any local or distant metastasis.

Stage 1- is characterized by localized tumour without the presence of lymph node metastasis. Depending on the tumour size, it can be sub-classified as Stage 1a (tumour smaller than 2cm) and Stage 1b (bigger than 2cm).

Stage 2- similarly to Stage 1 it can be subdivided into two categories depending on the extent of observed metastasis:

- Stage 2a- the extension of the tumour is not restricted to the pancreas, however no involvement of superior mesenteric artery (SMA), celiac axis or lymph done metastasis can be detected

- Stage 2b- the extension of the tumour is not restricted to the pancreas with regional lymph node metastasis

Stage 3- is characterized by extensive tumour, which burdens involve superior mesenteric artery or celiac axis and lymph node metastasis

Stage 4- is characterized by the occurrence of any of abovementioned events accompanied the presence of distant metastasis

1.4 Biology of pancreatic cancer

Pancreatic cancer is a very complex disease, which development is accompanied by reprogramming events driven by accumulating mutations.

1.4.1 Development of PDAC

The development of histologically defined tumour precursors called pancreatic intraepithelial neoplasias (PanINs) precedes the formation of the invasive cancer. It is well documented that following a pancreatic injury or inflammation, acinar cells undergo an acinar to ductal metaplasia (ADM), which, in normal conditions, is a reversible process (23). However, combined with abnormal activity of KRAS or growth factor signalling pathways, ADM may arise as a precursor of PanINs (18, 24, 25).

PanINs are classified into three stages (PanIN1, PanIN2, PanIN3), characterized by accumulating abnormalities reflected in the histologic modification (Figure 1.2).

- PanIN1, which may be further classified into PanIN1a and PanINb, is characterized by overlapping of histological features with non- neoplastic tissues
- PanIN2 stage is characterized by abnormalities observed at the cellular level, including enlarged nuclei and loss of cellular polarity.
- PanIN3 is a final precursor stage preceding the conversion into fully invasive tumour and is characterized by complete loss of cell polarity.

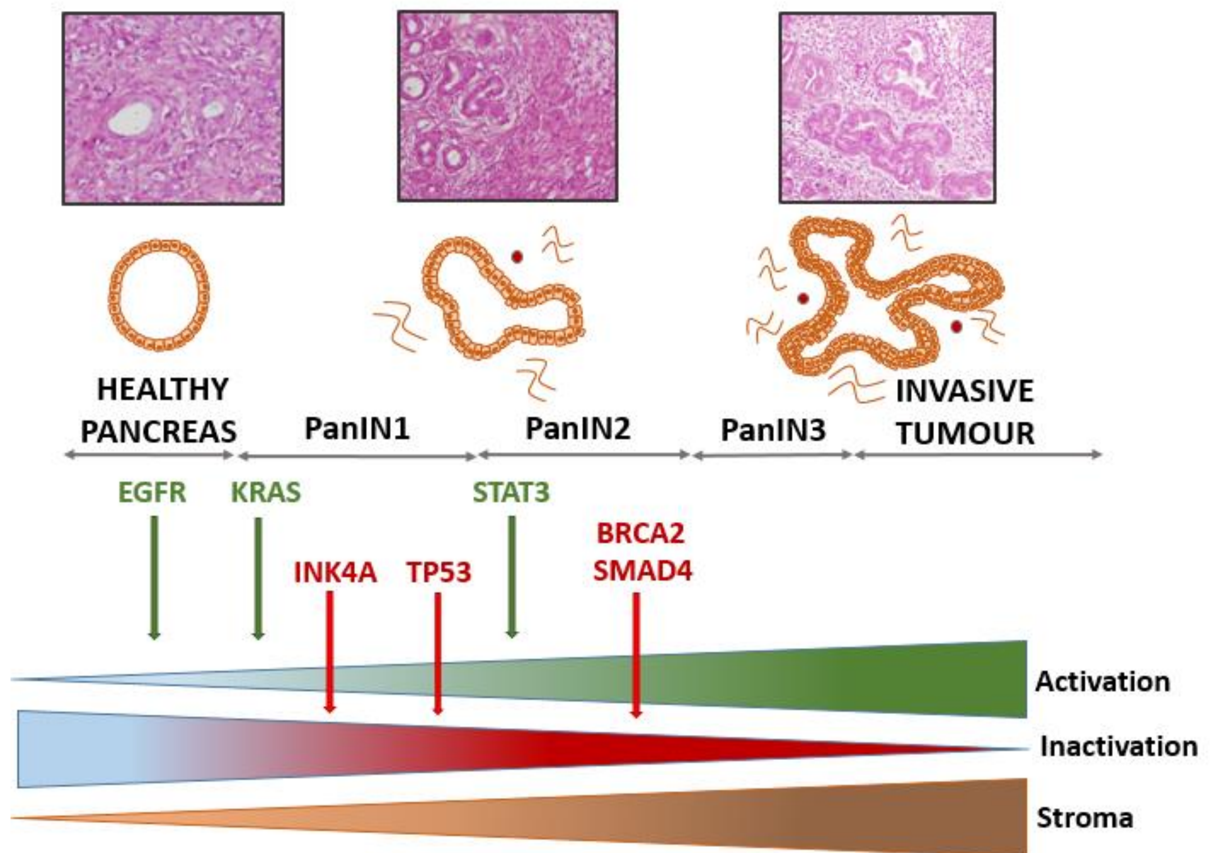

Figure 1.2 Development and histopathology of invasive PDAC through consecutive PanIN stages driven by activation (green) or inactivation (red) of key genes

These changes are accompanied by the progression of genetic and morphological changes that drive the observed ductal reprogramming (26).

1.4.2 Mutational heterogeneity of PDAC

Pancreatic cancer tissues are characterized by high complexity and heterogeneity that is reflected phenotypically and at the genetic level. The analysis of a series of pancreatic ductal adenocarcinomas and the use of the genome map led to the establishment of the protein-coding mutations prevailing in PDAC. Oncogenic *KRAS* and suppressor genes *TP53, SMAD4* and *p16/CDKN2A* were confirmed as the main genes, which dysregulation leads to the onset and acceleration of PDAC development. Additionally, mutations in between 26 and 67 genes, leading to

dysregulation of around 12 signalling pathways are found across different pancreatic tumours (Table 1.1) (27-30). Recently, observed mutations were classified into four different groups defined by distinct phenotype, mutational landscape and prognosis: squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX) (31). Based on genetic profiling, classification of patients into these groups may provide valuable information on the prognosis and potential curative treatments for the patients.

Gene	Function	Notes
	Main mutations	
KRAS	MAPK signalling	Oncogene
P16/CDKN2A	Cell cycle regulation	Tumour suppressor
SMAD4	TGFβ signalling	Tumour suppressor
TP53	DNA damage response	Tumour suppressor
	Additional mutations	
AKT2	PI3K signalling	
BRAF	MAPK signalling	
BRCA2	DNA repair	
CDK6	Cell cycle regulation	
GATA6	Transcription factor	
MAP2K4	MAPK signalling	
MET	Growth factor signalling	
MYC	Cell cycle regulation	
TGFBR1	TGFβ signalling	

Table 1.1 Selected mutations observed in PDAC development

Most pancreatic tumours carry *RAS* mutations (95% of all cases), among which the most distinctive is a point mutation in the 12 codon in the *KRAS* gene (85% of all *KRAS* mutations) (32). The membrane-bound GTP-ase protein encoded by *KRAS* gene is activated by the family of Epidermal Growth Factor Receptors (EGFRs). When mutated, KRAS protein gains oncogenic activity through continuous activation of downstream signalling pathways (e.g. MAPK/ERK, PI3K/Akt). This, in turn, enhances the proliferative signals in the cells, influencing their invasiveness, growth as well as changes their metabolism and environment. Although *KRAS* mutated pancreatic adenocarcinomas are prevailing among all diagnosed cases, *KRAS* wild-type tumours, harbouring other mutations, e.g. *BRAF* have been noted. Oncogenic KRAS activation is the earliest event in PDAC onset, detected in circa 90% of low grade PanINs. However, mutation of KRAS is not sufficient for progression of the lesions to fully invasive tumours. During PDAC progression, KRAS mutations accumulate, together with other alterations that pile up progressively. *TP53* is another important gene, which alterations are detected in around 75% of PDAC cases. Mutations of *TP53* are observed in later stages of PanIN, suggesting its involvement in PDAC progression to invasive tumours rather than its initiation. Loss of p53 results in genetic instability, leading to heterogeneity and cytogenic rearrangements (33). Inactivation of *CDKN2* gene, encoding protein involved in cell cycle regulation is observed in high percentage (~90%) of intermediate stages of PDAC progression (PanIN2/3) (34). Moreover, inactivating mutations of *SMAD4/DPC4* are observed in around 55% of cases (35). Additionally, a smaller fraction of PDAC cases is characterized by additional alterations if several genes, including *BRCA2, MLH1* or Breast Cancer 2 gene, contributing to high heterogeneity pf PDAC tumours (36-38).

Consequently, aberrations in multiple signalling pathways, involved in cell proliferation, survival and invasion are observed. As an example, JAK/STAT pathways, directly contributing to cell proliferation and apoptosis are found upregulated in PDAC, accelerating its progression (39). Moreover, a dysregulation of the Notch pathway has been widely reported in PDAC patients and was correlated with increased resistance of the tumours (40, 41). Similarly, overactivation of the components of Hedgehog pathway were observed during PDAC progression. In particular, Sonic hedgehog (SHH) and its downstream effectors have been reported

as important players in desmoplasia, contributing to enhanced disease progression and reduced drug delivery (42, 43).

Additionally, upregulation of several receptors has been observed in a variety of PDAC specimens. In particular, increased expression of human epidermal growth factor receptor (EGFR or ErbB1) was observed (44). EGFR is a member of ErbB receptor family, which activation leads to triggering of multiple signalling cascades (e.g. Ras/PI3K/Akt or MAPK/ERK), contributing to regulation of key cellular functions, such as cell cycle, cell survival and differentiation (45). Therefore, overexpression and enhanced activation of EGFR, detected in majority of PDAC samples, upregulates signalling pathways of crucial importance for PDAC progression.

1.4.3 STAT3 signalling in PDAC development

One of the earliest events in PDAC onset is also stimulation of the Signal Transducer and Activator of Transcription (STAT) signalling via increased phosphorylation of the STAT3 protein (46). In the embryonic development of the pancreas and pancreas homeostasis, STAT3 family members are present in the inactive state and are dispensable for its proper function (47). However, their constitutive activation is observed in a range of malignancies, including PDAC (48). Activation of STAT3 proteins, enhanced during tumorigenesis consists on phosphorylation of the tyrosine residues, which triggers the dimerization of the protein and its nuclear localization, where it subsequently contributes to the transcription of a variety of genes. It has been demonstrated that the main mechanism of STAT3 activation during malignancies is mediated by the Janus (JAK) family of tyrosine kinases or src kinases, which are activated by the growth factor or cytokine receptors (49, 50). Particularly, in PDAC it was proposed that stromal interleukin 6 (IL6) induces phosphorylation of STAT3 by binding and activating the gp130 receptor, frequently overexpressed in PDAC specimens (51). Despite the clear regulation of STAT3 signalling through gp130-mediated IL6 activation, it has been recently shown that other mechanisms exist in PDAC that participate in STAT3 activation in a gp-130 independent manner. Although STAT3 activation, together with *KRAS* mutation is one of the earliest events in PDAC tumorigenesis (52) contributing to PDAC onset and progression, no KRAS-regulated

STAT3 activation could be demonstrated. Nevertheless, the dependence of STAT3 continuous activation on mutation or deletion of *TP53*, another key gene for PDAC progression, has been recently documented. The activity of STAT3 is fundamental for PDAC progression and has been reported in high portion of PDAC cases at all stages of disease progression and promotes its progression by induction of cell invasion and metastasis (53, 54). The importance of STAT3 signalling in PDAC development is demonstrated by the fact that its activation is required for acinar-to-ductal metaplasia (ADM) one of the earliest events in PDAC carcinogenesis (55). The depletion of STAT3 in *KRAS* mutated mice resulted in significant reduction in ADM initiation and PanINs progression, but it also impaired tumour progression to the invasive stage, pointing to the role of STAT3 activity in the development of PDAC at all stages of the disease. Similarly, its inhibition significantly reduced PDAC cell growth and cancer progression (56). In addition, the involvement of STAT3 in apoptotic machinery has been demonstrated, showing the correlation between STAT3 phosphorylation and activity of Bcl-xl, an anti-apoptotic protein. In fact, the inhibition of STAT3 phosphorylation with the use JAK inhibitor (AZ960) was shown to down-regulate Bcl-xl in PADC cell lines, which, in turn, induced apoptosis. Other STAT3-regulated processes that contribute to tumorigenesis include energy metabolism, cell differentiation and regulation of inflammation. Moreover, the role of STAT3 in PDAC stroma and tumour-stroma interplay has been also suggested. Continuous STAT3 activation was indicated to support a pro-tumorigenic environment through myeloid-suppressor cells activation. On the other hand, it has been shown that the inhibition of STAT3 signalling reduced stromal density and vascularization via sonic hedgehog inhibition. In addition, STAT3 inhibition reduced the expression of cytidine deaminase, a key enzyme in the degradation of cytidine analogues such as gemcitabine. In fact, STAT3 inhibition via blocking of JAK activity combined with gemcitabine treatment resulted in the reduced cell resistance and the dramatic increase in gemcitabine efficiency in a mouse model (52). Therefore, targeting the STAT3 pathway in PDAC is an attractive strategy for PDAC therapy, leading to stroma reprogramming and reducing tumour chemoresistance.

1.5 Hallmarks of pancreatic cancer

One of the main hallmarks of cancer is the increased proliferative rate of the malignant cells. Uncontrolled propagation of cancer cells requires enhanced nutritional supply and a favourable environment to maintain the increased growth rate and the expansion of the tumour. Therefore, during tumorigenesis cancer cells are subjected to a series of pathological changes that adapt them to unfavourable conditions and support their increased nutritional needs (57).

1.5.1 Cancer metabolism

Tumour cells, proliferating faster than normal cells, have higher anabolic demands. Hence, tumour cells re-programme their metabolism to be able to supply cells with crucial components (58, 59). One of the remarkable features of cancer cells metabolism is the higher rate of glucose uptake and dependence on aerobic glycolysis as the source of energy, a phenomenon called as 'Warburg effect' (60). Although less efficient than aerobic oxidative phosphorylation in ATP production, glycolysis is advantageous for cancer cells since it allows them to survive under harsh hypoxic conditions and to form their own acidity buffer, which promotes cells invasiveness. Moreover, cancer cells use glycolysis intermediates as the source of energy and for synthesis of other molecules, including nucleotides or lipids, necessary for their growth (61, 62). Apart from glucose, many cancer types suffer from glutamine addiction. Utilized as a source of carbon and nitrogen, glutamine is used in nucleotide synthesis, supporting cancer cell growth and propagation (63, 64). Similar to glucose conversion, glutaminolysis leads to increased levels of pyruvate in cancer cells that in turn is converted to citrate, suppling PDAC cells with an additional carbon source for lipid synthesis (65, 66). PDAC cells are additionally characterized by enhanced turnover of lipids. The role of lipids in the development of metabolic diseases, including cancer has been widely documented. It has been documented that TP53, a tumour suppressor frequently dysregulated in malignant tissues including pancreatic cancer, regulates lipid homeostasis and metabolism and that its mutations increase

the synthesis of lipids in cancer cells (67, 68). Elevated lipid metabolism supplies cancer cells with nutrients essential for quickly proliferating cancer cells. Lipids can be used by cancer cells as an energy source, facilitating cell proliferation. In addition, as building blocks of cell membrane (mainly phospholipids and cholesterol), they facilitate the division of rapidly proliferating cells. As main membrane components and energy source, lipids also ensure appropriate cell energetic homeostasis. Phospholipids, sterol esters, triacylglycerols and the enzymes responsible for their synthesis (e.g. phospholipases) are crucial players in cell physiology. Interestingly, their abnormal synthesis and activity is reported in different malignancies, especially in tumorigenesis. Moreover, levels of fatty acids (FAs), phospholipids precursors, are remarkably increased in pancreatic cancer cells, and their overexpression is important for protection of cancer cells from oxidative stress and maintenance of high proliferative rates (69-73). Cholesterol is another essential metabolite for PDAC cell survival. Being one of the building blocks in cell membrane, mainly lipid rafts, cholesterol affects membrane integrity. It also facilitates the interaction with membrane-bound proteins, leading to the activation of phosphorylation cascades (74). Therefore, depending on its availability, cholesterol levels highly influence the growth and division of cancer cells (75). More importantly, some of the lipids, especially phospholipids, act as signalling molecules contributing to the activation of signalling pathways that influence cancer cell proliferation and survival.

Apart from lipids, the enzymes involved in their synthesis are upregulated in malignant specimens and actively contribute to cancer progression. In addition, it has been documented that their increased levels were correlated with poor prognosis of PDAC patients. As an example, fatty acid synthase (FASN) is typically upregulated in cancer cells and correlates with poor prognosis for the patients (76). It has been shown that FASN inhibition decreases cell proliferation and tumour growth, at the same time inducing apoptosis. Cyclooxygenase 2 (COX-2) is another molecule, which elevated expression was reported in substantial portion of PDAC cases (45-75%) (77, 78). In PDAC, COX-2 is involved in the synthesis of prostaglandins from phospholipid-derived arachidonic acid, influencing cell metabolism. Therefore, COX-2 inhibition,

e.g. with non-steroidal anti- inflammatory drugs has been investigated for the counteraction of the inflammation- related PDAC progression (79).

Altogether, malignant transformation of pancreatic cells is characterised by the increased dependence on glucose and glutamine consumption, and the increased synthesis, extracellular uptake and metabolism of lipids (80). This broad repertoire of genetic and metabolic remodelling allows PDAC to increase its proliferative ability and to survive under harsh conditions. Thus, targeting the metabolic machinery in PDAC represents a potential strategy to reduce the survival and growth of malignant cells, decelerating the disease progression.

1.5.2 Apoptosis

Another mechanism sustaining the high proliferating rates of cancer cells and supporting their survival is the ability to escape from apoptotic stimuli. Normal cells divide and proliferate in response to growth factor stimuli that are rigorously controlled in order to maintain proper cell functioning and homeostasis. However, cancer cells are able to escape this stringent control through several mechanisms, e.g. production of their own growth signals that stimulate their own proliferation or overexpression of growth factor receptors, which increases their responsivity (58). Another mechanism, by which human tissues maintain homeostasis is programmed cell death (apoptosis) aiming at the removal of damaged or mutated cells (81). There are three main ways for the induction of apoptosis, which involve intrinsic (called also mitochondrial), extrinsic (death receptor) and intrinsic endoplasmic reticulum pathways. The common mechanism of these pathways is the activation of the family of cysteine proteases called caspases, at the final stages of apoptotic signalling (82, 83). More precisely, caspases 3, 6 and 7, that are activated by both extrinsic and intrinsic pathways, cleave the inhibitor of deoxyribonucleases, leading to the induction of apoptosis. In addition, cleavage of substrate proteins, such as cytoskeletal proteins or DNAse inhibitors, changes the morphology of apoptotic cells (83).

In order to maintain their high proliferative potential, cancer cells activate several resistance mechanisms not detected in normal cells to reduce the responsiveness to death signals and evade the apoptotic machinery. These processes enable cancer cells to become self-sufficient and acquire the ability of uncontrolled growth. Interestingly, p53 mutations have been demonstrated to contribute to regulation of cell apoptosis in several malignancies (84).

There are several mechanisms, through which cancer cells may evade apoptosis, including reduced activity of caspase proteins, hindered death receptor signalling or imbalance in pro-apoptotic and anti-apoptotic signals (85). As an example, overexpression of anti-apoptotic proteins, like members of Bcl family, playing a key role in initiation of apoptosis or reduced expression of pro-apoptotic proteins, such as Bax or Bac changes the integrity of mitochondrial membrane (86, 87). This in turn induces the release of apoptotic regulators (e.g. cytochrome c) and cleavage of caspase protease family (88). It was demonstrated that members of Bcl-2 family, especially Bcl-xL are overexpressed in several cancers including PDAC and that its expression increases with tumour grading (89-92). It was also shown that overexpression of Bcl-xL correlates with aggressiveness of the tumours, poor prognosis and survival (93). In addition, it was proposed that both EGFR and STAT3 pathways, key players in PDAC development, are involved in the regulation of Bcl-xL expression and activity (94, 95). Moreover, expression of Bcl-xL was shown to assist the epithelial to mesenchymal transition (EMT) as well as cell invasiveness and migration, contributing to the increased metastatic potential of pancreatic tumours (96). Conversely, inhibition of Bcl-xL signalling leads to the increase in cancer cell senescence and apoptosis and, at the same time, a decrease in cell viability, providing a potential therapeutic approach for cancer treatment.

The existence of multiple mechanisms enabling malignant cells the escape from the programmed cell death affects their ability to survive and propagate in a hostile environment. Therefore, apoptosis is considered one of the major mechanisms of chemotherapy-induced cancer cell death (97, 98).

1.5.3 Epithelial to Mesenchymal Transition (EMT)

Cancer cell plasticity is another essential characteristic of PDAC, which enables PDAC cells to adapt their phenotype and metabolism to the changing environment (99, 100). These features contribute to the increased metastatic potential of PDAC cells. In order to colonize distant organs, cancer cells need to undergo a series of phenotypical and metabolic changes. Cell dissemination, extravasation, migration and colonization in distant organs requires a shift in cell phenotype. Epithelial to mesenchymal transition (EMT) is a cellular program, which allows a cellular transition from the epithelial state to the mesenchymal motile state (100, 101). The reverse mechanism, mesenchymal to epithelial transition (MET), takes place once the cells reach the metastatic niche, enabling them the re-colonization and development of metastatic tumours (102). Additionally, an intermediate partial EMT state called p-EMT, in which both states can be detected, was recently identified mainly for the circulating cancer cells, facilitating the motility of individual cells and cell clusters (103, 104). A complex machinery that involves transcriptional regulation of genes, supressing the epithelial and activating mesenchymal regulators governs EMT. Among many, SNAIL, ZEB1, Twist, SLUG, vimentin or claudins, are considered as the main players in EMT process and in metastasis (105, 106). In addition, the activity of EMT-related transcription factors (EMT-TFs) was shown to maintain the stem properties of cancer cells, reinforcing the essential role of EMT-TFs in cancer initiation and progression (107, 108). It was reported that the circulating pancreatic cells, which underwent EMT transition, possess stem-like characteristics, enabling them to survive under the changing and hostile conditions and efficiently metastasize (109, 110). The stem-like characteristics, together with metabolic changes and more mesenchymal character of the cells, apart from contributing to increased metastatic spread also induce chemoresistance in the cells (111). As an example, resistance to 5-FU or gemcitabine was linked with the activity of EMT-TF, SNAIL (112). Interestingly, the dissemination and invasion of the cells can be detected very early in the PDAC progression, even before fully invasive tumours can be noted, suggesting that the occurrence of EMT is one of the earliest event in PDAC development (113). Moreover,

inflammation processes and inflammatory environment accompanying the malignant transformation of the cells was suggested to promote EMT at the early PanIN stages of PDAC progression (114, 115). Although the involvement of EMT in cell dissemination was well documented, no evidence connecting EMT with colonization exists so far. I*n vivo* data investigating the role of particular EMT-TFs in metastatic spread did not provide convincing results, especially in PDAC (116, 117). This suggests that the EMT regulatory network is extremely complex and it is necessary for multiple EMT-TFs to function simultaneously in the regulation of metastatic spread.

1.5.4 Tumour microenvironment

One of the reasons for the dismal prognosis of PDAC is a high chemoresistance of the tumours arising because of heterogeneity and plasticity of PDAC tissues. An additional factor is the formation of a dense, diffuse stroma around the tumour bulk, which comprises about 80% of the tumour volume (118). Pancreatic stellate cells (PSCs), cancer associated fibroblasts (CAFs), immune cells, blood vessels and extracellular matrix (ECM) proteins (e.g. fibronectin, collagen, laminin, vimentin) form a dense environment that interacts with cancer cells through the expression of plethora of molecules (e.g. chemokines, EGFs, Cox-2), influencing tumour progression and invasion (119-121).

In a healthy pancreatic tissue fibrogenesis is a well-regulated process, controlling extracellular matrix turnover and maintaining proper architecture of the organ. It has been well established that pancreatic stellate cells are the key cells in fibrogenic process. In healthy pancreas they comprise a small proportion of the cells (4-7%) and remain in a quiescent fat-storing and vitamin A-storing phenotype (122). PSCs are converted into activated myofibroblast-like phenotype upon stimulation by extra or intracellular molecules, including cytokines, interleukins, growth factors or oxidative stress, which occur during pancreatic injury (123). Once activated, PSCs lose their ability to store vitamin A and commence to secrete high levels of ECM proteins, cytokines and growth factors themselves, which interact in a paracrine and autocrine

way with cancer and stromal cells stimulating the proliferation and remodelling the microenvironment, which leads to increased fibrosis (124). Thus, activation of PSCs involves several mechanisms such as paracrine activation with cytokines released by surrounding cancer and inflammatory cells and autocrine activation with own produced factors ensuring continuous activation. Given that PDAC cells express high levels of growth factors, e.g. PDGF or VEGF, the stimulation and activation of PSCs is remarkably enhanced in pancreatic cancer and leads to development of dense fibrotic tissue surrounding the tumour (125).

It has been demonstrated that activated stellate cells are the main source of fibrosis in pancreatic cancer and their presence was reported at the earliest stages of PDAC development (PanINs) (126, 127). Importantly, the correlation between the extent of PSC activation in PDAC stroma and patients' survival was reported (124, 128). Regulation of the activity of stromal fibroblast was linked with genetic dysregulation and activation of several cellular pathways occurring during tumorigenesis. In particular, one of the main mutations occurring during PDAC development, *TP53* dysregulation, was shown to influence desmoplasia. It was shown that mutation of *TP53* led to increased levels of IL-6, a well-established PSCs activator. Additionally, IL-6 increased phosphorylation rates of STAT3 proteins, key players in PDAC (129). Another important player in the development of PDAC stroma is the Hedgehog (Hh) signalling, enhanced in both tumour and stromal cells and crucial for PDAC progression (130). Alteration in at least one of the Hedgehog signalling genes (Smoothened (SMO), Sonic Hedgehog (SHH)) was correlated with the level of detected desmoplasia and PDAC progression (131). Vimentin or collagen are some of the stromal markers being able to stain fibroblast cells, whereas α smooth muscle actin (αSMA) is a marker for activated stellate cells and is remarkably enhanced in pancreatic desmoplasia (132).

It is now confirmed that PDAC microenvironment is a flexible entity, which composition and characteristics change upon tumour development. However, there have been conflicting data on the role of desmoplasia in PDAC progression and two-sided influence on tumour development has been suggested. While at the initial stages it suppresses tumour growth by restraining the available space, the interaction

of growing tumour with surrounding stroma modulates its characteristic and shifts it towards pro-tumorigenic phenotype. In return, by formation of a cancer-promoting environment, cancer stromal cells influence PDAC development. Therefore, the interactions between cancer and stroma cells reported in PDAC form of a feed-forward loop, which influences the survival and proliferation of epithelial and fibroblasts cells, perpetuating cancer progression and development of metastasis (133, 134). Epithelial cells secrete growth factors and cytokines providing continuous activation of PSCs. The transport of amino acids, acetate, lactate, lipids and TCA cycle intermediates was shown to contribute to the increased glycolytic rate observed in PDAC tumours (135). Moreover, EMC proteins secreted by stromal cells maintain proliferation of cancer cells and prevent their apoptosis (136). Additionally, it was demonstrated that cancer cell-induced autophagy occurring in cancer-associated fibroblasts (CAFs) led to increased levels of secreted alanine, which in turn provided fuel for tumour cells proliferating in low-glucose microenvironment (137). Elevated expression of EMT markers and enhanced cancer cell migration has been also associated with PSC activation (138).

The importance of stromal components in PDAC development and progression and the two-way interaction between epithelial and stromal cells shown *in vitro* has been also reported *in vivo*. It has been shown that PSCs alone are not sufficient to form the tumours in the xenograft animal models of PDAC. However, the addition of PSCs to PDAC cells remarkably increased the pace and the volume of tumours grown in xenograft mouse models of PDAC (both subcutaneous and orthotopic) (139, 140). Moreover, addition of PSCs significantly increased the number of metastatic nodules compared to injection of cancer cells alone (140). Interestingly, when injected, human activated stellate cells could be detected in murine liver metastasis, suggesting the involvement of the stromal cells in cancer cell migration and formation of the metastatic niche (141, 142). Additionally, an increased desmoplastic reaction was observed at the metastatic sites (120). On the other side, murine stellate cells could be detected in the stroma of pancreatic tumours in the orthotopic models, suggesting that the tumour, during its progression, recruits and activates the host stellate cells.

The importance of stroma in PDAC progression has been demonstrated. However, discrepancies exist so far regarding the potential of pancreatic stroma as therapeutic target in PDAC therapy. On one side, the formation of dense stoma around the tumour creates a protective coat around the tumour, providing it with the stimuli necessary for cell proliferation. In addition, the dense stroma decreases the micro-vascularity and, as consequence, restrains drug delivery to the tumour bulk. Therefore, it is tempting to speculate that depleting the stromal cells may present a potential anticancer strategy. Considering the involvement of Hedgehog (Hh) pathways in the interplay between tumour and stromal cells, targeting of the components of the Hh pathways has attracted attention as a potential strategy to disrupt tumour-stroma interactions. As an example, small-molecule inhibitor of Smoothened (IPI-926-03; Infinity Pharmaceuticals) in combination with gemcitabine was tested in clinical trials following promising *in vitro* results (143). When combined with gemcitabine or nab-paclitaxel, IPI-926 significantly increased drug delivery, prolonging survival in mouse models. However, deterioration of patients' outcomes due to the higher metastatic spread caused early termination of the clinical studies (144). Few other studies also demonstrated that stroma depletion led to development of more aggressive phenotypes and significantly shorter survival rates of the patients (145). A recent work evaluating the effects of modulation of Hh pathway activity in three different transgenic mouse models confirmed that inhibition of the Hh pathway accelerated the progression of the KRas- mutated PDAC (146). Therefore, results achieved so far are not clear-cut and indicate that different strategies in targeting desmoplasia in PDAC need to be investigated. Compounds targeting other stroma- related factors, such as connective tissue growth factor (CTGF) or angiotensin II type 1 receptor are currently also investigated. Additionally, targeting non-cellular stroma compartments, such as hyaluronic acid (HA) e.g. with PEGPH20 (a PEGylated recombinant hyaluronidase) showed promising preliminary data. Combination therapy of PEGH20 with gemcitabine (147), or ABRAXANE (148) is currently in progress.

Collectively, the indispensability of stroma in PDAC progression has been widely documented. However, the dual role of stroma needs to be considered while designing new therapeutic approaches targeting PDAC stroma. In addition, further

studies are necessary to evaluate the conflicting nature of PDAC stroma and determine its function in certain tumour stages or context.

1.5.5 Hypoxia

Fast proliferating cells and dense tumour microenvironment lead to poor tumour vascularisation and perfusion. As a consequence, insufficient blood supply and low oxygen levels cause hypoxia, which also characterizes the aggressive phenotype of PDAC (149). In addition, PDAC tumours and the surrounding stroma are able to produce anti-angiogenic factors, such as endostatin, which further reduce O_2 delivery to the tumour (150, 151). It has been shown that compared to normal tissues, which typically receive 30-50 mmHg of oxygen pressure, in solid tumours, including pancreatic cancer, these levels remarkably drop to 2.5 mmHg (152).

Cancer cells evolve in response to environmental conditions, such as reduced O_2 levels, in order to adapt and survive in ever-changing conditions. Deprivation of cancer cells of the oxygen stabilizes a protein called hypoxia inducible factor 1α (HIF1α), overexpressed in more than 85% of PDAC cases and correlated with overall poor prognosis of the patients. Following stabilization, HIF1α binds to HIF1β, forming a dimer that is transferred to the nucleus to bind with hypoxia-responsive genes (153). All these processes are induced to counteract the effects of low oxygen levels within the cancer cell environment, maintain ATP production and promote cell proliferation. HIF1α activity has been also linked with invasiveness and metastatic potential of PDAC as well as reprogramming of cell metabolism (154, 155). Interestingly, hypoxia and increased expression of HIF1α in cancer cells activates glucose transporter 1 (GLUT1). Increased glycolysis induced by HIF1α activity protects the cells from apoptosis in hypoxic conditions, when both oxygen and glucose supply to the cells is limited (156). Similarly, enhanced levels of glutaminase 2, converting glutamine to glutamate was observed in PDAC cells, enabling to maintain FAs levels in the hypoxic regions (157). The combination of hypoxic conditions and KRAS mutation-induced metabolic changes increases the aggressiveness of PDAC (158). In

addition, as the dense stromal reaction is responsible for creating an hypoxic environment, which, in turn activates HIF1α, this protein has been proposed as a mediator of cancer cell-microenvironment interactions (159). On one side, the dense stroma surrounding the tumour compresses the capillaries, decreasing the amount of delivered O_2, contributing to hypoxia. On the other side, hypoxia induces the activation of PSCs through enhanced secretion of Sonic Hedgehog (SHH) (160). A feed-forward loop formed by abovementioned interactions promotes hypoxia and hypoxia-mediated PDAC progression. Additionally, the role of HIF1α in the promotion of PDAC chemoresistance (161, 162), through upregulation of several anti- apoptotic (Bcl-xl) or pro-survival (PI3K/Akt) pathways adds to the complex role of hypoxia in PDAC progression (163, 164).

1.6 Therapies for Pancreatic cancer

This chapter is adapted from the parts of the following article:

Adamska A, Domenichini A, Falasca M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. Int J Mol Sci. 2017 Jun 22; 18(7): 1338; doi: 10.3390/ijms18071339

The whole published review is attached at the end of the thesis, with adapted sections highlighted in yellow. The author contribution form stating my contribution is attached at the end of the thesis.

In the last decades, significant improvements have been achieved in the screening and therapy of different solid cancers, highly incrementing patients' chance for cure. Nevertheless, despite the advancement in pancreatic cancer research, the mortality to incidence ratio has not experienced significant revision over the last few decades. Despite the remarkable effort made towards the improvement of PDAC patients' perspectives, no progress in its treatment has been achieved in last decades. Treatment options for pancreatic ductal adenocarcinoma are rather limited and highly depend on disease stage (Figure 1.3). Therefore, proper diagnosis and accurate staging allow for better prognosis and highly influence treatment choice and patients' chance of survival.

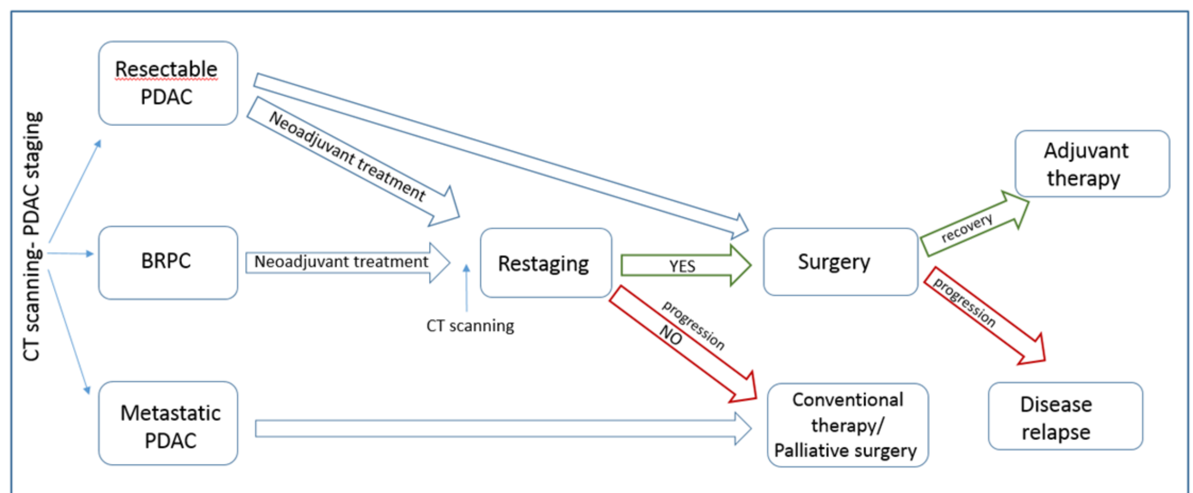

Figure 1.3 *Therapeutic options for PDAC patients*.

Figure 1 in the publication: **Adamska A**, Domenichini A, Falasca M. Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies. Int J Mol Sci. 2017 Jun 22; 18(7): 1338; doi: 10.3390/ijms18071339

Currently, surgical resection of the pancreas with microscopically free margins remains the only realistic and potentially curative option for pancreatic cancer patients, even though it is restricted to earlier disease stages. In general, the operability status is dictated mainly by the extent of venous involvement. However, the choice of surgery and its extent is imposed by not only tumour localization and extension, but also by surgeon expertise and by patient's performance status (PS), which is one of the major prognostic factors. For patients that are eligible for resection (resectable, borderline resectable), available surgical options are: pancreaticoduodenectomy (head/body of the pancreas and nearby organs are removed), distal pancreatectomy (tail, body and spleen), total pancreatectomy (whole pancreas and nearby organs) or palliative surgery (stent or bypass), which may alleviate symptoms of biliary and gastric outlet obstruction (165). Unfortunately, at the time of diagnosis, less than 20% of patients have a resectable tumour (166). The remaining patients present tumours, which are already too widespread to be surgically removed. At this stage of the disease retroperitoneal and perineural infiltration, hematogenic dissemination and angioinvasion are observed. In particular, cancers of the body and tail of the pancreas are often detected at the late stage and they usually present major vessels involvement, such as hepatic artery or celiac axis (167). Therefore, even despite the lack of metastasis, they are usually classified as unresectable. Despite low percentage of patients undergoing surgery, the chance of survival for surgical patients has significantly increased in the last few decades. Despite considerably high postoperative complications, the mortality rates do not exceed 5% (168). The effectiveness of surgery and patients' long-term survival depends partially on lymph-node infiltration but also on surgeon expertise and centre volume. Unfortunately however, even after successful resection the median survival time is 20 months, with 25% 5-year survival rate (169). The majority of resected patients suffers from tumour recurrence (~40%) within 6-24 months post-surgery

(170), highlighting the necessity for preoperative/postoperative therapies in order to achieve more effective treatments. Surgery followed by adjuvant therapy has been shown to provide slight, but significant survival benefit for non-metastatic patients in several phase III studies. So far, gemcitabine and 5-FU-based postoperative chemoradiation has been considered as standard of care, improving median OS of 2-5 months (171, 172). However, adjuvant therapy remains a controversial field, with results obtained in clinical trials ranging from definite survival benefit (173) to negative impact on patients' OS (172). In addition, almost 60% of resected patients presents early tumour progression or prolonged recovery, disabling planned postoperative treatment. Therefore, if no distant metastasis has been detected during cancer diagnosis and staging, the recommended first line treatment is neoadjuvant chemotherapy. The therapy aims to enhance drug delivery and tumour oxygenation and minimise tumour burden, which may result in downstaging and more definite surgical resection (174) and reduce the risk of tumour implantation during pancreatectomy (175). Preoperative treatment might also avoid the delay between diagnosis and start of postoperative treatment, usually caused by patient's prolonged recovery, and enable treatment of early micrometastasis (176). Currently, FOLFIRINOX-based preoperative therapy is being tested for improved efficacy, mainly in down-staging tumour burden, as well as targeting micrometastasis. However, neoadjuvant treatment also raises several concerns, such as disease progression during preoperative treatment or possible increase in surgical complications. Nevertheless, many clinical trials are still ongoing in order to combine the best neoadjuvant agents with postoperative adjuvant therapies (mostly exploring FOLFIRINOX and ABRAXANE-based options), hoping to obtain most prominent improvement in survival of patients with resectable or borderline resectable tumours.

Once metastasized, pancreatic cancer prognosis is poor. Chemotherapy treatment remains the main option for patients with advanced and metastatic tumours. Radiation, in combination with chemotherapy, is another option for unresectable, metastatic cancer (177). Nonetheless, the effects achieved by both approaches are mainly a mildly increased survival rate and lowered cancer-related symptoms.

Moreover, due to elevated toxicity, combination chemotherapy, which is associated with slightly better outcomes, is limited only to patients with a good performance status (PS). Since 1997, gemcitabine has been accepted as a reference first-line therapy drug for patients with a good performance status (178). Its advantage over previously used 5-FU has been reported in different individual studies. In a comparative phase III study (n=126) of single agent gemcitabine and 5-FU, a clinical benefit response was experienced by 23.8% of gemcitabine-treated patients compared to 4.8% of 5-FU-treated patients (178) with median survival time of 5.6 and 4.4 months. Following the positive results obtained by gemcitabine treatments, studies on more intensive and effective combination therapies, composed of gemcitabine and different cytotoxic and biological agents (e.g. cisplatin, epirubicin, fluorouracil, and gemcitabine (PEFG), S-1, erlotinib or capecitabine) have been developed. Unfortunately, despite an acceptable toxicity profile and increased response rates, significant improvement in overall survival (OS) over single-agent gemcitabine was rarely observed (179-183). More complex treatments would potentially increase the patient anti-tumour response. However, they are associated with higher toxicity and greater incidence of adverse effects (184). A significant response to a combination of gemcitabine and albumin-bound paclitaxel (nab-paclitaxel, ABRAXANE) was observed in patients with advanced pancreatic cancer (185, 186). A synergistic effect of the drug combination was attributed to the improvement in the intratumoral delivery of both gemcitabine and paclitaxel, facilitated by fused albumin (187). The effects of this combination treatment, in a phase III trial (n=861), significantly surpassed the single-agent gemcitabine therapy in all tested parameters. The median OS time of 8.5 months and 6.7 months was noted in ABRAXANE-gemcitabine and gemcitabine groups, respectively. Unfortunately, the positive response to this therapy was accompanied by a considerable increase in occurrence of adverse events, including grade 3 or 4 neutropenia, leukopenia, neuropathy, febrile neutropenia, or fatigue (188). Nevertheless, the increase in patients survival rates, at all time points, was a base for FDA approval and establishment of ABRAXANE-gemcitabine as the first-line therapy option for patients with advanced and metastatic pancreatic cancer. Recently, based on the proven advantageous and synergistic activity of its particular components

(189-192), a multidrug combination (irinotecan, oxaliplatin, fluorouracil, and leucovorin) called FOLFIRINOX has been shown to be an effective first line therapy, especially for patients with metastatic pancreatic cancer. The anti-tumour effect in patients with advanced cancer was shown in a phase I trial (193) and confirmed in a phase II-III study, which explored patients' response to FOLFIRINOX and single-agent gemcitabine (194). The superiority of FOLFIRINOX over gemcitabine was recognised in all efficacy parameters, including OS (11.1 months vs. 6.8 months), progression-free survival (PFS) (6.4 vs. 3.3 months), and 1-year survival rate (48.4% vs. 20.6%), which presented statistically significant improvement. Unfortunately, the safety profile of FOLFIRINOX treatments was not favourable (194). Despite elevated adverse effects, introduction of FOLFIRINOX and ABRAXANE to PDAC therapeutic repertoire brought new hope for patients and investigators. However, due to elevated toxicity, combination chemotherapy, which is associated with slightly better outcomes, is limited only to patients with a good performance status (PS).

Considering the wide variety of signalling pathways dysregulated in pancreatic cancer and triggering its progression, targeted therapies have emerged as a possibility to augment available therapeutic strategies. This approach has been successfully implemented in the treatment of different solid tumours, with imatinib mesylate (Gleevec) being the first FDA approved targeted treatment of metastatic gastrointestinal tumours in 2002 (195). Since then this therapeutic approach has been widely used and many targeted drugs for e.g. colorectal, melanoma or non-small lung cancer have been approved (196, 197). However, due to the heterogeneous nature of pancreatic cancer and complex stromal interactions, most of the targeted therapies failed to exhibit any clinical benefit compared to standard treatment. The only exception was erlotinib, an epidermal growth factor receptor (EFGR) inhibitor which, in combination with gemcitabine, showed moderate but statistically significant (2 weeks) improvement in patients' survival (198) giving the basis for FDA approval. Variety of pre-clinical trials explored the potential of inhibition of genes and signalling pathways key in PDAC tumorigenesis. Molecules targeting EGF receptors or KRAS- induced pathways (e.g. Gefitinib, Everolimus or Trametinib), HER-2 (Lapatinib), Notch pathway (Demcizumab) or JAK-STAT signalling (Ruxolitinib) alone or in combination with chemotherapy have been investigated. However, although

many of the studies on targeted PDAC therapies showed promising results in preclinical or clinical settings, most of them failed during phase II/III trials. Nevertheless, numerous phase I/Ib studies are still ongoing with many of them showing encouraging results, enabling to move on to phase II/III trials. Induction of an anti-tumour immune response has been shown to be extremely effective in different advanced stage cancer types. However, immunotherapy trials in PDAC have shown conflicting results so far. Collectively, conventional cytotoxic treatments applied so far, both chemotherapy and radiotherapy have been rather unsuccessful in improving patients' chances for survival, offering marginal benefits. Single agent gemcitabine, as well as its combinations, failed to provide expected results, prolonging life expectancy moderately. Similarly, disappointing effects were achieved with multimodality treatments (e.g. FOLFIRINOX) and targeted therapies. Therefore, there is a pivotal need for development of novel, effective strategies aiming to advance current therapeutic possibilities. Improvement in the field of targeted, more personalized therapies and introduction of novel strategies is of high importance.

1.7 ABC transporters

This chapter is presented in the form of the published review article:

Domenichini A, **Adamska A**, Falasca M. ABC transporters as cancer drivers: Potential functions in cancer development. Biochim Biophys Acta Gen Subj. 2019 Jan; 1863(1): 52-60; doi.org/10.1016/j.bbagen.2018.09.019

The last version prior to submission is presented. The published version is attached at the end of the thesis. References 199-304 in the thesis bibliography correspond to the references 1-107 in the published publication. The author contribution form stating my contribution to the publication is attached at the end of the thesis.

ABC transporters as cancer drivers: potential functions in cancer development

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Abstract

Background: ABC transporters have attracted considerable attention for their function as drug transporters in a broad range of tumours and are therefore considered as major player in cancer chemoresistance. However, less attention has been focused on their potential role as active players in cancer development and progression.

Scope of review: This review presents the evidence suggesting that ABC transporters might have a more active role in cancer other than the well know involvement in multi drug resistance and discusses the potential strategies to target each ABC transporter for a specific tumour setting.

Major Conclusions: Emerging evidence suggests that ABC transporters are able to transport bioactive molecules capable of playing key roles in tumour development. Characterization of the effects of these transporters in specific cancer settings opens the possibility for the development of personalized treatments.

General significance: A more targeted approach of ABC transporters should be implemented that considers which specific transporter is playing a major role in a particular tumour setting in order to achieve a more successful outcome for ABC transporters inhibitors in cancer therapy.

Keywords: ATP-binding cassette (ABC) transporters; cancer biology; chemoresistance; cell signalling; lipid transport.

1. Introduction

ATP-binding cassette (ABC) transporters belong to the most conserved protein superfamily, expressed from eukaryotes to vertebrates. Because of their ubiquitous expression, ABC transporters play crucial roles in the functioning of all the organisms. ABC transporters utilize the energy derived from ATP hydrolysis in order to translocate specific substrates or regulate the activity of membrane channels. In the majority of ABC transporters, ATP hydrolysis is mediated by two nucleotide-binding domains (NBDs), which closely interact with two transmembrane domains (TMDs). Conformational changes occurring at the level of NBDs, upon ATP hydrolysis are further transmitted to TMDs, which bind a specific substrate and translocate it across the biological membranes (199).

The human ABC transporters superfamily lists 48 members distributed into seven subfamilies (ABCA-G). Usually localized in cellular plasma membrane, ABC transporters have been also reported to be expressed in the membranes of mitochondria, Golgi and endoplasmic reticulum (200). Being responsible for the translocation of several substrates across these membranes, including steroids, phospholipids, glycolipids or xenobiotics, ABC transporters are engaged in diverse physiological processes such as membrane homeostasis, lipid trafficking, cell signalling, cell detoxification and drug resistance (201).

Despite the fact that a lot of emphasis has been placed on investigating the role of ABC transporters as protective pumps from exogenous compounds, xenobiotic excretion has been recently suggested not to be the primary function of these proteins (202, 203). Various other physiological roles have been assigned to ABC transporters; *inter alia* export of fatty acids, cholesterol, peptides and sterols, as well as defence against oxidative stress, detoxification and antigen presentation (Figure 1.4) (204). Notably, it

has been shown that some members of this superfamily are able to translocate endogenous lipids to actively influence lipid homeostasis, lipid trafficking and signalling. These are crucial processes for cell functioning and, more importantly, involved in the development of multiple pathologies (205). As the confirmation of the importance of ABC transporters in human physiology, the mutations or failure of nearly 50% of known ABC transporters are considered as the molecular basis of a plethora of human diseases (Table 1.1) (206).

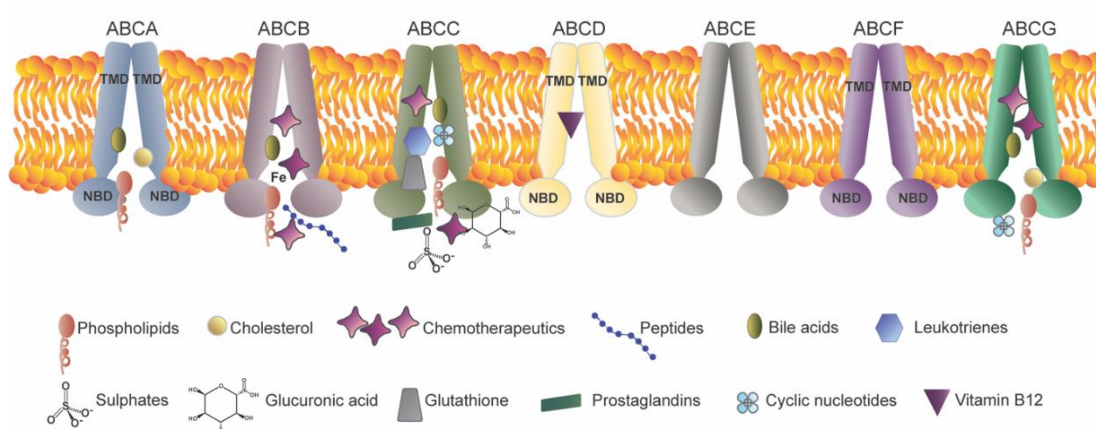

Figure 1.4 Family of ABC transporters *ATP-binding cassette family of transporters utilise the energy derived from the hydrolysis of ATP to translocate a variety of lipophilic endogenous substrates outside the cells. TMD, transmembrane domain; NBD, nucleotide-binding domain; Fe, iron.*

In this review, we summarize all emerging evidence that suggests that ABC transporters play a more active role in cancer biology and progression. We will also suggest that revised strategies should be carried out to target these molecules in disparate cancer settings. This will make possible to obtain better results than those achieved so far focusing only on their involvement in drug efflux.

2. ABCA subfamily

Most of the ABC transporters in this subfamily are involved in lipids transport and homeostasis and in the regulation of membrane trafficking and function (207). ABCA1 is involved in reverse cholesterol transport from the cells to circulating high-density lipoprotein (HDL), as well as phospholipids transport to the plasma membrane (208). Similarly, an excessive cholesterol influx mediated by low-density lipoprotein (LDL), promotes overexpression of ABCA2, ABCA3 and ABCA7 proteins, suggesting that these transporters play a pivotal role in maintaining a healthy cholesterol homeostasis within the cells. Moreover, ABCA2 has been found to be highly expressed in neuronal cells where it regulates cholesterol homeostasis by modulating the expression of low-density lipoprotein receptor (LDLR) (209) and ABCA3 has been reported to efflux cholesterol in the alveolar cells (210). In addition, the cluster of highly conserved ABCA5-related transporters including ABCA6, ABCA8, ABCA9 and ABCA10 is also involved in cholesterol and lipid efflux (211). Interestingly, ABCA4 mediates transport of molecules essential for retinal photoreceptor cells. ABCA4 has been found expressed predominantly in photoreceptors, where it transports retinal and other vitamin A derivatives, suggesting a key role in the visual process.

2.1. ABCA subfamily and role in disease

In the ABCA subfamily, defective ABCA1 is linked to Tangier disease, characterized by lack of circulating high-density lipoprotein (HDL). In this recessive condition, a mutation of the *ABCA1* gene disrupts the outflow of free cholesterol, causing a toxic accumulation of cholesteryl esters (CE) within the cells (212). ABCA7 is involved in the autoimmune disease affecting exocrine glands, known as Sjögren syndrome (213). Furthermore, due to its role in the transport of Vitamin A and derivatives in photoreceptor cells, *ABCA4* mutations are linked to various form of retinopathies, like retinitis pigmentosa and retinal degeneration (214). Different members of the ABCA

subfamily such as ABCA1, ABCA2, ABCA5 and ABCA7 seem to play a role in the pathology of neurodegenerative disorders and in particular Alzheimer's disease (215). ABCA12 is a lipid transporter expressed by keratinocytes and different mutations of the *ABCA12* gene account for different types of congenital ichthyoses, including the most severe form, called harlequin ichthyosis (215).

Transporters of the ABCA subfamily have also been linked to tumour progression and poor prognosis. ABCA2 plays a role in drug efflux and thus it seems to be responsible for multidrug resistance in different cancer types such as lung cancer and estrogen-dependent cancers (216, 217). In addition, overexpression of ABCA2 together with ABCA3 correlate with poor prognosis in infant acute lymphoblastic leukaemia (218). Similarly, amplification of the *ABCA13* gene is reported to confer poor prognosis to gastric adenocarcinoma where it increases the risk of developing lymphnode metastases (219). Elevated *ABCA13* mRNA levels are also linked to reduced overall survival in patients with metastatic serous ovarian carcinoma (220). This evidence indicate a possible role of ABCA13 in tumour metastasis and invasion (215).

3. ABCB subfamily

The ABCB subfamily is the most diversified, containing full and half transporters, with specificity for a wide range of substrates such as iron, peptides and drugs. The most characterised and the first described ABC transporter is ABCB1 (also known as P-glycoprotein or multidrug resistant protein 1), a widely expressed protein with a broad spectrum of substrates and known to be responsible for the development of chemoresistance in cancer cells (200). Other members of ABCB family, e.g. ABCB4 or ABCB11, exhibit higher substrate specificity, transporting phosphatidylcholine and bile salts. The endoplasmic reticulum membrane half-transporters ABCB2 and

ABCB3 participate in MHC I-dependent antigen presentation (200). ABCB4 is a transporter involved in lipid homeostasis. Predominantly expressed in the liver, ABCB4 mediates the transport of phosphatidylcholine from the canalicular membrane of hepatocytes to the biliary tree, reducing the toxicity of bile salts (221). ABCB6-8 are yet to be fully characterized; nevertheless, together with ABCB10, they are speculated to be mitochondria-localised transporters involved in the transport of metals, especially iron, across mitochondrial membranes, contributing to tightly regulate iron metabolism and homeostasis. These mitochondrial transporters also translocate peptides, proteins and heme across mitochondrial membranes (200). Furthermore, ABCB8 and ABCB10 seem to be involved in protection of cells from oxidative stress. ABCB8 has been reported to function as an ATP-dependent potassium channel (K_{ATP}) in rat cardiomyocytes, where it contributes to ablate oxidative stress damages leading to cell death (222, 223). ABCB10 is highly expressed in tissues exposed to elevated oxidative stress, like haematopoietic tissue, and in the heart where it plays a pivotal role in protecting cells from mitochondrial oxidative damage (224, 225). Transporters of the ABCB family also play a role in intracellular peptide transport (e.g. ABCB2 and ABCB3) and antigen presentation, DNA repair and chromosome recombination (226).

3.1 ABCB subfamily and role in disease and cancer progression

Mutations in *ABCB4* and *ABCB11* are responsible for progressive intrahepatic cholestasis (PFIC) (227). Nevertheless, ABC transporters are attracting interest as key players in carcinogenesis and their activity often correlates with cancer progression and aggressiveness. As an example, ABCB1 is the best characterised multridrug resistance protein, being the first human ABC transporters to be cloned (200, 228).

ABCB1 is known to transport a variety of hydrophobic drugs outside the cancer cells thus conferring chemoresistance to numerous tumour types, such as breast cancer, pancreatic cancer, lung cancer, hepatocellular carcinoma and neuroblastoma, leading to treatment failure and consequent tumour relapse (200, 229). Bebawy and colleagues highlighted a novel mechanism in which membrane microparticles (MPs), mediating inter-cell communication, can transfer ABCB1 from chemoresistant cells to sensitive ones. The latter are thus able to acquire drug resistance properties, and this non-genetic acquisition of multidrug resistance could explain metastatic spread and instruction of malignant cells in distant sites (229, 230). ABCB1 expression has been associated with tumour phenotype in colorectal cancer and soft tissue sarcomas, and its overexpression has been also linked with the progression of lymph node metastases. ABCB1 expression was also reported to be induced and elevated in chemoresistant breast and ovarian cancers (231, 232). Furthermore, ABCB1 is involved in the resistance to apoptosis, which is one of the hallmarks of cancer cells. In fact, inhibition of ABCB1 transporter results in cell cycle arrest and induction of apoptosis in leukaemia and colon cancer (233), whereas its overexpression leads to cells being less responsive to apoptotic stimuli (234). Platelet activating factor (PAF) activity has also been associated with ABC transporters, in the regulation of apoptosis. ABCB1 activity exporting PAF has been reported to enhance the anti-apoptotic signals by increasing the activity of proteins as BCL-2 or BCL-xl. Therefore, inhibition of PAF release may enable to make the cells more vulnerable to apoptosis (235). Moreover, ABCB5 is responsible for interleukin 1b (IL1b) secretion, inducing the pro-inflammatory CXCR1 pathway (236).

4. ABCC subfamily

The ABCC subfamily is most know for containing the majority of drug transporters and multidrug resistance proteins (MRPs), as well as the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7), important regulator of chloride ion export (235). ABCC transporters are also involved in lipid trafficking. As an example, ABCC1 exports lysolipids, such as sphingosine-1-phosphate (S1P) and lysophosphatidilinositol (LPI), both important signalling molecules and intracellular second messengers in tumour cell proliferation (237, 238). Classes of lipids like prostaglandins, together with steroid conjugates, folate and cyclic nucleotides are amongst the different signalling molecules exported by ABCC4 (239). Together with leukotrienes, prostaglandins are responsible for the leak of vascular endothelium, contributing to cancer metastasis (240). In addition, enzymes involved in prostaglandins synthesis, such as cyclooxygenase-2 (COX2), are highly expressed during cancer-related inflammation, and ABC- transported prostaglandins and leukotrienes influence inflammatory responses, as shown in mice lacking ABCC1 gene (241). ABCC10 is known to act as a lipophilic anions transporter in physiological conditions, playing a role in detoxification processes.

Implication of various ABCC transporters in cell migration and invasion has also been reported. Most notably, migration of dendritic cells has been shown to be influenced by ABCC1 and ABCC4 activity in mice and human tissues respectively and their downregulation in vitro highly reduced dendritic cells migration (242). Members of this family, such as ABCC5 and ABCC8, transport nucleotide and nucleoside analogs. Interestingly, ABCC8 has been recently shown to play a role in releasing an important mediator of chemotaxis, cAMP, synthetised and stored in microvesicular bodies and microvesicles in *Dictyostelium discoideum* (243).

4.1 ABCC subfamily and role in disease and cancer progression

Mutation in the gene of cystic fibrosis transmembrane regulator protein (*CFTR/ABCC7*), results in the development of cystic fibrosis defined by defective pancreatic secretions (200). Mutated *ABCC2* causes the recessive liver dysfunctions known as the Dubin-Johnson syndrome, linked to a defect in the excretion of bile acids (244).

Due to their role in multidrug resistance and drug efflux, members of the ABCC subfamily are also known as multidrug-resistance proteins (MRPs) and are found overexpressed in many cancer types where they play a key role in disease development and tumour progression. ABCC transporters contribute to cancer chemoresistance and treatment failure by exporting different classes of drugs, from amphipathic anions and non-ionic lipophilic compounds, including doxorubicin-related drugs (ABCC1/MRP-1) to hydrophobic and amphipathic drugs conjugated with sulphates or glutathione and glucuronic acid (200, 202).

Many of ABCC transporters, e.g. ABCC1, ABCC2, ABCC3, ABCC4, ABCC6, ABCC10 and ABCC11 are able to export Leukotriene C4 (LTC4) outside the cells (245). Leukotrienes activate GPCRs, triggering signalling pathways, upregulated in several cancers that promote tumour cell proliferation and survival. Due to the proved contribution of LTC4 to pancreatic ductal adenocarcinoma (PDAC) progression, the leukotrienes-ABCC activated signalling pathways have been widely studied as potential drug targets. More specifically, ABCC1, ABCC2 and ABCC3 showed specificity towards leukotriene C4 translocation, whereas ABCC4 possesses the ability to transport prostaglandins and PGA_2 or thromboxane A_2 (200). In particular, in addition to the direct inhibition of specific ABCC transporters, the inhibition of arachidonate 5-lipoxygenase (ALOX5), an enzyme upstream of LCT4, has been demonstrated to be effective in PDAC mouse models (246). Therefore, the main

inflammatory prostaglandin- and leukotrienes-mediated pathways, together with arachidonic acid and COX-2, which are involved in their synthesis, are considered to play a fundamental role in cancer development. Arachidonic acid and COX2, are often found overexpressed in tumour samples, and, together with other phospholipids and molecules participating in prostaglandin and leukotrienes synthesis, they have attracted the interest of researches as potential pharmacological targets (247). In PDAC, the prostaglandin-mediated tumour progression involves the activation of PI3K-Akt signalling pathway, increased expression of the vascular endothelium growth factor A (VEGFA) and consequent stimulation of angiogenesis in support of the inflammatory environment (248). All these mechanisms contribute to promoting an inflammatory environment, supporting cancer progression.

Furthermore, ABCC10 seem to be involved in the development of chemoresistance in colorectal and breast cancer progression (211). In neuroblastoma patients, overexpression of ABCC1 and ABCC4 is predictive of poor clinical outcome. ABCC1 is involved in the development of chemoresistance, as well as playing a role in promotion of cell proliferation and resistance to apoptosis (249), while ABCC4 transports signalling molecules relevant for cancer progression, like leukotrienes and prostaglandins (250, 251). Similarly, ABCC2 and ABCC3 are known to contribute to progression and poor prognosis of non-small-cell lung cancer and breast cancer (252, 253). Nonetheless, despite many studies suggesting the involvement of ABCC transporters in cell migration, no direct relationship between their expression and metastatic potential has been established yet. Nonetheless, an elevated expression of ABCC1 and ABCC4 has been observed in the cells dissected from metastatic tissues and metastatic lymph nodes compared to the cells derived from primary tumours (254),

suggesting a possible contribution of individual ABC transporters in the metastatic spread.

5. ABCD subfamily

The ABCD subfamily comprises four members which are half transporters and function as homodimers, with ABCD1-3 mainly localized in peroxisomes membranes and known to translocate very long chain fatty acids (VLCFA) into these organelles (255, 256). ABCD4 instead, has been reported to be residing in the endoplasmic reticulum (ER) and lysosomes where it has been reported to play an important role in the release of Vitamin B12 into the cytosol (257).

5.1 ABCD subfamily and role in disease

Diseases associated with mutations of members of the ABCD subfamily mainly involve peroxisomal dysfunctions. Different mutations of *ABCD1* are associated with X-linked adrenoleukodystrophy, which results in the toxic accumulation of VLCFA in tissues. Defects in ABCD3 have been recently identified to be associated with hepatosplenomegaly, while *ABCD4* mutations have been found in disrupted Vitamin B12 metabolism (255).

6. ABCE and ABCF subfamilies

To date, very little information is available about members of the ABCE and ABCF subfamilies, although ABCE1 seems to be a highly conserved protein in prokaryotes and eukaryotes. ABCE1 is formed by only two nucleotide binding domains (NBDs) and therefore, missing the transmembrane domain (TMD), it does not function as a transporter. Instead, it plays a fundamental role in cell division and initiation of protein

translation (258, 259). Similarly, ABCF members do not function as transporters but seem to be involved in translational regulation (260).

7. ABCG subfamily

ABCG family members, especially ABCG1, are associated with the export of phospholipids and cholesterol, in particular from cholesterol-loaded macrophages to HDL acceptors (261). ABCG2 is known as breast cancer resistance protein (BCRP) and plays a role in multidrug resistance, although its physiological role has been also described in human kidney as a urate exporter (262). ABCG4 functions as a lipid exporter and localises mainly in the central nervous system, while ABCG5 and ABCG8 are mainly expressed in enterocytes, where they limit plant-derived cholesterol absorption, and in canalicular membrane of hepatocytes where they help exporting sterols through the bile ducts, back to the intestinal lumen (262).

7.1 ABCG subfamily and role in disease and cancer progression

Because of their important role in regulating cholesterol absorption in the gut and liver, mutations of the genes *ABCG5* and *ABCG8* in liver and gastro-intestinal (GI) tract cause toxic intracellular cholesterol loading in patients affected by sitosterolemia (263). Similarly, because of its role as urate exporter, mutations of *ABCG2* have been linked to the accumulation of urate crystals in the blood and development of gout (264). Nevertheless, ABCG2 is mostly known for its role in multidrug resistance, being first described as breast cancer resistance protein or BCRP (265). ABCG2 is found overexpressed in numerous drug-resistant cancers including breast, ovarian, liver, lung and melanoma and it correlates with poor prognosis. In addition, ABCG2 is found particularly overexpressed in a subpopulation of slow-cycling cancer-stem like cells with self-renewal capacity and high chemoresistance (266).

ABC transporter	Tissue expression (204, 267)	Natural substrates	Clinical significance (65, 200, 267, 268)	MDR involvement (269)
		ABCA		
ABCA1	Lung, colon, liver, brain, testicles	Phospholipids, phosphatidylcholine, phosphatidylserine, sphingomyelin, cholesterol (270)	Glioma, lung, testis, liver, colorectal, pancreatic, breast, renal cancer, Tangier disease	Cisplatin
ABCA2	Nervous system	Cholesterol (271)	Alzheimer's disease, melanoma, breast, breast, liver, colon cancer, leukaemia	Mitoxantrone, estramustine, methotrexate
ABCA4	Photoreceptors	Vitamin A, phosphatidylethanolamine (270)	Autosomal-recessive disease Stargardt macular dystrophy, fundus flavimaculatus, cone-rod dystrophy, retinitis pigmentosa, age-related macular degeneration, breast, ovarian cancer	None identified
ABCA7	Bone marrow Brain, kidney, colon, lung pancreas	Phosphatidylserine, β-amyloid peptides (270)	Melanoma, lung, cervical, stomach, endometrial, colorectal, pancreatic, breast cancer, Alzheimer's disease	None identified
		ABCB		
ABCB1	Brain, blood-brain barrier, colon, liver, kidney, testis, placenta, small intestine, pancreas	Steroids, bile acids, lipids, bilirubin, platelet activating factor (272)	Ovarian, breast, colorectal, kidney, adrenocortical cancer, AML	Daunorubicin, epirobicin, doxorubicin, colchicines, paclitaxel, docetaxel, vincristine, vinblastine, imatinib

ABCB4	Liver	Phosphatidylcholine (273)	Liver, lung, pancreatic, renal cancer, melanoma, soft tissue sarcoma	Daunorubicin, digoxin, **paclit axel**, vinblastine
ABCB5	Liver, testicles	Interleukin 1b (274)	Renal cancer, melanoma	5- fluorouracil, doxorubicin, ininotecan, topotecan, camptothecin, mitozantrone
		ABCC		
ABCC1	Kidney, colon, pancreas, lymph nodes, liver, testis, brain, blood-brain barrier, breasts, spleen,	Lysophosphatidylinositol (LPI) , leukotriene C4, prostaglandins, sphingosine-1-phosphate (275), glutathione, glutathione disulphide (276)	Breast, lung, ovarian or prostate cancer, neuroblastoma	Anthracyclines , vinca alkaloids, camptothecins, daunorubicin, imatinib, etoposide, vincristine, vinblastine, methotrexate
ABCC2	Brain, lymph nodes, liver, colon, kidney, lung, testis, breasts, pancreas	Bilirubin, leukotriene C4 (200), glutathione, glucuronate and sulfate conjugates (277)	Colorectal, liver, lung, gastric cancer, Dubin-Johnson syndrome	Doxorubicin, carboplatin, cisplatin, irinotecan, epirubicin, paclitaxel, vinblastine, topotecan, vincristine
ABCC3	Pancreas, liver, lymph nodes, lung, adrenal glands, colon, testis, spleen, small intestine	GSH (272), prostaglandins, leukotriene C4 (LT_4) (278)	Pancreatic, liver, lung, colorectal, stomach, renal, breast cancer	Etoposide, methotrexate, teniposide, vincristine
ABCC4	Brain, testis, colon, kidney adrenal glands, pancreas, liver, ovary, lung, spleen,	Cyclic nucleotides, prostaglandins, trombocane A2, steroids, GSH conjugates, folate, urate (279)	Prostate, renal,liver, lung, breast, ovarian, stomach cancer, neuroblastoma	5-Fluorouracil, 6-mercaptopurin e, , Irinotecan, methotrexate, gemcitabine, **t opotecan,** vinblastine

	breasts, skin, heart			
ABCC5	Lymph nodes, pancreas, kidney, testis, brain, colon, liver, heart, muscles	Cyclic nucleotides (cAMP and cGMP), folic acid, glutamate conjugates, N-acetylaspartylglutamate, hyaluronan (280)	Lung, urothelial, breast, cervical, renal, liver, pancreatic cancer, glioma	Gemcitabine, methotrexate, 6-mercaptopurine, doxorubicin, 5-fluorouracil
		ABCG		
ABCG1	Pancreas, liver, colon, kidney, brain, lung, lymph nodes, testis	Phospholipids, cellular sterols (281)	Lung, renal, breast, endometrial, prostate, colorectal, cervical, pancreatic cancer, glioma	Doxorubicin
ABCG2	Intestine, testis, colon, placenta, liver, kidney, small intestine	Phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine (282), Sphingosine 1-phosphate GSH, androgens, haem, flavonoids, HIV-1 protease inhibitors (283)	Liver, testis, prostate, renal, non-small-cell lung cancer, glioma, Alzheimer's disease	Daunorubicin, doxorubicin, irinotecan, mitoxantrone, methotrexate, epirubicin, etoposide
ABCG4	Brain, endocrine, testis, colon, liver, kidney	Esmosterol and amyloid-β (284)	Glioma, melanoma, thyroid, head and neck, renal, testis, ovarian, endometrial, non-small-cell lung cancer	None identified

Table 1.2 *Summary of main ABC transporters families, physiological role and role in disease progression and development of multi-drug resistance in different cancer types. The table indicates tissues where each transporter is mainly expressed and substrates exported in normal (neutral) conditions. The clinical significance indicates diseases associated with the mutated or defective ABC transporters. In addition, for each transporter overexpression in specific cancer types is listed. Overexpression of a particular ABC transporters in this table is linked to a poor prognosis. (Table 1 in presented publication).*

8. ABC transporters beyond chemoresistance

Tumour chemoresistance represents a major challenge in the treatment of malignancies and several ABC transporters play a pivotal role in the development of multidrug resistance (MDR). MDR is characterised by upregulation of membrane-associated ABC transporters among which the most widely investigated are P-glycoprotein ABCB1 (MDR-1), multidrug resistance protein ABCC1 (MRP1) and breast cancer resistance protein ABCG2 (BRCP)(202). Overexpression of multidrug resistance transporters in cancer patients correlates with poor prognosis and lower survival rates mostly due to the failure to respond to chemotherapy. It has been hypothesised that the drug efflux mediated by ABC transporters in chemoresistance mechanisms is the result of their ability to export a diverse array of endogenous compounds and signalling molecules and, concomitantly, chemotherapy drugs (285). Nevertheless, the mechanisms at the base of this process are still unknown and it is yet to be investigated whether cancer cells do overexpress MDR proteins in response to chemotherapy.

The role of ABC transporters in tumorigenesis depends on their involvement in the secretion of bioactive molecules and the transport of lipids that contribute to the activation of important signalling pathways leading to cancer progression. Lipid transport by various members of the ABC transporter family suggests an active role of these proteins in cancer progression, beyond drug resistance mechanisms. Work conducted by our group has investigated the role of ABCC1 in the transport and release of LPI in the extracellular milieu where, interacting with G-protein coupled receptor 55 (GPR55), activates signalling pathways involved in cancer progression (286, 287). Our understanding of the role of ABC transporters in cancer is still very limited. However, we speculate that the ABC transporters play a key role in transporting lipids, prostaglandins, leukotrienes and other signalling molecules to promote cancer progression and, coincidentally, broad -spectrum transporters confer chemoresistance

by exporting therapeutic drugs. Cancer cells overexpress ABC transporters and this often correlates with poor prognosis and increased tumour aggressiveness, but the mechanisms regulating ABC transporters overexpression are still mainly unknown. The majority of patients' databases are based on the level of mRNA expression and only few data are available at the protein level. This creates a discrepancy between mRNA and actual protein levels because overexpression of ABC transporters is often regulated post-transcriptionally by miRNAs (288). At the gene level, it is important to outline that overexpression of ABC transporters in cancer cells, just as metabolic reprogramming, is driven by oncogenes. In neuroblastoma *MYCN* regulates the expression of ABCC1 and ABCC4 (289) while *P53,* together with *P63* and *P73,* seems to be involved in the regulation of ABCB1 expression (290).

9. Role of ABC transporters in cancer biogenesis

Oncogene-driven metabolic reprogramming is characterised by enhancement of glycolysis at the expense of oxidative phosphorylation. This process guarantees a rapid, although less efficient, ATP production, with the main advantage of reducing the generation of potentially damaging reactive oxygen species (ROS), thus promoting rapid cancer cell proliferation. In cancer metabolic reprogramming, lipid metabolism plays an important role for tumour progression as lipids are used not only as signalling molecules activating tumorigenic pathways, but also as building blocks to sustain enhanced biogenesis and anabolic processes leading to tumour cell proliferation. Cancer cells have a distinctive plasma membrane lipid composition, which is different from normal cells, and here we argue that ABC transporters play an important role in maintaining this structure. Membrane lipid composition in malignancies is a unique signature not only distinguished from normal non-cancerous cells, but that also allows

to discriminate between different tumour types, from benign compared to malignant cancers, and to identify the cancer stages, whether localised or metastatic (291). Other than being responsible of maintaining the lipid homeostasis, studies conducted using *Saccharomyces cerevisiae* have demonstrated that ABC transporters contribute to support plasma membrane asymmetry and stability (292, 293). Acting as lipid flippases, some ABC transporters regulate the level of membrane fluidity by increasing the transport of unsaturated fatty acids, thus decreasing membrane fluidity and permeability (282, 294). This important function has been primarily reported for ABCB1 and ABCB3, ABCC1 and ABCG2, because of their ability to flip phospholipids, mainly phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, across the lipid bilayer (205). Similarly, other than just transporting cholesterol, ABCA1 and ABCG1 activity has been linked to the translocation of phospholipids across the plasma membrane (282). The asymmetrically arranged lipids forming the lipid bilayer may be shifted across the membrane during differentiation or in pathological conditions, such as apoptosis, causing the loss of asymmetry (295).

Furthermore, cholesterol metabolism, is upregulated in cancer, which could explain the reason why ABC transporters are often overexpressed in cancer cells. Cholesterol is a fundamental component of the peculiar plasma membrane of cancer cells, but it is stored in large amounts also within the cells and it correlates with tumour aggressiveness in breast and prostate cancers (296, 297). The extrusion of cholesterol from cancer cells might have the same autocrine or paracrine role proposed for signalling molecules. At the same time, proliferating tumour cells release signalling molecules that influence the surrounding tissues and cells in order to create a tightly regulated microenvironment that supports and sustain cancer progression, mainly

providing nutrients for an increased anabolic demand. Phospholipids, together with sphingolipids, are synthesized form fatty acids, which levels in turn are controlled by ATP availability, a simultaneous determinant of ABC transporter activity.

10. ABC transporters and tumour microenvironment

Tumour progression is far from being a merely enhanced proliferative capacity of malignant cells. In the past two decades it has become evident that cancer development and propagation is a complex and heterogeneous process, involving extensive metabolic reprogramming and remodelling in order to create the tumour microenvironment (298). Tumour microenvironment not only provides support to tumour proliferation, but also acts as a physical and biochemical barrier for chemotherapy. Cancer-induced remodelling of the microenvironment and consequent tumour migration to distant sites for metastatic progression is mediated by an extensive network of autocrine and paracrine cell-to-cell communication. Extracellular vesicles (EVs) are secreted in abundance by cancer cells and play a pivotal role in this communication network (299, 300). EVs are classified according to their biogenesis and include microvesicles (MVs) derived from blebbing of the plasma membrane and exosomes, derived from late endosomes. EVs are shed by all cells in the body, including cancer cells, and they are cargoes transporting an array of signals that promote tumour progression, migration and establishment of distant metastatic niches (300). It has been shown that ABC transporters are present in the membranes of exosomes and MVs. MVs blebbing from chemoresistant human acute lymphoblastic leukaemia cells can transfer ABCB1 to recipient sensitive cells that acquire multidrug resistance (230). Moreover, multi-drug resistance proteins seem to be involved in transporting and packing chemotherapy drugs into vesicles that are then exported

outside the cells (301). It has been hypothesised that in cancer cells EVs, MVs and exosomes, transport signals that are then released and promote migration and invasion. This release of chemotactic signals from EVs has been shown to involve ABC transporters (300). In particular, a mechanism of release has been demonstrated with a recent study by Kriebel and colleagues investigating the role of cAMP released from shed microvesicles in *Dictyostelium discoideum.* Authors showed that MVs synthetise and secrete the cAMP, promoting chemotaxis, via the ABC transporter ABCC8 (243). Similar mechanisms might regulate the release of signals from tumour EVs to promote extravasation and metastatic spread to distant sites as well as reprogramming of cells in the tumour microenvironment. In solid malignancies characterised by a dense desmoplastic stroma, like pancreatic cancer adenocarcinoma (PDAC) and breast cancer, the tumour microenvironment contributes to the development of chemoresistance which, in turns, enhances the chance of tumour relapse. Desmoplastic stroma is composed by a heterogeneous array of cell types among which tumour associated macrophages (TAMs) are known to overexpress MRP1 (ABCC1) and MRP3 (ABCC3), thus further contributing to both tumour development and chemoresistance (302). Cancer-mediated reprogramming of the tumour microenvironment though EVs and transfer of MDR also includes remodelling of the immune system in order to escape the organism immune response and enhance cancer survival and progression (303). Resident macrophages are the first-line immune response to malignant cancer cells, although tumours activate mechanisms to elude this surveillance. Jaiswal and colleagues have elegantly demonstrated how EVs shed by multidrug resistant breast cancer cells can bind inflammatory macrophages and impair their migration and engulfing activity. Instead, impaired macrophages are phagocytised by tumour cells scavenging for nutrients. In addition, pro-inflammatory

cytokines released by impaired macrophages can act as attractant stimuli for extravasation of cancer cells and to further recruit TAMs in the establishment of a metastatic niche (303). These findings suggest a role of ABC transporters in mediating the paracrine signals involved in tumour microenvironment remodelling by transferring MDR to chemo-sensitive neighbouring cells, as well as immune elusive response by reprogramming macrophages activity.

11. ABC transporters and cancer cell energy balance

A particular aspect of ABC transporters functioning in cancer cells needs to be carefully considered. These transmembrane proteins need ATP to function and the more ABC transporters are expressed in a cell, the more ATP is required. Actively proliferating cancer cells are characterized by a rapid metabolic rate and have been reported to rely mainly on glycolysis for energy production, thus, in cancer cells, ATP is a limited and precious resource. Utilisation of this resource by an increased number of ABC transporters in cancer cells must confer a selective advantage, promoting tumour progression despite scarcity of ATP. We hypothesize that one of the strategies to maintain this balance is related to cancer stem-like cells (CSCs), a population of slow cycling cells characterised by self-renewal capacity and elevated tumorigenic potential, that contribute to tumour relapse due to an enhanced chemoresistance (304). Cancer stem-like cells are quiescent, compared to the fast-growing bulk of the tumour, they rely more on oxidative phosphorylation rather that glycolysis and, as discussed in our recent review, CSCs overexpress ABC transporters (266). Oxidative phosphorylation provides the cells with more ATP (32 molecules per molecule of glucose) compared to glycolysis (two molecules) which could sustain the elevated expression and activity of ABC transporters. It is therefore evident that more

investigation on energy balance in cancer cells is required to explain the importance of maintaining a costly set of ATP-dependent multidrug transporters when energy availability is a crucial element necessary to sustain the high demand of metabolic power of cancer cells.

12. Concluding remarks

In conclusion, in this review we have discussed the role of ABC transporters in cancer progression and highlighted how their role in multi-drug resistant mechanisms strongly depends upon their physiological function in cancer cell biology. Despite the expanded knowledge on the molecular characterization of ABC transporters and their involvement in chemoresistance, the specific substrates and the roles of the majority of these proteins are still elusive. In addition, it is worth to note that individual ABC transporters might have different functions in diverse cellular context and diseases (205). Consequently, we hypothesized that the role and the substrate of a specific ABC transporter might differ in a certain cancer setting compared to his normal physiological function. Some ABC transporters members, are important exporters of lipids, including fundamental signalling molecules promoting cancer progression, cancer associated inflammation and tumour-stoma crosstalk (303). We propose that overexpression of ABC transporters in cancer cells has a function beyond chemoresistance, which needs to be addressed and revisited. ABC transporters are energetically expensive to maintain for cancer cells that are fast proliferating and mainly relying on glycolysis for the production of ATP, thus we propose that the reason why tumour cells are overexpressing these transmembrane proteins should be further investigated. Moreover, it is paramount to understand the role of ABC transporters in the cancer-associate stroma in solid tumours and the tumour-

microenvironment interaction mediated by signalling lipids and other signals excreted by ABC transporters, as well as the role of cancer stem-like cells that overexpress multidrug resistance proteins and play a role in tumour relapse and metastatic spread. In addition, the genetic and epigenetic mechanisms regulating ABC transporters expression are still unknown, especially in the reciprocal interplay between cancer cells, stroma and immune system. Finally, in order to implement a personalized treatment targeting a specific ABC transporter, we need reliable and clinically validated assays to detect the expression of ABC transporter at the protein level. Subsequently, we need more specific and less promiscuous inhibitors that efficiently target a specific transporter and possibly resulting in less toxic effects.

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1.8 G-protein coupled receptors (GPCRs)

1.8.1 Structure and functions of GPCRs

Among other features, cell membranes are responsible for the maintenance of cellular homeostasis and for the specific transduction of biochemical signals from the outside to the inside of the cells through specific transmembrane proteins. G protein-coupled receptors (GPCRs), also called 7TM (7 transmembrane domain) receptors, are transmembrane proteins that form one of the largest families of proteins (305). GPCRs can be classified in 4 subfamilies (class A rhodopsin-like, class B secretin-like, class C metabotropic glutamate/pheromone and frizzled), depending on the their structure (306). Overall, GPCRs are composed of seven transmembrane helices, with the N-terminus localized outside the cell and C-terminus in the cytoplasm. Upon activation, GPCRs transduce external signals into the cellular responses, activating multiple signalling pathways involved in the regulation of variety of physiological processes (307, 308). G-proteins are molecules capable of hydrolysing GTP that are localised at the plasma membrane. Upon receptor activation, they bind to the cytoplasmic part of the receptor and act as secondary messengers, transducing the signal to downstream molecules (309, 310). G proteins consist of three subunits designated as α, β and γ. In a basal state the αβγ trimer is bound to a GDP molecule through the α subunit. After ligand binding, the receptor is activated and conformational changes of GPCRs increase their affinity towards the G protein. The trimer complex binds to the receptor followed by the release of GDP molecule. Subsequently, GTP binding to the α subunit causes the dissociation of the complex, releasing a GTP-bound α subunit and the βγ complex, which can then activate or inactivate downstream signalling cascades, ultimately affecting cell function. The hydrolysis of GTP to GDP and P_i allows the α-GDP subunit to bind the βγ complex, returning to the basal state and terminating the signal transduction (Figure 1.5) (311).

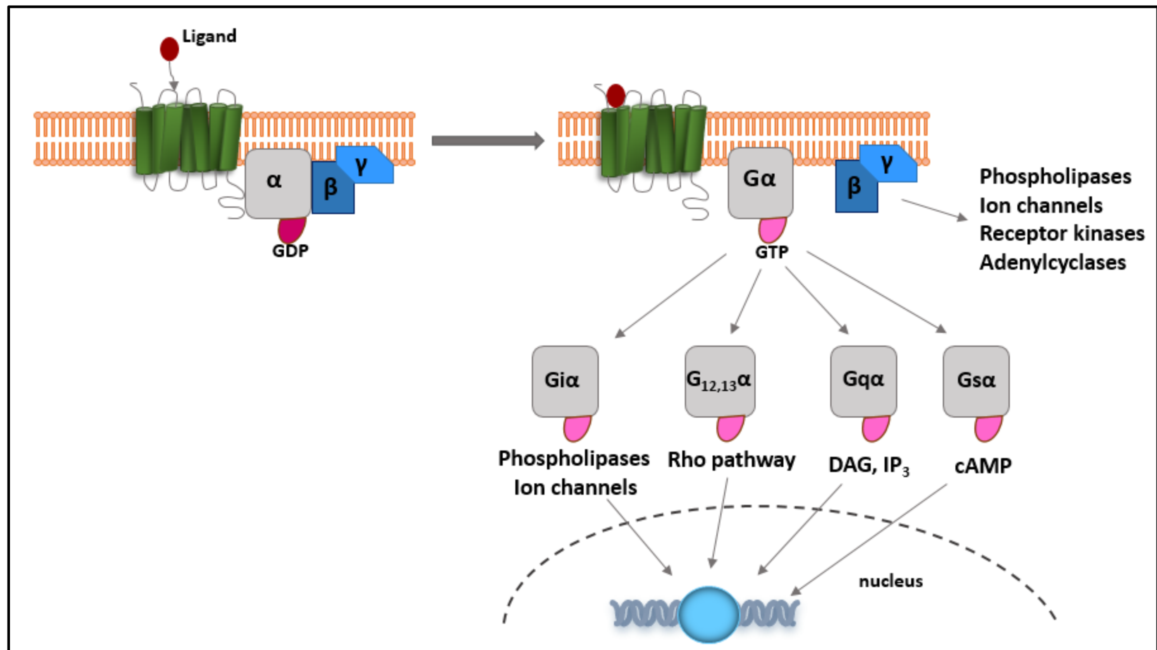


Figure 1.5 Mechanism of activation of G-protein coupled receptors

The functions of GPCRs are tightly regulated and the desensitization of the receptor through phosphorylation of the intracellular domain is observed after prolonged exposure to the ligand. Depending on a G-protein subunit type ($G\alpha_s$, $G\alpha_{q/11}$, $G\alpha_{i/0}$, $G\alpha_{12/13}$), different downstream effectors are affcted (312). Most of the GPCRs are able to activate more than one Gα subtype, however, the increased affinity towards one is usually shown. The main pathways that are regulated by G proteins are those activated by cAMP ($G\alpha_s$, $G\alpha_{i/0}$), phospholipase C (PLC) and Rho/ROCK kinases ($G\alpha_{12/13}$), which activation/inhibition by the G proteins regulates the phosphorylation of downstream proteins (313-315). Additionally, G-proteins stimulated by activated GPCRs gate ion channels that regulate calcium, sodium, potassium and chloride current through the plasma membrane (316). Stimulation of GPCRs leads to the activation of a plethora of signalling pathways, essential for cell functions, such as mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3K), Janus kinases or extracellular signal regulated-kinase (ERK). These pathways are implicated in essential cellular functions, such as regulation of cell division, differentiation, metabolism or angiogenesis, which dysregulation leads to the development of several pathological conditions. Functionally, GPCRs are involved in regulation of a

wide variety of physiological processes, including vision, smell or pain perception (Figure 1.6).

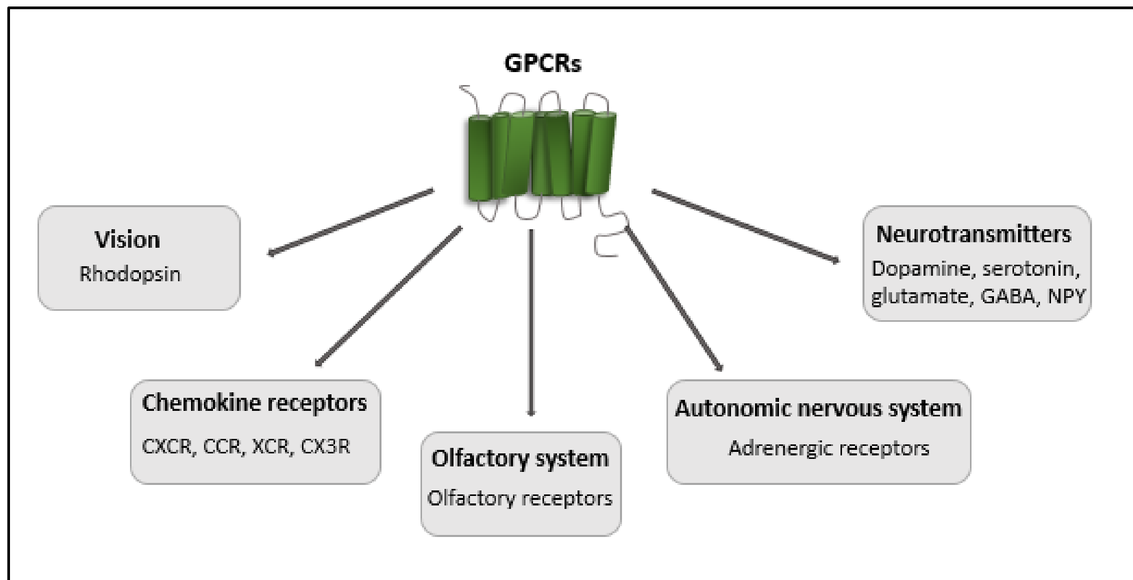


Figure 1.6 Role of GPCRs in human physiology

1.8.2 GPCRs in human disease

Out of ~800 known GPCR receptors, more than half are associated with light, smell or taste perception. The remaining GPCRs are involved in a plethora of physiological processes, which dysregulation leads to a variety of human malignancies (317, 318).

GPCRs have been implicated in the pathogenesis of various diseases (319) including:

- Metabolic disorders: Five neuropeptide ligands of GPCRs, which regulate appetite, have been suggested as important players in physiology of obesity, which include ghrelin system, melanocortin system, melanin-concentrating hormone system, neuropeptide A/B system, orexins/hypocretins system or bile-acid receptor TGR5. Similarly, other classes of GPCRs, playing roles in glucose homeostasis and pancreatic function (incretin receptors, neurotransmitter receptors, free-fatty acid-binding GPCRs) have been suggested to influence the pathophysiology of type 2 diabetes.

- Cardiovascular diseases: Chronic activation of several GPCRs, such as angiotensin, adenosine, adrenergic or endothelin receptors have been implicated in the development of heart disease and are being explored as potential drug targets in cardiac-related diseases.
- Immunological disorders: Several groups of GPCRs have been linked to inflammation in different human malignancies, inter alia chemokine receptors, involved in migration of leukocytes during disease-related inflammation. Other groups of GPCRs that contribute to inflammation processes are eicosanoid receptors, histamine receptors, protease-activated receptors (PARs), sphingosine-1-phosphate receptors, neurokinin receptors or cannabinoid receptors.
- Neurodegenerative disorders: The involvement of GPCRs in neurodegenerative disorders, including Alzheimer's disease, Parkinson's disease or Huntington's disease was also demonstrated.
- Osteoporosis: Two groups of GPCRs have been linked with susceptibility to osteoporosis, which regulate calcium homeostasis: calcium-sensing receptor and parathyroid hormone receptor.
- Cancer: The overexpression of several GPCRs in cancer specimens suggests their essential function in cancer pathophysiology. A variety of GPCRs have been implicated in the development of cancer, through their involvement in the processes crucial for tumorigenesis. Induction of cell proliferation, modulation of angiogenesis, cell differentiation and apoptosis, impact on the inflammatory environment of the tumours and cell migration induced by e.g. chemokine networks, are all mediated by GPCRs activity and are considered as hallmarks of cancer. Therefore, aberrant expression of GPCRs was shown to favour progression of the disease (320, 321) and presents a potential drug target in cancer therapy (321).

All these features make this family of proteins one of the main drug targets in cancer therapy, with almost 40% of all available therapies directed at GPCRs (322).

1.8.3 Role of GPR55 in cancer

GPR55 is a rhodopsin-like member of GPCRs identified in 1999 and considered as an orphan receptor until 2007. GPR55 has been initially classified as putative cannabinoid receptor, as it was demonstrated that various cannabinoid substances (e.g. anandamide, methandamide) could activate GPR55 (323). However, its low identity with the canonical cannabinoid receptors CB1 and CB2 (14% and 15% homology respectively), as well as some controversy regarding endocannabinoid-mediated activation of the receptor, led to the investigation of its alternative ligands (324, 325). The homology of GPR55 with several lysophosphatidic acid-sensitive receptors was reported. In 2007 Oka et al identified lysophosphatidyloinositol (LPI), a bioactive lysolipid showing no affinity towards CB1 or CB2 receptors, as the main endogenous ligand of GPR55 (326). Interestingly, other related endogenous lysolipids, such as LPA, lysophosphatidylserine, lysophosphatidylcholine did not exhibit stimulatory activity on GPR55 (326, 327). Upon LPI stimulation, $G\alpha_{12/13}$ and $G\alpha_q$ associated with GPR55 activate a phosphorylation cascades, which actively participate in cell proliferation (328). Proteins such as PLC, PKC, MAPK, ERK1/2, PI3K/Akt or Rho are stimulated by GPR55 activation and cause the increase in intracellular Ca^{2+} levels, which in turn induces several transcription factors, including NF-κB, NFAT or CREB (323, 327, 329, 330). However, complete GPR55-induced signalling network remains to be elucidated. All these signals lead to activation of many pathways important for cell proliferation, cell division, differentiation and apoptosis. Moreover, dysregulation of abovementioned pathways commonly accompanies neoplastic cell transformation. GPR55 is expressed in many tissues, including central nervous system, lungs, bone, kidney, liver or pancreas (324, 331). Especially, pancreatic islets and insulin-secreting β cells are rich in GPR55, which activation induces insulin secretion and increases glucose tolerance in *in vivo* models. Considering the expression profile of GPR55, the main physiological procsses, in which GPR55 may be involved include regulation of energetic homeostasis and bone resorption in osteoporosis (332, 333). mRNA GPR55 expression was also reported in the central nervous system. The role of the receptor in vasculature was additionally

demonstrated, suggesting its involvement in angiogenesis. Neuropathic pain and related inflammation are other conditions influenced by GPR55 and neutrophil migration and functions were additionally shown to be induced by GPR55.

Recent evidence have demonstrated that GPR55, and its ligand LPI,is overexpressed in different cancer types. In particular, ovarian, breast, brain, liver, skin and prostate cancers, in which LPI-induced activation of GPR55 influences the proliferation and invasion of cancer cells have been characterized by increased GPR55 levels. As an example, overexpression of GPR55, e.g. in human embryonic kidney (HEK293) or T98G (human glioblastoma) cell lines resulted in the increased proliferative abilities of the cells (315). Additionally, GPR55-LPI axis was demonstrated to play essential role in progression of ovarian and prostate cancer, through activation of an autocrine loop involving cPLA2 and ABCC1 transporter (334). LPI release by the cells through ABCC1 transporter remarkably induced the proliferation of ovarian and prostate cancer cells. On the contrary, knockdown of GPR55 in these cells significantly reduced LPI-induced effects. In addition, genetic silencing of the receptor with siRNA molecules decreased the viability of breast cancer (EVSA-T) (335). Similarly, in glioblastoma knockdown of GPR55 resulted in significant decrease in tumour growth (336). Similar effect of GPR55-mediated promotion of cell proliferation was also noted *in vivo*. Interestingly, the increased expression of GPR55 in these tumours correlated with their aggressiveness, with higher levels of GPR55 detected in histologically more advanced tumours and associated with lower survival rates (315). Additionally, GPR55-LPI axis was demonstrated to play essential role in progression of ovarian and prostate cancer, through activation of an autocrine loop involving cPLA2 and ABCC1 transporter (334). LPI release by the cells through ABCC1 transporter remarkably induced the proliferation of ovarian and prostate cells. On the contrary, knockdown of GPR55 in these cells significantly reduced LPI-induced effects. Furthermore, it was demonstrated that high expression of GPR55 correlates with enhanced proliferation, aggressiveness and metastasis of the tumours. The involvement of GPR55 in anchorage-independent growth, an essential feature of metastasis progression, has been shown for several cancer types. Furthermore, the involvement of GPR55 in cell migration and angiogenesis of endothelial cells suggests

that GPR55 may regulate malignant neoangiogenic processes (337, 338). Migration of breast cancer cells was also shown to be regulated by GPR55 activity (335). In addition, regulation of immune responses, an important component of tumour development, as well as inflammatory pain has been suggested to be mediated by GPR55 activity (339).

1.8.4 Antagonists of GPR55 as potential anti-carcinogenic agents

Proven the importance of GPR55 in tumorigenesis, the potential of its pharmacological inhibition was explored. Beginning in late 1990s', increased evidence of anticancer effects of cannabinoid treatment has been presented in *in vitro* and *in vivo* studies (340, 341). Anti-proliferative effects as well as induction of apoptosis and autophagy has been attributed to cannabinoid treatment. It has been previously evidenced that different cannabinoids may exhibit anti-tumour effects through their activity of cannabinoid CB1 and CB2 receptors (342, 343). Increasing evidence suggested that cannabinoid ligands might also participate in the regulation of GPR55 activity (331). Particularly one of the non-psychoactive members of cannabinoids family, cannabidiol (CBD), has been shown to exert significant anti-tumour effects (344). Inhibition of breast cancer tumour growth was shown in a xenograft model following CBD treatment (345). It was also demonstrated that CBD activity interferes with cell invasion, as it was proved in the case of A549 lung cancer cells (346). Suppression of angiogenesis, e.g. in lung cancer tissues was also attributed to CBD activity. In addition, CBD-mediated downregulation of EMT markers suggests its potential effects on cell invasion and metastasis (347, 348). Furthermore, it also seems to have the chemo-preventive effect (349). However, due to its considerably low affinity towards CB_1 and CB_2 receptors, the mechanisms of action, through which it exerts its functions, was not well defined for a long time. Based on the affinity of GPR55 with endocannabinoid system, GPR55 agonists and antagonists have been explored and the specificity of CBD in GPR55 inhibition was demonstrated, showing its antagonistic effects (332, 335). By targeting GPR55, CBD reduced growth of cancer cells, with little or no effect in non-malignant samples (345), which makes it a potent

GPR55 antagonist. The screening of several synthetic molecules identified a potent and selective small molecule inhibitor of GPR55, CID16020046, which shows no affinity towards CB_1 and CB_2 receptors. CID16020046 treatment impedes GPR55-mediated cellular effects (e.g. LPI-induced ERK1/2 phosphorylation or calcium mobilization) at low micromolar doses (IC_{50} of 0.21 µM in HEK-GPR55 cells) (350). Therefore, both CBD and CID16020046 are the main antagonists used in GPR55 inhibition in cancer research.

1.9 Models of Pancreatic cancer

Despite the significant amount of successful *in vitro* studies and pre-clinical validation of PDAC-targeting therapies, most of results could not be translated into clinical trials. One of the reasons is the lack of proper pre-clinical validation of data in more complex *in vitro* and *in vivo* models of the disease. *In vitro* 2D experiments can serve as an initial evaluation of conducted studies; however, they do not reflect the real genetic and environmental complexity that is characteristic for PDAC. Tumour cell-cell interactions, contact with surrounding microenvironment and the genetic heterogeneity are difficult to recapitulate in the conventional cell culture models. Hence, in order to study the biology of the tumour and carry out the preclinical assessment of developed therapies, more advanced preclinical models of the disease are essential. No significant advances in PDAC therapy could be achieved without implementation of three-dimensional (3D) culture methods and *in vivo* models, which allow retaining the tissue-specific phenotype, mimicking the studied disease. Therefore, several models of pancreatic cancer have been developed over the years, including organoids, spheroid culture, transplantation mouse models and genetically engineered mouse models (GEMMs).

1.9.1 3D models of pancreatic cancer

3D cell culture methods have been developed to enable the high-throughput studies in the conditions recapitulating the *in vivo* settings. Different methodological strategies have been implemented to study 3D cultures that serve as a bridge between cell culture and *in vivo* models, providing more insight than standard *in vitro* culture methods. Importantly, increased power of drug efficacy and toxicity analysis achieved by the implementation of 3D cell-based models allows reducing the number of animals required for the studies.

The simplest models of the 3D cultures include multicellular tumour spheroids established from conventional cell cultures (351-353). Cancer cells are grown in spheres in the conditions promoting cell-cell adhesion and interactions, such as matrigel or agarose gel, which allow the cells to form colonies and respond to tested therapies in a more complex environment (354). These techniques are based on the ability of cancer cells to grow, proliferate and form colonies independently of the surrounding conditions, whereas normal cells require contact with the extracellular matrix for expansion. Therefore, soft agar colony formation assays are widely used techniques applied to assess the malignant capability of cells. Apart from the distinction of the cells with tumorigenic potential form the normal cells, colony formation assays allow for quantitative analysis of cell response to different conditions *in vitro*. Although these models do not fully recapitulate the genetics and histology of the tumours, due to the simplicity of genetic manipulation and high clonality they provide a good method for the initial high-throughput testing of novel therapies. Organotypic multicellular spheroids and explant cultures, established from dissociated cancer tissues, provide more valuable tool due to the reproducibility of tumour histology and microenvironment (355, 356). Recently, the tumour-derived organoid *ex vivo* models were also developed (357) by growing primary cells in matrigel in specific growth-stimulating conditions. These models represent a powerful informative tool for the study of organogenesis and disease development (358).

In addition, tumorspheres (tumour-derived spheroids) enriched in cancer stem-like cells were proposed as another model in cancer research. It is now well established that within the heterogeneous bulk of the tumours exists a small fracture of dedifferentiated cells, mainly at the invasive front, capable of self-renewal and differentiation referred to as cancer stem cells (CSCs) (359, 360). Discovered in 1990s, the presence of CSC was reported in a variety of cancers, contributing to their aggressiveness, chemoresistance and recurrence (361-363). Therefore, investigation of CSCs in PDAC is of high clinical importance. Due to inconsistence in the establishment of universal cell-surface markers distinguishing stem cells form the bulk of tumour cells (proposed CD133, CD44, ALDH) (364, 365), the metabolic and functional characteristics of CSCs are used for the verification of the studied cell populations. Among others, the ability of CSCs to grow in spheroid cultures in the absence of serum is an unequivocal feature (366, 367). The establishment of CSCs spheroid cultures from different cancer cell lines and tissues has been recently involved in the cancer research model repertoire and was documented as a valuable tool in the analysis of tumour heterogeneity and the efficacy of developed therapeutic approaches (368).

1.9.2 Mouse models of pancreatic cancer

1.9.2.1 Xenograft models

The earliest *in vivo* models used in cancer research were transplantation (xenograft) mouse models, which used human or murine cancer cells implanted into immunodeficient mice (Figure 1.7) (369). Depending on the injection site, xenograft models can be sub-classified into subcutaneous, peritoneal, intravenous or orthotopic. In the subcutaneous model, cells are injected between the dermis and muscles, usually on the flank or on the back of the mouse. Orthotopic models are generated by injecting cancer cells in their organ of origin. These mice develop metastases, more closely mimicking the disease with respect to tumour progression

and clinical response than subcutaneous model (370, 371). However, they are technically more challenging, time, and money consuming. Despite several advantages of xenograft models in the investigation of tumour biology and response to therapies, these models present several drawbacks. In order to implant human cells into mice organism avoiding immune reaction, immunodeficient “nude” or “scid” mice are used (372). Therefore, although the model allows for successful cell implementation, the lack of a competent immune system, which important role in PDAC development has been widely described, limits the proper evaluation of tumour responsiveness. Similarly, the tumour microenvironment, which is very heterogeneous and complex in PDAC and accounts for its aggressive nature and resistance, is highly restricted in the subcutaneous xenograft models, creating another obstacle in proper evaluation of obtained data. Another disadvantage is that the cells used for creating the xenografts models are previously cultured *in vitro*, which may lead to the loss of genetic and phenotypical heterogeneity observed in the tumours. Therefore, the xenograft models lack the complexity observed in PDAC, restraining the complete evaluation of the tumour behaviour. On the other hand, the reasonably short timeframe for the initial evaluation of tumour responsiveness to different drugs makes this model a valuable tool in cancer research (373). An alternative to abovementioned model is the syngeneic model, which consists on the injection of murine cancer cells into the mice, and consequently does not require the use of immunodeficient mice, therefore preserving the immune system. However, it should be taken into account that the limited number of available murine cell lines creates analogous constraint in tumour complexity. Humanized mouse model has been also developed to retain the immune system in the xenograft models (374).

1.9.2.2 Patient- derived xenograft models (PDX)

Patient- derived xenograft (PDX) mouse models emerged as more representative and more complex way to assess tumour biology and responsiveness to therapies. Derived from human tumour tissues during surgical resection and engrafted subcutaneously into immunocompromised mice, PDX models provide more reliable tool in terms of mutational heterogeneity of the tumours, providing valuable

information on the tumour responsiveness to the drugs in the development of personalised medicine (375, 376). Therefore, the design of more accurate clinical trials and development of novel efficacious treatments for individual patients might be carried out using this model. PDX mouse models are increasingly applied in cancer research and drug development studies. However, similarly to cell-based xenograft models, lack of natural tumour environment and tumour-stroma interactions do not fully recapitulate the nature of the disease. Further advancement of the PDX models, by inserting the tumour fragment to the subrenal capsule (SRC) instead of subcutaneous implantation of the tissues, provided models characterized by faster tumour growth, increased tumour perfusion and higher metastasis rates (377). However, the complicated and invasive nature of the surgical procedure and still the necessity to use immunocompromised animals, represent remarkable drawbacks of this approach. Establishment of less invasive surgery methods for the development of PDX models could increase the feasibility of the model. Fine-needle aspiration biopsy performed during the diagnosis has been proposed as the tissue source for PDX model development (378). This would enable faster determination of tumour responsiveness to selective therapies, before surgical intervention. Collectively, the implementation of the PDX models in pancreatic cancer research, although still in very early stages, provided a valuable tool in novel drug testing, raising the hope for more effective preclinical trials (379). In fact, by screening of the wide repertoire of PDX models it was demonstrated that reproducible association of tumour genotype and response to selective drugs could be predicted (380).

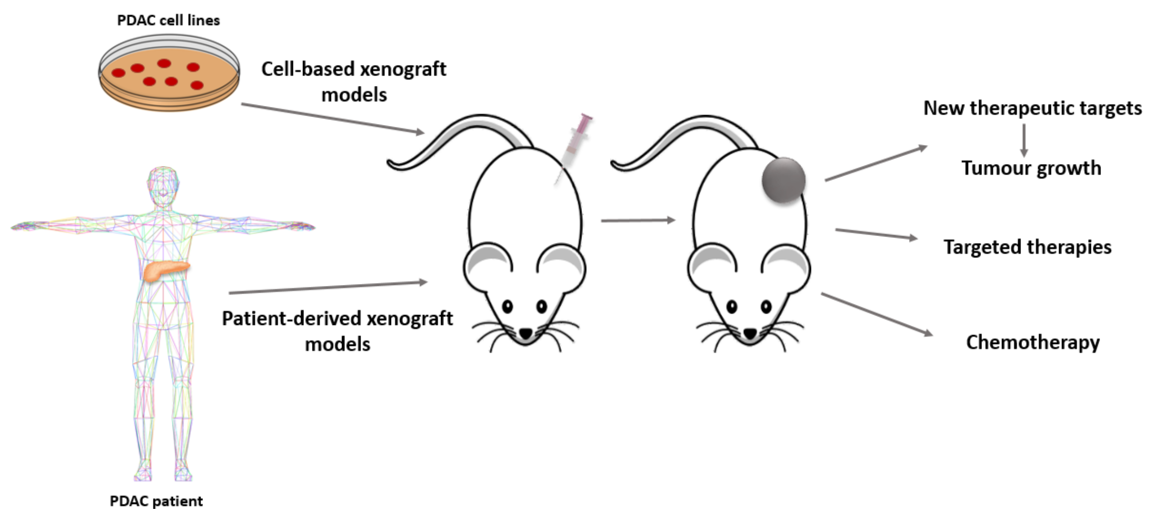


Figure 1.7 Establishment and potential of xenograft mouse models of PDAC

1.9.2.3 Transgenic models

All described drawbacks of the available animal models of PDAC led to the development of transgenic mouse models. In these models, mutation-driven tumours are developed in the pancreas under organ-specific promoters (381, 382). These models provided a valuable tool to explore the proteins and mutations essential for PDAC development. Additionally, the importance of distinctive proteins and signalling pathways in the response to different therapies may be assessed (383, 384). The main application of the transgenic models is the validation of novel drug targets, drug safety, efficacy and pharmacokinetics in a complex environment (383). The first developed transgenic mouse model of pancreatic cancer was based on the high prevalence of the pro-oncogenic KRAS alterations leading to the development of the KC mice, in which LSL-KRAS^{G12D} knock-in mutation is induced by the Cre recombinase under pancreas-specific promoter (Pdx-1-Cre) (385). Although KC mice closely recapitulated disease progression through all the PanIN stages, the progression to fully differentiated and invasive tumours was very slow and with low penetrance (386). Therefore, the combination of different transgenes was considered to generate models that will more accurately progress to invasive and metastatic tumours. Up to date, around 30 different genetically modified models of

PDAC have been developed. Mutations in several pathways altered in PDAC was investigated, including TGFβ (387), INK4A (388), Notch (389) or SMAD4 (390, 391). However, no full recapitulation of the metastatic disease could be achieved.

The development of the KPC mouse model, combining *KRAS* activation and *TP53* suppression under the Pdx-1-Cre promoter provided a mouse model characterized by the spontaneous development of the pre-tumorigenic lesions with full progression through consecutive PanIN stages and development of complete invasive and metastatic disease with 100% penetrance, reproduced the key features of the human PDAC (392). Mainly, the development of a rich tumour microenvironment with inflammatory infiltrate is developed around the tumours. The development of PDAC in the KPC model closely mirrors human disease and is so far the most representative model of PDAC available. The newborn mice present healthy pancreas, which as the mice get older starts acquiring histopathological characteristics of the precursor lesions and developing an invasive tumour at around 65-80 days of age. At this age, developed tumours with dense desmoplastic reaction, accompanied by development of ascites, cachexia or jaundice are observed. Additionally, the EMT transition of the cells in the primary tumours leads to the metastatic spread of the disease and the development of metastatic niche mainly in the liver, lungs and lymph nodes of the KPC mice (393). Moreover, resistance to gemcitabine and short survival rates have been shown, the characteristics observed in human PDAC. In addition, several variations of the KPC mice were developed, such as KPCY mode, in which LSL-Rosa26-YFP transgene is incorporated, allowing for monitoring of location and spread of the pancreatic cells by tracing of the yellow fluorescent protein (YFP) (113). KPC-derived cell lines were also proposed as a good model for initial screening of tested therapies. Interestingly, recent high-throughput analysis of a variety of PDAC cell lines and patient-derived tissues placed the KPC- derived cell lines in an aggressive squamous subtype, based on detected mutations (394). All these characteristics make KPC mouse model the most clinically valuable tool in the investigation of the PDAC biology and preclinical validation of developed therapies.

The comparison between described mouse models of PDAC is presented in Table 1.3.

Animal model	Advantages	Disadvantages
Xenograft mouse models	-Quick and efficient method for assessment of the tumour response to targeted therapies and chemotherapies -Ease of cell genetic manipulation prior or after implantation -Fast and easy -Fairly inexpensive -High throughput	- No sufficient tumour heterogeneity, instability of used cell lines - Lack of functioning immune system -Lack of metastases -Lack of tumour environment
PDX mouse models	-Similar histology to the tumour -Substantial heterogeneity of the tumours, resembling patient's mutation repertoire -Chance of metastasis (orthotopic model) -Allow for the analysis of targeted therapies and patient- specific therapies	-Lack of tumour environment (subcutaneous) -Low throughput -Limited expansion -Limited to biopsy samples and resectable tumours
Transgenic mouse models	-Competent immune system -Presence of full tumour environment -Tumour development mimicking the stages of human disease -Tumours arise in the pancreas -Development of metastasis -Used to defining the key mutations in disease development -Allow the mechanistic insight to the development of pre-malignant and malignant lesions -Reliable tool in drug validation	-Costly and time consuming -Development of the tumour is hard to assess without the use of sophisticated instruments -Tumour development is slow and variable

Table 1.3 Comparison of several available mouse models of PDAC

Thesis hypothesis and aims

Pancreatic cancer is a devastating disease, characterized by high aggressiveness, early metastatic spread and high chemoresistance leading to the lack of effective therapeutic approach. In addition, high heterogeneity of pancreatic cancer is one of the reasons for the lack of success in implementation of targeted therapies so far. Therefore, it is of high importance to identify novel therapeutic targets and new, more potent therapies that can be implemented for the broad cohort of pancreatic cancer patients. The original research from our group demonstrated the existence of an autocrine loop in ovarian and prostate cancer, involving ABCC1 and GPR55, which activity, involving the release of lysophosphatidylinositol (LPI) by ABCC1 and LPI-induced activation of GPR55, contributes to increased cancer progression.

The main hypothesis of my project was to investigate the existence of similar mechanisms in pancreatic cancer and to verify its potential as a novel therapeutic target in PDAC therapy. In addition, the understanding of the mechanisms governing the expression of the proteins involved in the potential loop and their influence on cancer cell proliferation, survival and cell signalling was explored. To verify that hypothesis I aimed to deliver on the following objectives:

1. To investigate to role and pharmacological potential of GPR55 in PDAC progression *in vitro* and *in vivo*
2. To identify the ABC transporter responsible for LPI release in pancreatic cancer and to investigate its role in PDAC progression
3. To verify the pharmacological potential of the ABC transporter *in vitro* and *in vivo*
4. To investigate the existence of the ABC transporter-LPI-GPR55 axis and to analyse the potential of the targeting of both components of the proposed loop
5. As a side project, I also aimed to investigate the role of tumorspheres in PDAC chemoresistance

If successful, the results obtained during the course of this study could provide a pre-clinical validation of the pharmacological inhibition of novel targets in PDAC therapy and promote a further development towards human clinical trials.

2. Materials and methods

2.1 Cellular work

2.1.1 Cell culture

Cell line	Source	Organism	Organ/Disease /Metastatic site	Culture conditions
AsPC-1	ATCC (CRL-1682™)	*Homo Sapiens*	Pancreas/PDAC/ Ascites	RPMI-1640 media (Sigma® Cat# R8758)
HPAFII	ATCC (CRL-1997™)	*Homo Sapiens*	Pancreas/PDAC/ Ascites	MEM/EBSS media (HyClone-GE Cat# SH3024401)
CFPAC-1	ATCC (CRL-1918™)	*Homo Sapiens*	Pancreas/PDAC/ Liver	IMDM media (HyClone-GE Cat# HYCSH3025902)
SW1990	ATCC (CRL-2172™)	*Homo Sapiens*	Pancreas/PDAC/ Liver	RPMI-1640 media (Sigma® Cat# R8758)
Bx-PC3	ATCC (CRL-1687™)	*Homo Sapiens*	Pancreas/PDAC/ Liver	RPMI-1640 media (Sigma® Cat# R8758)
Capan-2	*ATCC (HTB-80™)*	*Homo Sapiens*	Pancreas/PDAC/ Liver	McCoy 5A media (HyClone™ Cat# SH30200.01)
hTERT-HPNE	ATCC (CRL-4023™)	*Homo Sapiens*	Pancreas	70% DMEM no glucose media (Sigma Cat#. D-5030 25% M3 Base Incell Corp. Cat# M300F- 500
HPDE	Prof H. Kocher (Queen Mary University of London)	*Homo Sapiens*	Pancreas	Keratinocyte-SFM culture media (Gibco)+ EGF+ bovine pituitary extract (Life Technologies)
BJ	ATCC (CRL-2522™)	*Homo Sapiens*	Skin (fibroblasts)	MEM/EBSS media (HyClone-GE Cat# SH3024401)
CAFs	Neuromics #PC00B5	*Homo Sapiens*	Pancreas (fibroblasts)/ PDAC	Pancreatic Stellate CAF Maintaining Media (Neuromics Cat# PC00B5)
HEK293T	ATCC	*Homo Sapiens*	Embryonic kidney	DMEM media (Sigma® Cat# D5796)

	(CRL-11268™)			
KPC	KPC mouse	*Mus Musculus*	Pancreas/PDAC	DMEM media (Sigma® Cat# D5796)
PZR1	Owen Sansom	*Mus Musculus*	Pancreas/PDAC	DMEM media (Sigma® Cat# D5796)
PZPR1	Owen Sansom	*Mus Musculus*	Pancreas/PDAC	DMEM media (Sigma® Cat# D5796)
PZPflR	Owen Sansom	*Mus Musculus*	Pancreas/PDAC	DMEM media (Sigma® Cat# D5796)

Table 2.1 List and characterization of cell lines used for this study.

Authenticated cell lines were purchased from ATCC (VA, USA): AsPC-1 (ATCC® CRL-1682™), HPAFII (ATTC® CRL-1997™), CFPAC-1 (ATCC® CRL-1918™), BxPC-3 (ATCC® CRL-1687™), Capan-1 (ATCC® HTB-79™), Capan-2 (ATCC® HTB-80™), hTERT-HPNE (ATCC® CRL-4023™) and BJ (ATCC® CRL-2522™). Authenticated Human Immortalized Pancreatic CAF-stellate cells (CAFs) were purchased from Neuromics, #PC00B5 (Edina, MN, USA). All cell lines were cultured at 37°C, 5% CO_2, 95% air, in conditions recommended by the manufacturer's guidelines. Cell media were supplemented with 10% (v/v) foetal bovine serum (FBS, Bovogen (Cat # SFBS-F)), 1% (v/v) penicillin/streptomycin (PS, Sigma®, #P4333) and 2nM L-Glutamine (Sigma® G7513) unless stated otherwise. Murine primary cell lines (PZR1, PZPR1, PZPflR) were kindly provided by Owen Sansom (Beatson Institute, Glasgow, UK) and grown in complete DMEM media. Murine cells were authenticated for TP53 status by Western blot analysis. Cells were cultured for a maximum of 10 passages before fresh cells were thawed. All cell lines were regularly tested (every 3 months) for the presence of *Mycoplasma* by PCR. Cells were cultured in *Mycoplasma*- free conditions.

2.1.2 Cell cryopreservation

Cells were collected at the early passage (1-4) at the confluence of 80-90%. After removing the growth media, cells were washed with PBS and detached from the culture plate with trypsin-EDTA (0.25%) for 5-10 minutes at 37°C. Trypsin was stopped by adding equal volume of culture media, cells were collected and centrifuged at 1100 rpm for 5 min, RT. The supernatant was discarded and the remaining cell pellet was resuspended with a volume of freezing media (growth media supplemented with 5% DMSO) containing approximately 10^6

cells/ml. Cell suspension was placed in cryovials (Thermo Scientific) and gradually frozen at 1°C/minute using isopropanol as the alcohol bath at -80°C for at least 24h before storing in the liquid nitrogen (vapor phase).

2.1.3 Tumorspheres culture

Pancreatic cancer cell lines were cultured according to previously described methods. At ~80% of confluency, cell media was replaced with media without FBS and cells left to grow in the serum-free conditions for the following 7 days. After that time, the supernatant with floating cell spheroids was collected and the remaining cells were detached by trypsinization. Detached cells were combined with the supernatant, spun down at 200x rcf and washed twice with HBSS. Pelleted cells were resuspended in 1 ml of Accutase™ (STEMCELL™ Technologies) and incubated at RT for 10 min in order to disaggregate cell spheroids. Then the cell suspension was passed through 25g needle to further dissociate the cell clusters. The single cell suspension was then resuspended in a stem cell growth media (DMEM/F12 enriched with N2 and B27 supplements; R&D Systems). Cells were grown in low-adherence conditions with the addition of growth factors: 1µM EGF and 2µM FGF_2 in ultra-low attachment flasks (Cornig) and split every few days. For media change or cell splitting, the cell suspension was spun down at 200x rcf and cells were resuspended in fresh stem cell media supplemented with EGF and FGF_2.
For the tumorsphere growth assay, tumour spheroids were collected from the growth flask, spun down at 200x rcf and disaggregated in 1 ml of Accutase for 10 min at RT. Dissociated cells were counted with the use of Neubauer chamber with trypan blue exclusion. Cells were seeded in 24-well low attachment plates at the density of 25.000 cells per well. The following day, cells were treated in duplicate with appropriate drugs at increasing concentrations and left to grow for 5 days. After that time, spheroids from each well were collected in a separate Eppendorf tube, spun down at 200 x rcf and resuspended in 50 µl of Accutase for 10 min at RT. Cells were counted with trypan blue exclusion. Each experiment was performed at least in triplicate.

2.1.4 Establishment of the primary cell culture

Primary cell line (KPC cells) was established from the pancreatic tumours of the KPC transgenic mouse model of PDAC. Tumour tissue was minced and digested in 5mg/ml collagenase P (Roche) in complete DMEM media at 37°C for 90 min on a rocking shaker. Cells were disaggregated with the use of 5 ml serological pipette tip and separated by filtering the cell suspension through 100 µm and 40 µm filter. Single cell suspension was washed twice with cold PBS (pelleting at 200g for 10 min, breaks off). Subsequently, red blood cells were lysed for 5 min at RT. The remaining tumour cells were washed with PBS once more and spun down. Cells were plated and cultured in DMEM media supplemented with 10% (v/v) FBS, 1% (v/v) PS and 2nM Glutamine. Cells were used for up to 4 passages. Cells at early passages were also cryopreserved in FBS supplemented with 10% DMSO and stored in liquid nitrogen.

2.1.5 Gene silencing

2.1.5.1 Transient siRNA transfection with the use of Darmafect 1 transfection reagent (Dharmacon®)

Cells were seeded in a 6-well plate at the density of $2.5x10^5$ cells per well and let to adhere overnight. On the following day, transfection was performed when cells reached 40-60% confluence. For each transfection, two solutions were prepared (solution A, solution B). Solution A contained siRNA (from 20 µM stock) and base cell growth media (not supplemented with FBS nor PS) up to 200 µl. Solution B contained Darmafect 1 reagent in the same volume as used siRNA solution and the same media up to 200 µl. The optimal working concentration of the siRNA used was established at 75 nM, which equals 7.5 µl of siRNA stock solution per one 6-well plate (2ml). Two different siRNA sequences were used for silencing of each protein. As a control, scrambled negative siRNA (siSCR) was used at the same concentration as used silencing siRNA sequences. Both solutions were incubated in dark at room temperature for 5 minutes before transferring both into sterile 1.5 ml Eppendorf tube. Created mix solutions was incubated in the dark at room temperature for

20 min. In the meantime, the media was aspirated from the cells and replaced with 1.6 ml of fresh media supplemented with 10% FBS but not containing penicillin/streptomycin solution. After 20 minutes, each of the mix solution was added to separate wells and incubated at 37°C for 24 hours. All the procedures were performed with sterile DNA/RNA-free filtered tips and sterile DNA/RNA-free Eppendorf tubes. On the following day, the media from each well was replaced with fresh complete media and cells were left to incubate at 37°C, 5% CO_2/95% air. Used siRNA sequences are presented in Table 2.2

Used sequences:

Gene	Nomenclature	Sequence	Manufacturer/ Catalogue number
GPR55	siGPR55-1	GAAUUCCGCAUGAACAUCA	Darmacon®/ J-005581-06
GPR55	siGPR55-2	Sense: AGGUGUUUGGCUUCCUCCUUCCCAU Antisense: UGGGAAGGAGGAAGCCAAACACCU	-
ABCC3	siABCC3-1	Sense: CGCUGAUCUUACAACACUATT Antisense: UAGUGUUGUAAGAUCAGCGAC	Quiagen / Hs_ABCC3_6
ABCC3	siABCC3-2	Sense: UGAUCAGGUUUAUCUCCAATT Antisense: UAGUGUUGUAAGAUCAGCGAC	Quiagen / Hs_ABCC3_15
ABCC3	siABCC3-3	GCACACCGGCUUAACACUA	Darmacon®/ J-007312-05
ABCC3	siABCC3-4	GGACAAAGGAGUAGUAGCU	Darmacon®/ J-007312-06
TP53	siTP53-1	GAAAUUUGCGUGUGGAGUA	Darmacon®/ J-003329-14
TP53	siTP53-2	GUGCAGCUGUGGGUUGAUU	Darmacon®/ J-003329-15
Negative control	siSCRL	-	Ambion/ # 4390843

Table 2.2 List of siRNA sequences used in this study.

For the miRNA transfection, the following miRNAs were used: has-miR-34b-3p (Ambion, # MC12727) and has-miR-34c-5p (Ambion, # MC11039) at the working concentration of 75µM. Optimal times at which cells were harvested post transfection were determined for each cell line and targeted gene:

GPR55:

- AsPC1, HPAFII- 72h

ABCC3:

- AsPC1, HPAFII- 48h
- CFPAC-1- 29h

P53:

- HEK293T, SW1990- 48h

miR-34c, miR-34b:

- AsPC1, HPAFII- 72h

2.1.6 Cell viability assay

PDAC cell lines were plated at a density of $5x10^4$ cells per well in 12-well or 25.000 cells per well in 24-well tissue culture plates and incubated overnight. The following day cells were treated in duplicate with increasing concentrations of drugs of interest or drug combinations (Cannabidiol- GWpharma, CID-Sigma, Gemcitabine- Sigma, S3, Paclitaxel- Sigma, Docetaxel- Selleckchem #S1148, Carboplatin-Sigma #C2538, Trametinib- Selleckchem #S2673). Drug vehicle (DMSO, Sigma) was used as a control for each of the treatment. After 72 hours, cells were detached by trypsinization and counted manually using a Burker chamber with the Trypan blue to exclude dead cells. Cells were counted with the use of a light microscope at 10x magnification and the number of cells was counted according to the formula:

Number of cells/ml= average number of cells per square x10000.

Cell number in the control wells was considered as the 100% of cell viability. All experiments were performed in triplicate for statistical analysis. Accordingly, 48-72h post performed transfection, cells were collected and counted manually with Trypan blue exclusion, comparing the viability of siRNA- transfected cells with control siRNA (siSCR).

2.1.7 MTT assay

Pancreatic cancer cell lines (AsPC1, HPAFII, CFPAC-1) were seeded in a 96-well plate at the density of 3.000 cells/well and incubated overnight at 37°C. The following day, cell media was removed and replaced with fresh media containing the studied drugs at increasing concentrations. Drug combinations were also applied to the cells. Cells were left to grow in the presence of the drugs for the following 72h. After that time, the media was removed and cells were incubated with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolim bromide) solution (5 mg/ml stock) at working concentration of 0.5mg/ml in fresh cell growth media for 2 hours at 37°C. The reaction was stopped when the majority of the cells was stained. MTT solution was then aspirated and the plate was left to dry for several hours. Stained cells were then resuspended in 70 µl of DMSO and mixed well. The absorbance was read with the use of a plate reading spectrophotometer at the 570 nm.

2.1.8 Colony formation soft agar assay

To validate the effectiveness of the investigated therapies in a 3D model, cells were grown in anchorage- independent manner in the agarose gel. Soft agar colony formation assay is a widely used technique applied to assess the malignant transformation of cells. It based on the ability of cancer cells to grow and form colonies independently of the environment, whereas normal cells require contact with the extracellular matrix for expansion. Apart from the distinction of the cells with tumorigenic potential form the normal cells, colony formation assay allows for quantitative analysis of in vitro cell response to different conditions. Two agarose layers, varying in the matrix density were created to allow cells to grow in tumour-like environment and prevent cell from adhering to the culture plate. The bottom layer, composed of 1.2% noble agar and 2x concentrated RPMI growth media (supplemented with 20% FBA and 2% PSG) mixed in a 1:1 ratio, was poured in 6- well plate and left to solidify. $1x10^4$ of cells were resuspended in 0.75ml of 2x RPMI supplemented with the corresponding treatments. Similarly, $1x10^4$ cells harvested 24h following gene silencing were mixed with 0.75ml of 2x RMPI. Cell suspension was then mixed with 0.6% noble agar at 1:1 ratio and placed on top of the

bottom layer. RPMI complete cell growth media was placed on top of the layers and changed weekly. Cells were grown in the agarose for 4 weeks. After that time, formed colonies were fixed with the use of 10% Acetone/Methanol, stained with 0.05% crystal violet and counted. All experiments were performed in triplicate for statistical analysis.

2.2 Biochemistry

2.2.1 Western blotting

2.2.1.1 Gel preparation

The gel pouring glasses (1.5 mm) (Bio-Rad Laboratories) were assembled in the Bio-Rad gel casting device. 8%, 10% or 12% polyacrylamide separating gels were prepared, poured between the glasses, covered with isopropanol and left to polymerise for at least 20 min at RT. Following that time, IPA was removed from the above of polymerised gel and washed with water to remove remaining traces of IPA. 6% stacking gel solution was then prepared and poured between the glasses on top of the separating gel. Immediately, 10-well or 15-well separating combs were placed in the stacking solution and the gel was left to solidify for the following 15 min. Prepared gels were used immediately or stored in 4C for up to one week.

2.2.1.2 SDS-Page electrophoresis and Western blotting

Protein expression analysis was performed by western blotting. PDAC cell lines were plated at a density of $2.5x10^5$ cells per well in 6-well tissue culture plates, incubated overnight and treated with 10µM S3. Cells were collected after 24h (CFPAC-1) or 48h (AsPC1, HPAFII) and lysed in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% SDS, 0.5% sodium deoxycholate (SDC), 1% Triton X-100) supplemented with protease inhibitor cocktail (Sigma Aldrich) and phosphoSTOP phosphatase inhibitor (Sigma Aldrich). Cells were sonicated with the use of water bath sonicator and insoluble material was

removed by centrifugation at 25,000*g*. Prepared lysates were quantified for protein content using Millipore protein quantification cards. Samples were boiled with LDS sample buffer at 37°C for 10 min (for ABCC3 and GPR55 immunoblotting) or 95°C for 5 min, and resolved in SDS-PAGE gel under constant voltage of 120 V. Gels were then transferred on the nitrocellulose membrane with the use of semi-dry BioRad system at constant 400mA for 1 hour. Blocking was performed with 3% BSA/TBS-T 0.05% solution for an hour at RT and was followed by membrane incubation with primary antibodies overnight at 4°C. The following day, membranes were washed with TBS-T 0.05% three times and incubated with secondary HRP-conjugated antibody for an hour at RT. Excess secondary antibody was removed by washing three times with TBS-T 0.05% and once with TBS. Membranes were developed with the use of ECL Start Western Blotting Detection Reagent (GE Healthcare) and the signal was detected with BioRad ChemiDoc Imaging system. Antibodies used for wester blotting are presented in Table 2.3. Immunoblots were quantified using ImageJ and Image Lab 5.2.1 software.

Antibody	Manufacturer/ Catalogue number	Western Blot conditions	IHC conditions
ABCC3	Santa Cruz, # sc-59612	1:1000 in 3% BSA/TBS-T	1:50, pH 9.0 Tris/EDTA antigen retrieval
ABCC3	Invitrogen, # PA5-23653	1:1000 in 3% BSA/TBS-T	1:25 pH 9.0 Tris/EDTA antigen retrieval
GPR55	Cayman, # 10224	1:50000-1:100000 in 3% BSA/TBS-T	N/A
pSTAT3 Y705	CST, #9131	1:1000 in 3% BSA/TBS-T	1:400, pH 9.0 Tris/EDTA antigen retrieval
Total STAT3	CST, #9139	1:1000 in 3% BSA/TBS-T	N/A
HIF1α	Novus Biologicals, #NB100-479	1:1000 in 3% BSA/TBS-T	1:100, pH 9.0 Tris/EDTA antigen retrieval buffer
Vimentin	CST, #5741	1:1000 in 3% BSA/TBS-T	1:100, pH 6.0 Citrate antigen retrieval buffer
Cleaved Caspase 3	CST, #9661	1:1000 in 3% BSA/TBS-T	1:400, pH 6.0 Citrate antigen retrieval buffer
Phosphor-p44/42 ERK1/2	CST, #4370	1:2000 in 3% BSA/TBS-T	N/A
α-SMA	Abcam, #ab5694	1:1000 in 3% BSA/TBS-T	N/A
Bcl-xl	Novus Biologicals	1:1000 in 3% BSA/TBS-T	pH 6.0 Citrate antigen retrieval buffer
Vinculin	CST, #13901	1:1000 in	N/A

		3% BSA/TBS-T	
Tubulin	CST, #2148	1:1000 in 3% BSA/TBS-T	N/A
GAPDH	CST, #5174	1:2000 in 3% BSA/TBS-T	N/A
β actin	CST, #4970	1:2000 in 3% BSA/TBS-T	N/A
α actinin	CST, #3134	1:1000 in 3% BSA/TBS-T	N/A

Table 2.2 List of antibodies and conditions used for Western blotting and immunohistochemistry analysis in this study.

2.2.2 Apoptosis assays- Caspase 3/7 activity

Cells were seeded at the density of 1x10^4 cells per well in a 96-well plate and left overnight at 37°C. On the following day, media was replaced with fresh media containing 10μM S3 and Caspase 3/7 reagent (1:1000) (Essen Bioscience) accordingly to manufacturer's instruction. Cells were incubated at 37°C in the IncuCyte Life Cell Analysis Imaging System (Sartorius) with scanning interval set at 2h. Cells were monitored for up to 72h. Each experiment was performed in triplicate. After ABCC3 knockdown, cells were collected 24h post-transfection and reseeded at the density of 1x10^4 cells per well in a 96-well plate. After overnight incubation, cell media was replaced with fresh media containing Caspase 3/7 reagent (1:1000). Cells were incubated at 37°C in the IncuCyte Life Cell Analysis Imaging System (Sartorius) with scanning interval set at 2h. Cells were monitored for up to 72h. Each experiment was performed in triplicate.

2.2.3 Apoptosis assay- Annexin V

Cells were seeded in a 6-well plate at the density of 2.5x10^5 cells per well and left to attach overnight at 37°C. The following day, appropriate cell treatments were performed. 48 hours

post- treatment cells were harvested. Collected cells were washed with PBS once and once with 1x Annexin-V binding buffer (MACS Miltenyi Biotec). Subsequently, $1x10^6$ cells were resuspended in 400µl of binding buffer containing 1ul of Annexin-V stain (MACS Miltenyi Biotec) and incubated in the dark for 20 min. Then 5µl of PI staining was added to the solution for the exclusion of necrotic cells and stained cells were immediately analysed using FACS Canto II.

2.2.4 Cell cycle analysis

Cells were seeded at the density of $2.5x10^5$ cells per well in a 6-well plate and left to grow overnight at 37°C. The following day, cells were treated with appropriate drugs. Cells were collected 24, 48 and 72 hours post-treatment by trypsinization and washed twice with ice-cold PBS. $1x10^6$ cells were resuspended in 100 µl of ice-cold PBS and cells were fixed with ice-cold 70% ethanol solution, slowly added to the cell suspension. Fixed cells were stored at -20°C for maximum of a week before cell cycle analysis. Cells were washed twice with cold PBS by spinning down at 850g for 10 min. RNA was removed from the sample by resuspension of the cells in 50µl of RNAse solution (100 µg/ml stock) and incubation for 20 min at 37°C. Subsequently, 200µl of Propidium Iodide (50 µg/ml stock) was added to the solution for the following 20 min. Cells were analysed using FACS Canto II. Data was collected for $2x10^4$ events.

2.2.5 Acute LPI stimulation

PDAC cells were seeded at the density of $2.5x10^5$ cells per well in a 6- well plate and left to attach overnight at 37°C. Cells were serum-starved overnight before stimulation with LPI (Calbiochem, cat# 440153). For the analysis of the activation of protein phosphorylation,

cells were stimulated with 1µM LPI for 8 min (pSTAT3 Y705) or 24h (HIF1α), harvested and lysed with RIPA buffer and analysed by western blot protein analysis as previously described. Each experiment was performed in triplicate and the results are presented as a mean ± SEM.

2.2.6 Two-colour Calcein transport assay and inhibition of ABCC3 by Sulindac and S3

Wild type ABCC3 cDNA encoded by recombinant pcDNA3.1 plasmid (pcDNA3-ABCC3) was a kind gift from Prof Susan Cole (395). pDsRed2-C1 (pDsRed) was from Clontech (Mountain View, California, USA).

HEK293T cells were cultured as adherent monolayers in Dubecco's Modifed Eagle Medium (DMEM) High Glucose (ThermoFisher scientific; Waltham, MA, USA) supplemented with 10% foetal calf serum (FCS). Transient transfection used polyethylenimine (PEI), as described previously (396). Briefly, 6.25 x 10^5 cells were seeded onto a T25 tissue culture flask and double transfected 24 hrs later with a transfection mix prepared from 7.5 µg pcDNA3-ABCC3 and 2.5 µg pDsRed in a 20 µl volume of 5% glucose and 17 µg of linear 25 kDa PEI (Sigma-Aldrich; Gillingham, Dorset, UK). The DNA/PEI complex was diluted in 5 ml DMEM and added to the cells. After a further 24 hrs the culture was supplemented with butyric acid to a final concentration of 2 mM to stimulate transcription. The cells were harvested after a further 24hrs in versene and aliquots (2 x 10^5 cells in 200 µl growth medium) incubated with Calcein-AM (0.1 µM; Invitrogen, UK) with 0 µM to 750 µM inhibitor (Sulindac or S3), for 20 minutes at 37°C. Stock solutions of Sulindac and S3 were prepared in DMSO; the vehicle had no effect on the transport assay (data not shown). The cells were then washed twice by pelleting at 160 x G and resuspension in 0.5 ml ice-cold DMEM minus phenyl red and supplemented with only 1% FCS. The cells were analysed using a FACScan flow cytometer (Becton Dickinson, NJ, USA). The population was gated for 10,000 single cells of normal size and granularity. Calcein content was measured in the FL-1 (green) channel, and red fluorescence from the expressed DsRed was measured in the FL-2 channel. Flow cytometry data were acquired using CellQuest Pro Software (BD Biosciences, San Jose,

CA) and analysed using FlowJo (Tree Star; OR, USA). Gating of the transfected and untransfected cell populations is described in Figure Y. ABCC3 transport activity was inferred from the fold difference in Calcein content of untransfected cells versus the transfected cells. To compare independent datasets the fold difference was normalised to 100% activity in the absence of inhibitor. Statistical analysis of the dose response from three biological replicate experiments was by GraphPad PRISM® V5.0 software with IC50 determined by non-linear regression analyses (Graphpad Software, CA, USA).

2.2.7 Cyclooxygenase assays

Cyclooxygenase activity was determined using the COX Fluorescent Inhibitor Screening Assay Kit (Cayman Chemical) according to the manufacturer's recommendation. Recombinant COX-1 or COX-2 were incubated with S3 for 20 minutes prior to the addition of arachidonic acid before initiating the assays. The fluorescent readout from the assay was measured using a Biotek Synergy H4 plate reader. Sulindac sulfide served as a positive control for the assay.

2.3 In vivo experiments

All animal experiments were performed accordingly to standards of national and institutional guidelines. The Curtin University animal care and use committee responsible for ethical compliance approved all animal procedures (AEC 2016 40). Xenograft work was approved by the Italian Ministry of Health (N.484/2016-PR) and was performed at the University of Chieti, Chieti, Italy. All animals were kept at 21°C in ventilated cages, with 12h light/ 12h dark cycle.

2.3.1 Drug preparation

S3 was administered to the animals at the dose of 25mg/kg. Drug was prepared and stored at RT, protected from light for maximum of 7 days.

- Vehicle (0.5% CMC/0.25% Tween 80) was prepared following the protocol:

Needed volume of water was warmed to 64°C. Calculated mass (g) of CMC was slowly added to warmed water, with stirring, until CMC got completely dissolved. Calculated volume of Tween80 was added under stirring conditions to cooled-down solution and stirred for the next 10 min.

- S3 preparation

Calculated weight (mg) of S3 was weighted. 3% $NaHCO_3$ was prepared, pH 7-8. 30.3 µl of NaHCO3 was used per 1mg of S3. Measured volume of NaHCO3 was warmed up. S3 was dissolved in warm NaHCO3 under stirring condition. Once the drug is completely dissolved, calculated volume of vehicle was added to the solution and stirred for next few minutes.

2.3.2 Xenograft mouse model

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* and the animals' health status was monitored daily. Xenograft work was performed at the University of Chieti, Chieti, Italy. Experiments were performed by Dr Emily Capone and Verena Damiani under the supervision of Prof Vincenzo de Laurenzi and Dr Gianluca Sala.

HPAFII xenograft: $3x10^6$ HPAFII cells, re-suspended in 200 µl of PBS, were injected subcutaneously in the right flank of female CD-1 nude mice. When tumours reached a volume of about 100 mm^3 mice were randomized in groups of six animals and treated via oral gavage with 25 mg/kg of S3 or vehicle (0.5% CMC/0.25% tween 80 in water) three times a week, for three weeks. Tumours were measured using a surgical caliper and the volumes were calculated according to the formula: tumour volume= (length * $width^2$)/2. Mice were sacrificed when tumour volumes reached 1500 mm^3.

2.3.3 Patient derived xenografts (PDXs) mouse model

Pancreatic cancer PDX mice were established by engrafting samples of primary pancreatic cancer obtained from patients after surgical resection (kindly provided by Dr Pierluigi Di Sebastiano, Department of Surgery, Unit of Surgical Oncology, SS. Annunziata Hospital, G.

D'Annunzio University, Chieti, Italy) into the right flank of 4-6 week old female CD-1 nude mice. PDXs were passaged twice (P1 and P2) by sequential reimplantation in CD-1 mice. When tumours reached a volume of 50 mm^3, animals bearing P2 PDXs were randomized into two groups (n=6) and treated via oral gavage with 25 mg/kg of S3 or vehicle three times a week, for three weeks and sacrificed as tumours reached 1000 mm3. Tumour volumes were measured by a caliper using the formula tumour volume = (length * $width^2$)/2. Mice were sacrificed as tumour volumes reached 1000 mm^3.

2.3.4 Transgenic mouse model of pancreatic cancer

$KRAS^{WT/G12D}$, $P53^{WT/R172H}$, $PDX\text{-}1CRE^{+/+}$ (KPC) transgenic mouse model of pancreatic cancer and KPC control mice were bred and provided by the Animal Research Centre (ARC, Murdoch, Western Australia). All mice were maintained on a C57BL/6 genetic background. Mice were generated according to the protocol established by Hingorani et al. in 2005 (392). Both male and female mice were used for experiments. Mice were ear-marked and genotyped by the provider. After reaching 80 days (predicted time of the commencement of tumour development), KPC mice were palpated daily to assess tumour presence. Mice were randomized into different treatment arms and subjected to treatment after the tumours reached palpable size.

ABCC3 targeting: Mice were treated daily by oral gavage with 25mg/kg S3 (n=6) or vehicle (0.5% CMC/0.25% tween 80 in water) (n=8) as a control. Additionally, third treatment arm was injected by tail vein with 60mg/kg of Abraxane (Abraxis BioScience) once a week (n=7). The combination arm (n=6) received 25 mg/kg S3 daily by oral gavage and 60 mg/kg Abraxane weekly by tail vein injection.

Animals were monitored daily and sacrificed when visible signs of pain and distress could be observed, such as significant weight loss (more than 15% of initial bodyweight), dehydration, development of ascites and breathing problems caused by developing lymphoma or pain. Mice were sacrificed by snipping of the main cardiac vein followed by organ perfusion through the heart. Pancreas, liver, spleen and lung tissues were collected

for further analysis. Survival of mice was plotted using a Kaplan-Meier curve and quantified using a log rank test.

2.3.5 Pharmacokinetic studies

Female C57BL/6 mice (S3-treated mice) and female athymic nu/nu mice implanted subcutaneously with human HT29 colon tumour xenografts (sulindac-treated mice) were acclimated in the laboratories prior to experimentation. The animals were housed in microisolated cages in a 12-hour light/dark cycle. The animals received filtered municipal water and sterilizable Harlan-Teklad TD8656 rodent diet ad libitum. Cages were changed twice weekly. The animals were observed daily and clinical signs were noted. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

Three mice were treated with 100 mg/kg S3 in 0.5% CMC/0.25% tween 80 in water once by oral gavage. Blood was collected at 30 min and 2 h (survival bleeding), and 5 h (terminal bleeding) following the S3 treatment. 12 mice were treated with 100 mg/kg sulindac in Maalox once by oral gavage. Blood was collected at 1 h, 2 h, 4 h or 8 h following the treatment (three mice per time point) following the sulindac treatment. Collected plasma was separated and frozen. Plasma concentration of S3 and sulindac sulfide (the active metabolite of sulindac) was analysed by LC-MS/MS.

2.3.6 Histopathological analysis

Pancreas and liver tissues were resected from sacrificed experimental mice and snap frozen in the optimal cutting temperature OCR resin (Tissue-Tek® O.C.T. Compound, Sakura® Fintek) or fixed in 10% formalin for 24h. After that time formalin fixed tissues were placed and stored at RT in 70% ethanol. Fixed tissue samples were embedded in paraffin by standard techniques and cut into 4µM sections using a Leica microtome. Histopathological analysis of collected tissues was performed by Hematoxylin and Eosin (H&E) staining. For

the deparaffinization of the tissue sections, slides were incubated in three changes of xylene, 5-10 min each. Re-hydration of the sections was performed by incubation in 100% ethanol three times for 3 min, followed by incubation of the sections for the same time in 70% and 50 % ethanol before placing slides in the distilled water. Slides were stained with Hematoxylin solution for 30-60 seconds, washed with running water for a minute and incubated in distilled water for further 5 min. Dehydration was performed in 95% ethanol, followed by three-time incubation in absolute alcohol 1 minute each. Counterstain with 1% alcoholic Eosin solution was carried out for 30-60 seconds. Slides were then washed in three changes of 100% ethanol and three changes of xylene before mounting of the section slides with xylene- based mounting medium.

2.3.7 Immunohistochemistry

Formalin fixed paraffin embedded tissues were deparaffinised using abovementioned protocol. Slides with tissues were placed in the appropriate heat- induced antigen retrieval buffer and boiled in the microwave at the maximum power for 1-2 min followed by constant boiling at the low power for the next 20 min. Tissues were cooled down in the retrieval buffer before washing 3 times with TBS. To reduce background staining, endogenous peroxidase activity was blocked with 0.3% H_2O_2 for 30 min followed by three washes with TBS. If enhancement of the signal using biotinylated antibodies was necessary, blocking of avidin and biotin was performed for 10 min each. Next, blocking of the proteins with was carried out in 5% BSA for 30-60 min. Immunohistochemistry staining with specific antibodies was performed overnight at 4°C. Used antibodies and antigen-retrieval conditions are presented in Table 2.3. The next day, following the washing of the remaining primary antibody with TBS, biotinylated secondary antibodies (1:500) were used to intensify the staining. The incubation was carried out for an hour at RT followed by tissue incubation with Avidin-HRP solution (Molecular Probes) (1:200) for the subsequent 30 min. All the steps were performed in a humidified chamber. Antibodies were developed with DAB chromogen (Sigma Aldrich) and the staining was monitored using the inverted microscope. All samples stained with the same antibody were developed at the same time in the same conditions. Tissues were counterstained with haematoxylin and fixed with xylene-based mounting media after re-hydration of stained sections.

2.3.8 TUNEL assay

For the analysis of apoptosis, Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was performed with the APO-BrdU™ TUNEL Assay Kit (Merck Millipore, # S7101). Briefly, tissue sections were deparaffinised using the previously described protocol. Tissues were then incubated with freshly prepared 20 µg/ml of proteinase K for 15min at RT. Enzyme was removed by 2 washes in distilled water for 5 min. Endogenous peroxidase activity was blocked by incubating the sections with 3% H_2O_2 for 5 min and washing the remaining solution twice in distilled water. Equilibration buffer was applied to the tissues for at least 10 minutes before section incubation with working strength TdT enzyme for an hour at 37°C. The reaction was quenched by placing the slides in the stop buffer for 10 min followed by three washes in PBS, 1 minute each. Subsequently, anti-digoxygenin conjugate was added to the sections for 30 min at RT and washed off with 4 changes of PBS. Peroxidase substrate (DAB,3,3'-diaminobenzidine) was used to stain the sections and the staining was monitored with the use of inverted microscope. Tissues were counterstained and mounted as previously described.

2.4 Statistics

Necessary sample size for each experiment was assessed based on previous work done in our laboratory. Statistical analysis of the results was performed by unpaired, two-tailed *t*-test (western blot and IHC quantification), multiple t-test (tumour growth) and one-way ANOVA (cell growth) assuming independent samples and normal distributions. A 95% confidence interval was used for statistics and $P < 0.05$ was considered significant. Survival of mice was plotted using a Kaplan-Meier curve and quantified using a log rank (Mantel- Cox) test. All results are presented as the mean ± SEM. All statistical analyses were performed using GraphPad PRISM® V6.0 software (Graphpad Software, CA, USA).

Buffers:

PBS 20x- 2l

-5.76g Na_2HPO_4
-320g NaCl
-8g KH2PO4
-8g KCl

TBS 20x- 2l

-96.8g Tris Base
-320g NaCl
-pH 7.6

RIPA 2x

-50mM Tris-HCl (pH 7.4)
-150mM NaCl
-1% NP-40
-0.1% SDS
-1mM EGTA
-5mM EDTA

Tris-glycine running butter (10x)- 2l

-60.55g of Tris base
-288g glycine
-100ml of 20% SDS

Tris/EDTA (antigen retrieval)

-10mM Tris Base
-1mM EDTA
-0.05% Tween-20
-pH 8.0

Citrate buffer (antigen retrieval)

-10mM Sodium citrate
-0.05% Tween-20
-pH 6.0

3. The potential of GPR55 as pharmacological target in PDAC therapy

3.1 Introduction

Overexpression of some GPCRs in human cancers has been previously described and the link between their expression and cancer progression has been indicated (397). In particular, receptors for lysophospholipids (e.g. sphingosine 1-phosphate or lysophosphatidic acid) were shown to prompt cell proliferation in several cancer types. For several years, the focus of our group has been placed on lysophospholipids and their role in cancer progression. Lysophosphatidylinositol (LPI) was investigated as a potential cancer driver, since the increased levels of this signalling molecule have been detected in several cancers (203). Following the recent identification of GPR55 as a specific receptor for endogenous LPI (326), GPR55 has been proposed as potential player in tumorigenesis. Activated by its endogenous ligand, LPI, GPR55 stimulates the downstream signalling cascades that enhance the proliferative rates and block the apoptotic stimuli in cancer cells. GPR55 activation by LPI was demonstrated in both cells with endogenous expression of the receptor and cells where expression of GPR55 was ectopically induced. These data suggest that LPI and GPR55 might play a key role in the regulation of cell proliferation in many types of cancer. It has been reported that high levels of GPR55 expression characterized several types of cancer and, in some cases, it has been shown that this remarkably correlated with tumour aggressiveness and invasiveness. As an example, it was shown by our group that in ovarian and prostate cancer, GPR55-LPI axis is critical for the regulation of cell proliferation and anchorage-independent cell growth (334). Similarly, GPR55 signalling was proposed to be involved in breast cancer cell migration (335). The receptor was found expressed 30-fold higher in the metastatic MDA-MB-231 cell line, compared to MCF-7, a low-metastatic cell line. The role of GPR55 in glioblastoma progression was additionally confirmed and a correlation between high expression of GPR55 and low survival of glioblastoma patients was observed (315). Moreover, the correlation between GPR55 overexpression and high

proliferative index was also detected in glioblastoma and breast cancer (315). Although the involvement of GPR55 has been documented for other cancer types, limited studies investigated the potential role of GPR55 in pancreatic cancer nor explored its pharmacological potential in PDAC targeted therapies. One study demonstrated a correlation between GPR55 expression and PDAC staging with significantly higher mRNA levels of GPR55 in PanIN2 and 3 compared to early stages of PDAC development (p=0.007) (315), suggesting an important role of the receptor in PDAC development. At the same time, overexpression of GPR55 in HEK293 cells was shown to enhance proliferative capacity of the cells. Importantly, database analysis (hgserver1.amc.nl) demonstrated correlation between GPR55 expression and survival probability, showing significantly increased survival of the patients characterized by low expression of GPR55 (Figure 3.1).

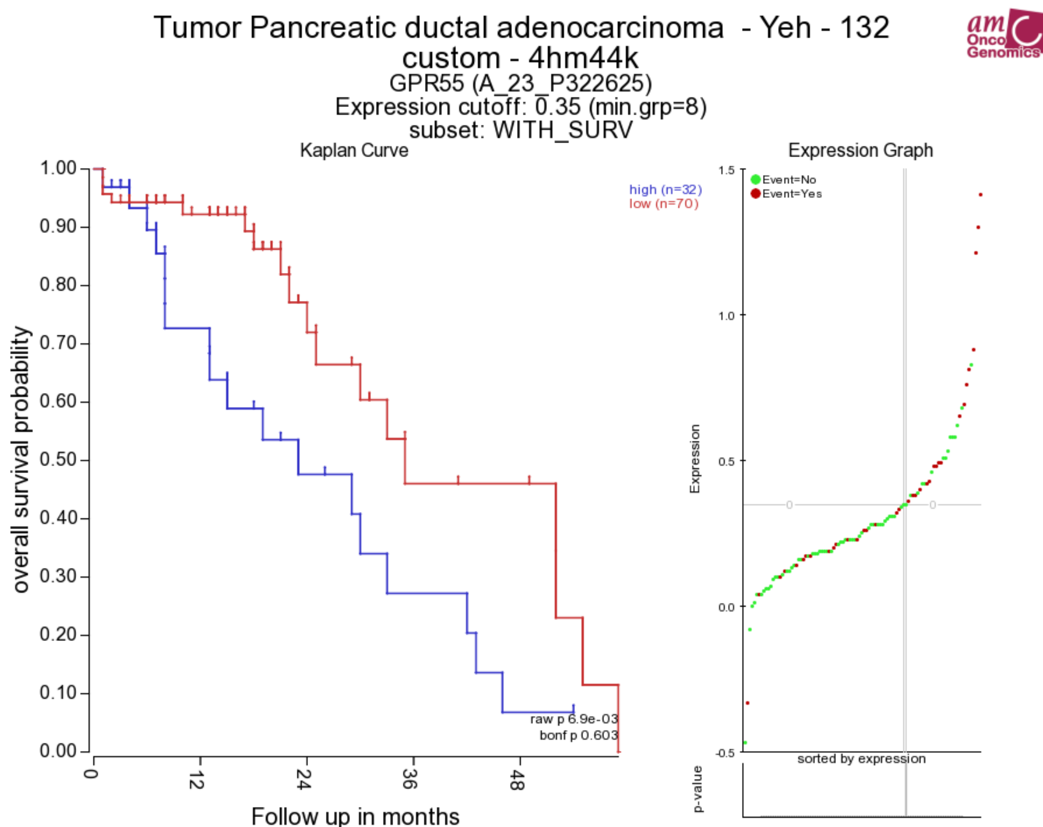


Figure 3.1 ***GPR55 expression correlates with survival.*** *Kaplan Meier curve comparing the survival probability of patients with low (red) and high (blue) expression of GPR55. Data was adapted from https://hgserver1.amc.nl/cgi-bin/r2/main.cgi; gene ID 9290.*

Therefore, the potential involvement of GPR55 in pancreatic cancer development and progression and its pharmacological potential in pancreatic cancer therapy remained to be explored.

3.2 Aims of the project

The main aim of this project was to evaluate the potential of GPR55 as a novel pharmacological target in pancreatic cancer therapy. For this purpose, we aimed to accomplish the following goals:

- Analysis of GPR55 expression in pancreatic cancer specimens, including cell lines and tumour tissues
- Analysis of the mechanisms regulating GPR55 expression in PDAC
- Analysis of the potential role of GPR55 in PDAC progression *in vitro* and *in vivo*
- Analysis of the mechanisms involved in GPR55-mediated regulation of PDAC progression
- Analysis of the effects of pharmacological inhibition of GPR55 on PDAC progression *in vitro* and *in vivo*

Dr Riccardo Ferro (Blizard Institute, Queen Mary University of London, UK) commenced the work on the project. My work contributed to the completion of the project, which resulted in the following publication, attached at the end of the chapter:

R Ferro, **A Adamska**, R Lattanzio, I Mavrommati, CE Edling, SA Arifin, CA Fyffe, G Sala, L Sacchetto, G Chiorino, V De Laurenzi, M Piantelli, OJ Sansom, T Maffucci, Marco Falasca *GPR55 signalling promotes proliferation of pancreatic cancer cells and tumour growth in mice, and its inhibition increases effects of gemcitabine*; Oncogene, 2018 (37); doi: 10.1038/s41388-018-0390-1

The data that I obtained for the publication, as well as additional unpublished results are presented in this chapter.

3.3 Results

3.3.1 GPR55 is overexpressed in PDAC

Elevated expression of GPR55 has been suggested to correlate with progression and aggressiveness of several human cancer types. Thus, in order to verify the potential involvement of GPR55 in pancreatic cancer development and progression, its expression levels in cancer specimens were initially studied.

3.3.1.1 Optimization of the anti-GPR55 antibody for Western blot analysis

Expression levels of GPR55 was analysed in a panel of pancreatic cancer cell lines and non- malignant pancreatic cell lines by Western blotting analysis. An optimization process for the detection of GPR55 expression was initially carried out. Several anti-GPR55 antibodies from different manufacturers (Novus, ThermoFisher, Cayman) were tested for their specificity and sensitivity in Western blot analysis. Due to the lack of specificity of the ThermoFisher antibody, resulting in the detection of multiple bands and hindering proper analysis of the results, the Cayman antibody was chosen as the most suitable for further studies. Subsequently, the optimal working concentration of the antibody needed to be evaluated due to the high intensity of the signal when the antibody was used according to manufacturer's instructions. Dilutions of the antibody ranging from 1:50 000 to 1:100 000 were chosen as the optimal for the identification of changes in GPR55 expression and all further Western blot experiments were performed with Cayman anti-GPR55 antibody at working concentration within this range. High intensity of the signal detected in the pancreatic cancer cell lines may confirm the enhanced expression of the protein in the PDAC specimens.

3.3.1.2 Verification of GPR55 expression in PDAC cell lines and tissues

Pancreatic cancer cell lines (AsPC1, HPAFII, CFPAC-1, BxPC3 and SW1990) and non-malignant pancreatic cells (HPDE, hTERT-HPNE) were studied for GPR55 expression. Western blot analysis showed that the receptor is expressed at the higher levels in the PDAC cell lines compared to the control cell lines (Figure 3.2). Interestingly, we observed that SW1990 cell line, which is the only cell line in the panel bearing wild type p53, was characterized by lower GPR55 levels, compared to the rest of PDAC cell lines, which suggests possible correlation between *TP53* and GPR55 expression.

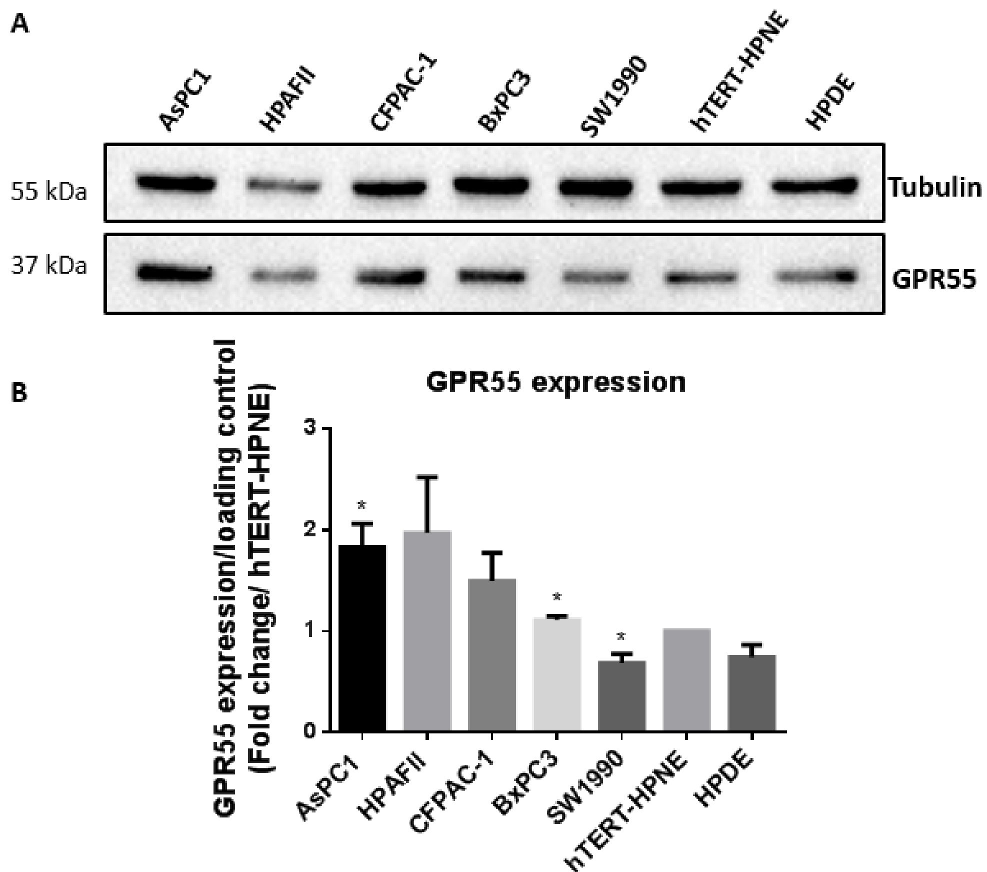


Figure 3.2 ***GPR55 is overexpressed in PDAC cell lines****. (A) Representative Western Blot presenting the panel of expression of GPR55 in PDAC cell lines compared to non-malignant pancreatic cell lines (hTERT-HPNE, HPDE); (B) quantification of the expression of GPR55 in the same cell lines presented as mean± SEM of 3 independent experiments, GPR55 expression in all the cell line is compared to the expression in the hTERT-HPNE cell line set as base level; *p<0.05*

Similarly, it was previously shown by our group that GPR55 mRNA levels are overexpressed in PDAC by comparison of the panel of PDAC cell lines to the control immortalized “non-malignant” pancreatic cells (HPDE, hTERT-HPNE) (Ferro R., Adamska A. et al. (398), Supplementary Figure 1a). Correspondingly, enhanced expression of GPR55 in PDAC was confirmed by immunohistochemistry analysis of human and murine pancreatic and pancreatic cancer tissues. Interestingly, strong ductal staining was observed in the PDAC specimens, whereas GPR55 expression in “healthy” pancreatic tissues could be only detected in the Islets of Langerhans (Ferro R., Adamska A. et al. (398), Figure 1a), consistent with the proposed role of GPR55 in the insulin and glucose homeostasis in healthy pancreas.

3.3.2 GPR55 regulates PDAC cell growth and clonal expansion

3.3.2.1 Knockdown of GPR55 reduces PDAC cell proliferation

Having confirmed the overexpression of GPR55 in PDAC cell lines and tissues, the potential role of the protein in pancreatic cancer cell proliferation and cancer progression was verified. Transient gene silencing of the receptor was performed in two PDAC cell lines (AsPC1, HPAFII) and the cell proliferative capacity was assessed after suppression of the receptor. Cells were transiently transfected with two specific siRNAs, according to the Materials and Methods section (Chapter 2.1.5). A non-targeting negative siRNA (siSCRL) was used as a control. 72 hours post transfection, the number of viable cells was counted and compared to the number of control cells. At the same time, in order to verify the efficiency of transfection, protein analysis was performed to test the reduced expression of GPR55 receptor in the transfected cells. A reduction in GPR55 expression was confirmed in the AsPC1 cell line (Figure 3.3-A), which resulted in a significant decrease in the number of viable cells reported after GPR55 silencing with both sequences (Figure 3.3-B) ($p=0.0040$ for siGPR55-1 vs siSCRL; $p<0.0001$ for siGPR55-2 vs siSCRL).

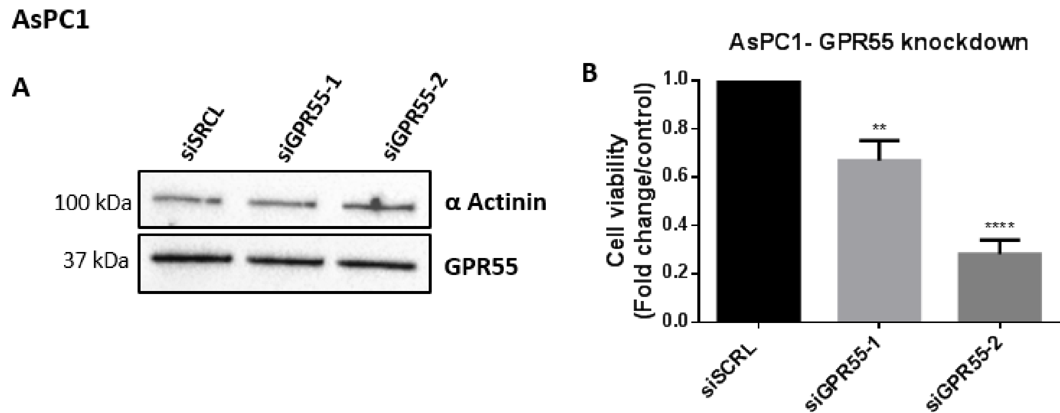


Figure 3.3 ***GPR55 regulates PDAC cell growth****. The effects of silencing of GPR55, confirmed by Western Blot analysis (A), on cell number (B) of AsPC1 cell line. Cell number is presented as mean ± SEM of 5 experiments; **p<0.01, ****p<0.0001.*

Similarly, silencing of GPR55 in HPAFII pancreatic cancer cell line (Figure 3.4-A) reduced cell proliferation, significantly decreasing the number of viable cells (p<0.0001 for siGPR55-1 vs siSCRL and p<0.0001 for siGPR55-2 vs siSCRL) (Figure 3.4-B).

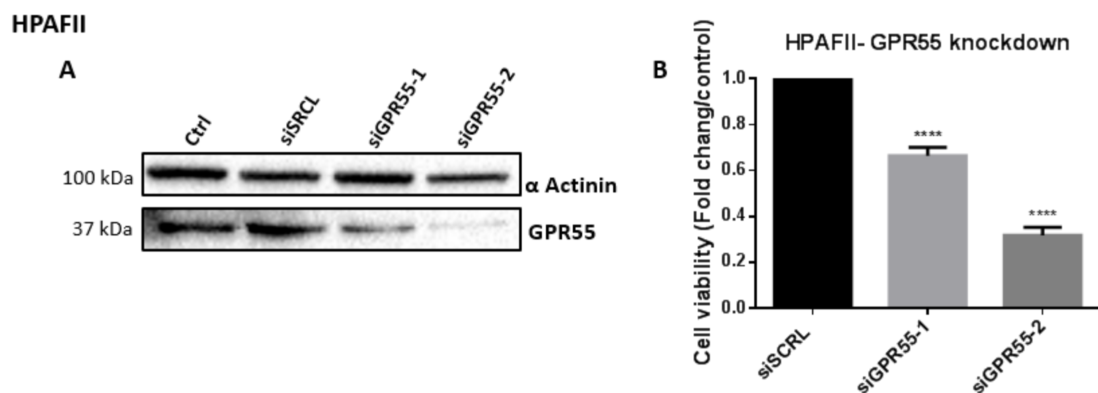


Figure 3.4 ***GPR55 regulates PDAC cell growth****. The effects of knock down of GPR55, confirmed by Western Blot analysis (A), on cell number (B) of AsPC1 cell line. Cell number is presented as mean ± SEM of 6 experiments; ****p<0.0001.*

Additionally, previous results obtained in PANC-1 pancreatic cancer cell line confirmed the remarkable reduction in cell growth in the PDAC cells with the

knockdown of GPR55. These results highlight the important function played by GPR55 in the regulation of PDAC cell proliferation.

3.3.2.2 Knockdown of GPR55 reduces clonal expansion of PDAC cell in vitro

To gain more insight into the GPR55-regulated cell growth and proliferation, the transfected cells were grown in the agarose matrix supplemented with complete cell growth media, which resembles the conditions present in the tumour microenvironment. Soft agar assay is used to analyse cancer cell grow in anchorage-independent conditions and other cell characteristics, like *in vitro* clonal expansion. Malignant cells possess the ability to grow and propagate in the *in vitro* three-dimensional (3D) anchorage-independent conditions, characteristic that cannot be attributed to non-neoplastic cells. Thus, soft agar assay may be used to assess the tumorigenic potential of the cells and to verify the pharmacological potential of drug candidates.

48 hours post GPR55 knockdown, cells were collected and an equal number of cells from each sample (10.000 cells) was seeded in the agarose matrix according to the protocol described in the Materials and Methods section (Chapter 2.1.7). Cells were allowed to grow for 4 weeks. The number and the size of the colonies with GPR55 knockdown was compared with the colonies formed by control cells. Consistent with the cell viability assay, the number of colonies formed after 28 days significantly differed between control and transfected cells in both AsPC1 and HPAFII. The significant reduction in anchorage-independent growth following GPR55 genetic silencing was demonstrated for AsPC1 cell line ($p=0.1476$ for siGPR55-1 vs siSCRL and $p=0.0053$ for siGPR55-2 vs siSCRL) (Figure 3.5-A). A more evident effect was observed in the HPFAII cell line, in accordance with the higher reduction of cell proliferation shown for this cell line after GPR55 knockdown. A significant reduction in the number of HPAFII colonies formed by the cells with silencing of GPR55 was demonstrated ($p<0.0001$ for siGPR55-1 vs siSCRL and $p<0.0001$ for siGPR55-2 vs siSCRL) (Figure 3.5-B). In addition, not only the number or formed colonies was decreased but also the size of individual colonies.

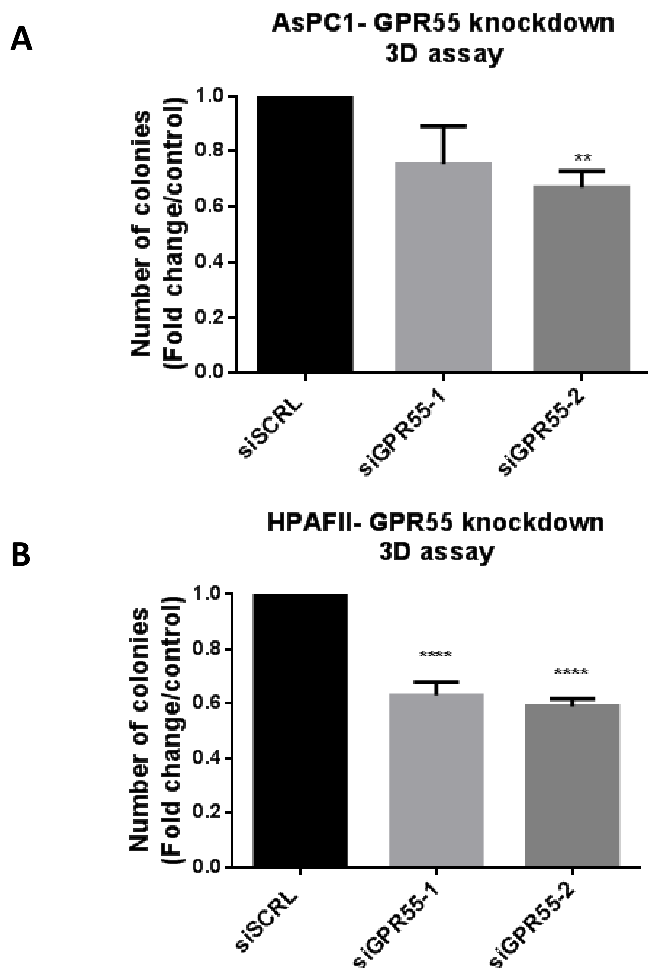


Figure 3.5 ***GPR55 influences clonal expansion of PDAC cells.*** *The effects of GPR55 silencing in AsPC1 (A) and HPAFII (B) cell line with two independent siRNAs (siGPR55-1, siGPR55-2) on the number of colonies compared to the control cells (siSCRL). The results are presented as mean ± SEM of 3 independent experiments; **p<0.01, ****p<0.0001*

3.3.2.3 Knockdown of GPR55 significantly slows down PDAC progression in vivo

The work performed previously in our group confirmed the involvement of GPR55 in PDAC progression *in vivo*. A transgenic mouse model of pancreatic cancer (KPC mice) was crossed with mice harbouring homozygous deletion of *Gpr55* (GPR55 KO), giving rise to a new mouse model, called KPCG (GPR55 KO KPC mice). Kaplan-Meier analysis comparing the survival of KPC and KPCG mice showed remarkable increase in the survival of the GPR55 KO mice, suggesting a crucial role of GPR55 in progression of

PDAC (Ferro R., Adamska A. et al (398), Figure 1b). In addition, IHC analysis of pancreatic tissues resected form both mice strains revealed a significant reduction in the Ki67 staining (marker for cell proliferation) in the pancreatic tissues resected from KPCG mice (Ferro R., Adamska A. et al (398), Figure 1c). This again confirms the role of GPR55 in the regulation of PDAC cell proliferation that influences the development of the disease.

In summary, the remarkable influence of GPR55 on PDAC cell proliferation and colonization *in vitro* demonstrated by my work and its crucial role in disease progression *in vivo* shown previously, underline the relevance of GPR55 in PDAC biology and suggest that this receptor is a potential drug target in PDAC therapy.

3.3.3 Expression of GPR55 is regulated by TP53 in PDAC

A crucial issue in cancer therapy is the stratification of patients based on their genetic background in order to predict their response to therapies. Therefore determining the genetic events that might regulate expression and activity of GPR55 was pivotal. Considering the main mutations occurring during PDAC carcinogenesis, we investigated if any of those events might affect GPR55 enhanced expression in PDAC. It is known that 50-70% of PDAC patients carry mutations in tumour suppressor *TP53* gene. Considering lower expression of GPR55 in SW1990 cell line, bearing WT p53, we investigated the potential correlation between p53 status and GPR55 expression.

3.3.3.1 Expression of GPR55 is negatively correlated with TP53 status

The *in vivo* studies performed previously in our group suggested the existence of a relationship between *TP53* status and GPR55 expression. Comparison of the survival of KC mice (characterized by *KRAS* mutation under Cre promoter and WT *TP53*) with KCG mice (KC mice crossed with GPR55 KO mice) did not show significant difference in the survival of the mice. However, the same experiment analysing the survival of KPC mice with KPCG mice (both with mutant *TP53*), described in previous paragraph

(3.3.2.3), demonstrated significant difference in the survival between the two mice populations.

Additionally, it was previously shown that the reintroduction of WT p53 into a PDAC cell line bearing *TP53* mutation (AsPC1) clearly downregulated levels of GPR55 (Ferro R., Adamska A. et al (398), Figure 3c). These data suggested a role of p53 in the regulation of GPR55 expression.

To fully investigate the relationship between GPR55 expression and different p53 status, I studied the levels of GPR55 in several mice cell lines bearing different *TP53* status: *TP53* WT (PZR1), *TP53* mutated (*TP53*$^{R172H/+}$, PZPR1) and *TP53* deleted (*TP53*$^{fl/+}$, PZPflR) (Figure 3.6).

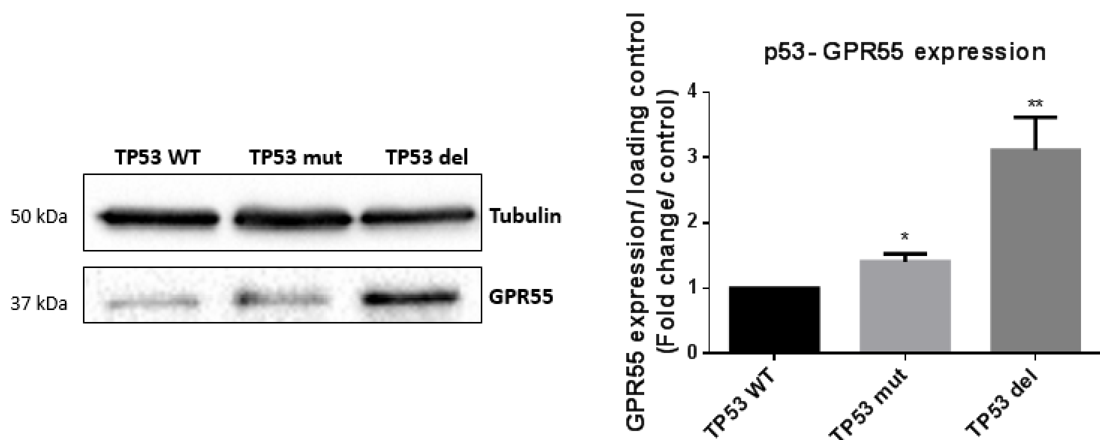


Figure 3.6. ***GRP55 expression correlates with TP53 status.*** *The analysis of GPR55 expression in the murine pancreatic cell lines bearing different TP53 status: wild type (TP53 WT), mutated (TP53 mut) and deleted (TP53 del). The results are presented as mean ± SEM of 3 experiments; *p<0.05, **p<0.01*

Analysis of GPR55 expression in these cell lines performed by Western blotting clearly showed an increase in GPR55 levels, which negatively correlated with dysregulation of p53 activity, with the highest levels of expression observed for cells with deleted p53. Interestingly, negligible levels of GPR55 were detected in the cells with expression of fully functional (WT) p53 protein. These data suggested that GPR55 expression is negatively regulated by wild type p53 protein. Additionally, direct silencing of *TP53* with the use of two specific siRNAs was performed in the cell lines

bearing wild type TP53: human embryonic kidney cell line HEK293T and pancreatic cancer cell line SW1990. The silencing efficiency, as well as the expression of GPR55 was analysed by Western blotting (Figure 3.7). In both cell lines, very low expression of GPR55 was noted in the control samples, confirming that the presence of wild type p53 represses GPR55 expression. On the other hand, *TP53* silencing resulted in significant increase in GPR55 expression after 48 hours of transfection in both tested cell lines.

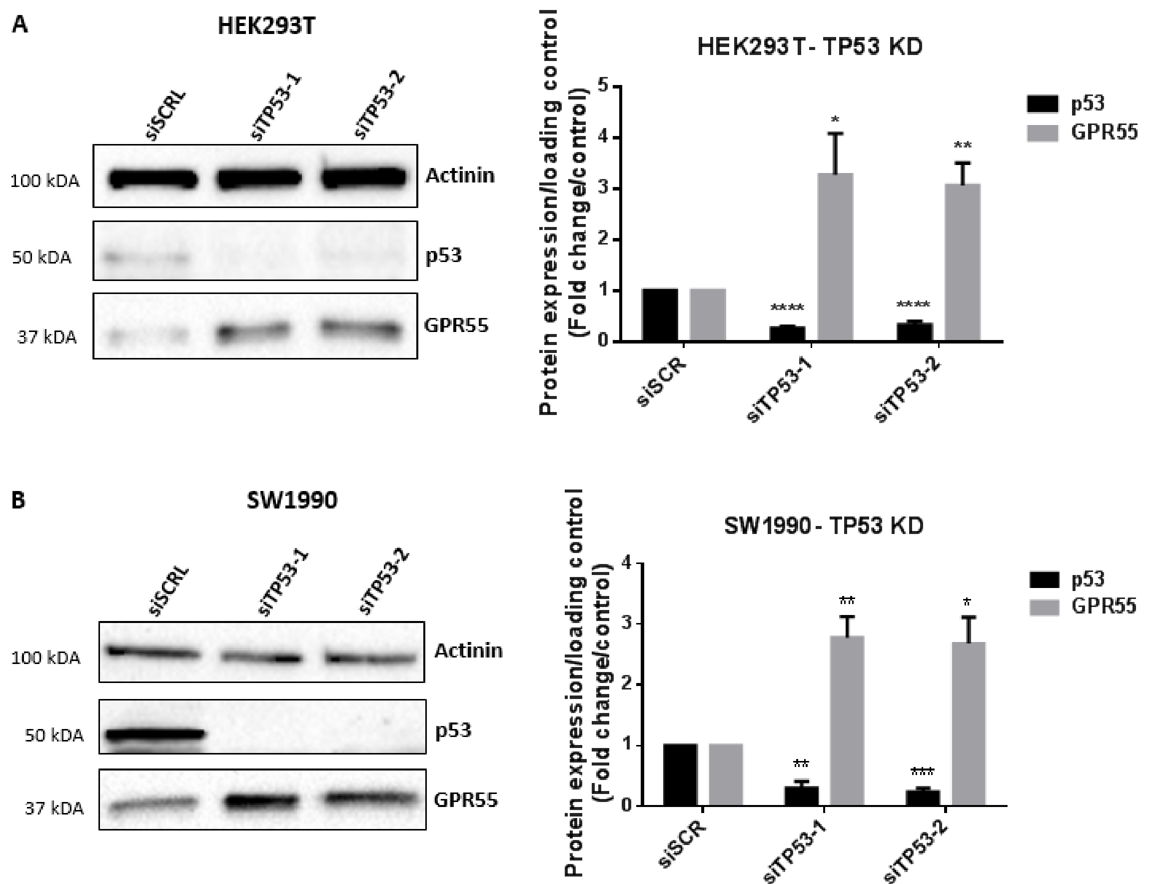


Figure 3.7 ***GRP55 expression is regulated by p53.*** *Western blot analysis of the effects of TP53 silencing in two cell lines bearing wild type TP53: HEK293T (A) and SW1990 (B) on the expression of GPR55. Quantitative analysis of GPR55 expression changes is presented as a mean ± SEM of 3 independent experiments; *p<0.05, **p<0.01, ***p<0.001 ****p<0.0001*

These results reinforce the hypothesis that the status of tumour suppressor p53 has a negative correlation with GPR55 expression. In healthy cells, the presence of fully active p53 blocks the ductal expression of GPR55. However, when p53 is mutated, as

reported in 50-70 % of PDAC cases, p53 loses its inhibitory effect, allowing the full expression and activity of GPR55 and facilitating PDAC progression.

3.3.3.2 p53 regulates GPR55 expression through miR-34b-3p

In order to gain more insight into the mechanisms, by which p53-mediated regulation of GPR55 occurs, we investigated the intermediate molecules that might participate in the p53-GPR55 axis. Publicly available database analysis indicated that a family of microRNAs, miR-34, is remarkably downregulated in PDAC patients and in the majority of PDAC cell lines. It is also known that miR-34 expression is regulated by p53, which mutation represses miR-34 expression. Therefore, we hypothesized that GPR55 expression might be regulated by p53 via miR-34. Interestingly, database analysis demonstrated that one member of miR-34 family, miR34b, shows affinity towards 3'UTR region of GPR55, suggesting the possible involvement of this miRNA in GPR55 regulation. In fact, reintroduction of miR-34b by transient transfection into two pancreatic cell lines with low levels of miR-34b (AsPC1, HPAFII) significantly decreased GPR55 levels in both cell lines (Figure 3.8).

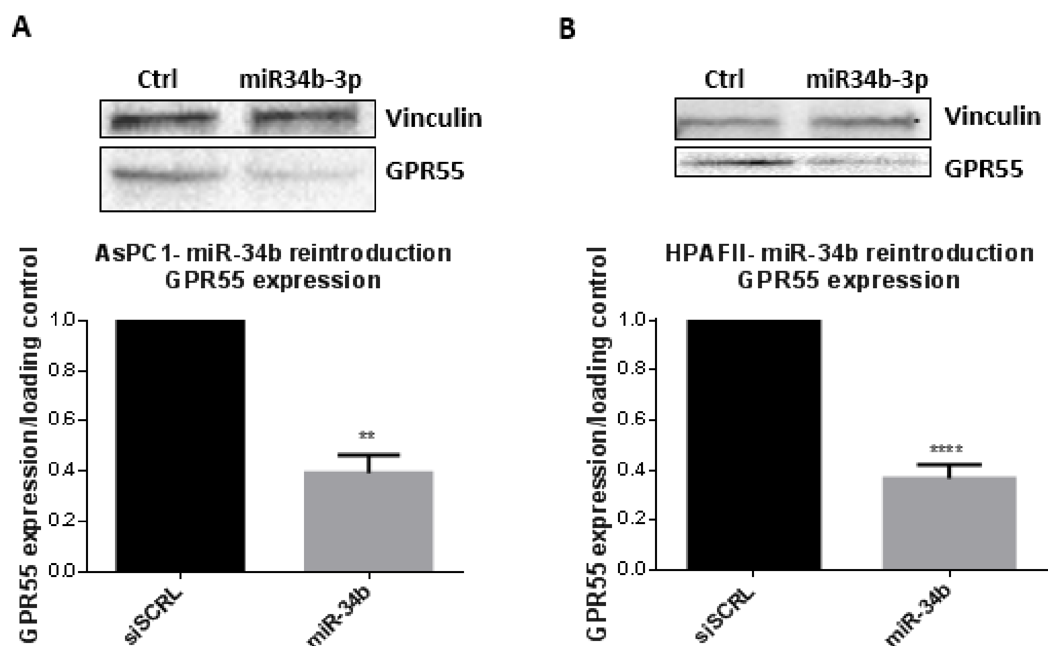


Figure 3.8 ***GRP55 expression is regulated by miR-34b.****The reintroduction of miR-34b into PDAC cell lines characterized by low miR-34b levels AsPC1 (A) and HPAFII (B) affects GPR55*

*expression levels. Results are presented as mean ± SEM of 3 (AsPC1) and 6 (HPAFII) independent experiments; **p<0.01, ****p<0.0001*

In parallel, a significant reduction in cell number was reported in both cell lines (Figure 3.9), supporting the hypothesis of a GPR55-mediated regulation of PDAC cell proliferation.

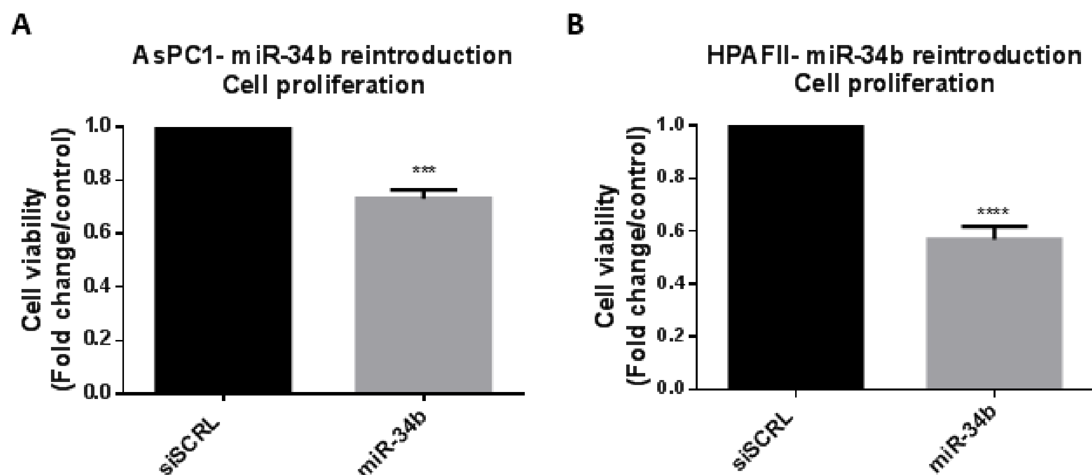


Figure 3.9 ***miR-34b regulates GPR55 expression and PDAC cell growth*** *The effects of reintroduction of miR-34b into AsPC1 (A) and HPAFII (B) cell lines on cell number. Results are presented as mean ± SEM of 3 independent experiments; ***p<0.001, ****p<0.0001*

This data suggests a microRNA-mediated mechanism by which p53 mutations regulate GPR55 expression. Indeed, WT p53 protein does not repress miR-34 and, consequently, the elevated miR34b levels inhibit GPR55 expression. On the other hand, in PDAC cells, p53 mutations impede miR-34b expression, unblocking GPR55 suppression.

3.3.4 Pharmacological inhibition of GPR55 influences PDAC progression

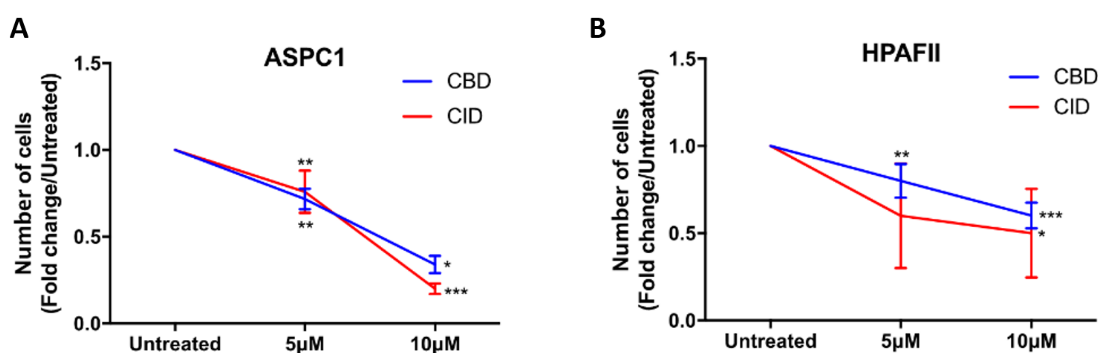
3.3.4.1 Selection of the potential inhibitor of GPR55 for PDAC therapy

Following the validation of the importance of GPR55 in PDAC progression, the pharmacological potential of GPR55 targeting on PDAC cell proliferation was investigated. The structural similarity of GPR55 to cannabinoid receptors and its proven affinity towards several endocannabinoids indicated that cannabinoids might be used as GPR55 antagonists. Indeed, it has been shown that several cannabinoids are able to affect GPR55 activity, although the majority of endocannabinoid compounds showed no pharmacological activity. A non-psychoactive compound, Cannabidiol (CBD), has been widely investigated as an anti-carcinogenic agent and its effectiveness in several cancer types has been demonstrated (399). Recently, CBD has been suggested to act as a GPR55 antagonist by counteracting the GPR55 responses to LPI in several cancers (331, 332). On the other hand, tetrahydrocannabinol (THC), a major psychoactive cannabinoid found in cannabis, was unable to affect GPR55 signalling (323). Thus far, the potential of CBD as GPR55 inhibitor has not been addressed in pancreatic cancer.

The optimization of a suitable inhibitor of GPR55 (verifying the efficacy of several cannabinoids, such as CBD (Cannabidiol), CBDV (Cannabidivarin) and THCV (Tetrahydrocannabivarin) (GW Pharma) and reaction conditions (10% FBS vs 2% FBS) previously performed in our group showed that CBD was the most specific compound and exhibited the highest effect on cell proliferative abilities in all tested cell lines (AsPC1, HPAFII, PANC1). Therefore, further experiments investigating the effects of the pharmacological inhibition of GPR55 in pancreatic cancer were performed using synthetic CBD. Additionally, a synthetic inhibitor of GPR55, CID16020046 (referred to later as CID) was tested as a positive control in comparison with CBD to verify the specificity of GPR55 pharmacological blockage in PDAC *in vitro* models.

3.3.4.2 Pharmacological inhibition of GPR55 significantly reduces PDAC cell growth

The effectiveness of CBD and CID treatment on the reduction of cell proliferation was verified in five PDAC cell lines: AsPC1, HPAFII, PANC1, BxPC3 and Capan2. Cells were seeded in 12-well plates and treated with increasing concentrations of the drug ranging from 0.5µM to 10 µM. As control, human pancreatic normal epithelial cells (hTERT-HPNE) were also treated with both compounds, to verify the specificity of investigated treatments. Cell number was determined after 72h by cell counting. Consistent with the results obtained after GPR55 silencing, the pharmacological inhibition of the receptor resulted in significant reduction of cell growth in a dose dependent manner. Following CBD treatment, a significant decrease in the number of viable cells was observed in all tested cell lines, in which the highest concentrations almost completely blocked cell proliferation. Similarly, treatment of AsPC1 and HPAFII cells with corresponding doses of CID decreased cell number to a similar extent (Figure 3.10). These results confirm the pharmacological potential of CBD in decreasing PDAC cell proliferation. They also show the specificity of CBD towards GPR55, as its effectiveness was comparable to commercially available GPR55 synthetic inhibitor.



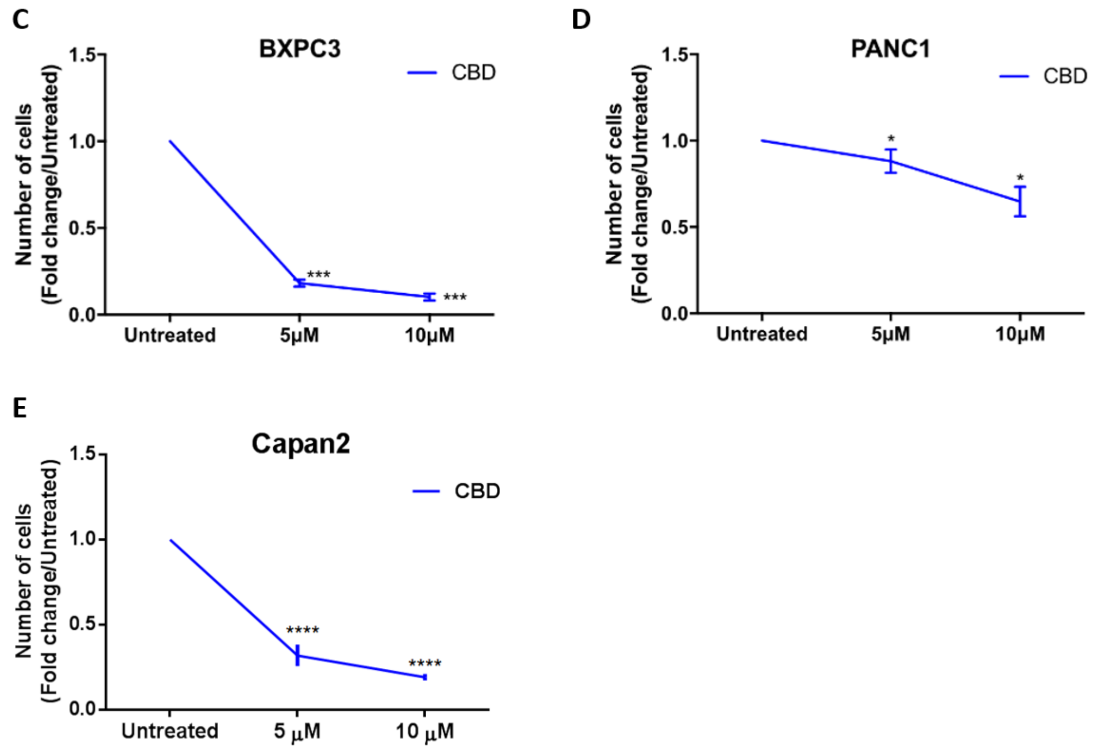


Figure 3.10 ***GPR55 inhibition reduces PDAC cell growth.*** *The effects of pharmacological inhibition of GPR55 with increasing doses of CBD and CID on cell number in PDAC cell lines: AsPC1 (A), HPAFII (B), BxPC3 (C), Panc1 (D) and Capan-2 (E). The results are presented as mean ± SEM of 3 independent experiments; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.* Supplementary Figure 5 in the following publication: R Ferro, **A Adamska**, R Lattanzio, I Mavrommati, CE Edling, SA Arifin, CA Fyffe, G Sala, L Sacchetto, G Chiorino, V De Laurenzi, M Piantelli, OJ Sansom, T Maffucci, Marco Falasca *GPR55 signalling promotes proliferation of pancreatic cancer cells and tumour growth in mice, and its inhibition increases effects of gemcitabine*; Oncogene, 2018 (37); doi: 10.1038/s41388-018-0390-1

Importantly, the treatment of hTERT-HPNE cells, a non-malignant pancreatic cell line, with both inhibitors: CBD and CID at the same doses as used for pancreatic cancer cells, showed no activity or a marginal effect at the highest dose for CBD (10µM; Figure 3.11- A, B).

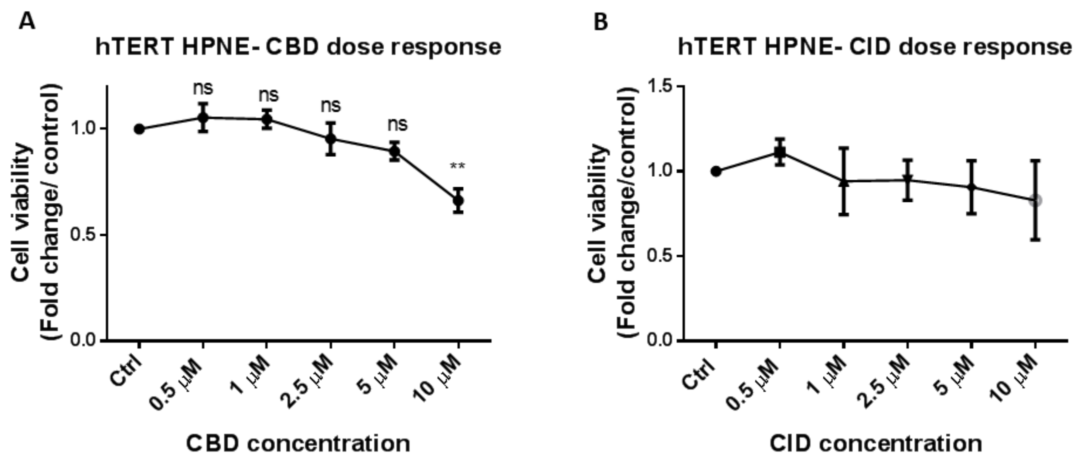


Figure 3.11 ***GPR55 inhibition is specific for PDAC cells.*** *Effects of GPR55 pharmacological inhibition with increasing doses of (A) CBD and (B) CID on the number of non-malignant pancreatic hTERT-HPNE cell. The results are presented as mean ± SEM of 3 independent experiments; **p<0.01*

This little or no effect correlates with the lower expression of GPR55 in these cell lines (Figure 3.2) compared to PDAC cell lines. These data therefore support the specificity of GPR55 inhibition for PDAC cells compared to non-malignant pancreatic cells.

3.3.4.3 Pharmacological inhibition of GPR55 significantly reduces clonal expansion of PDAC cells

To further validate the pharmacological potential of targeting GPR55 and its applicability in *in vivo* studies, the *in vitro* anchorage-independent growth of AsPC1 and HPFAII cells was tested upon treatment with increasing doses of CBD and CID. Cells were grown in agarose gel in the presence of increasing concentrations of each drug and the number of colonies was assessed after 4 weeks. Consistent with genetic downregulation of GPR55 expression and similarly to the cell number assay, a significant reduction in the number of colonies, as well as a decreased size of individual colonies, was detected following the inhibition of GPR55 activity in both tested cell lines. Similar effects were observed for both drugs and cell lines tested (Figure 3.12).

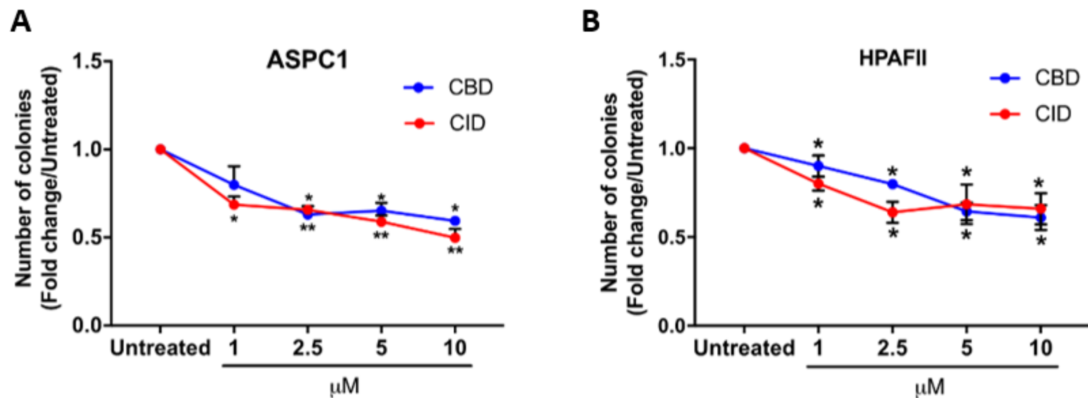


Figure 3.12 ***GPR55 inhibition reduces clonal expansion of PDAC cells.*** *Effects of GPR55 pharmacological inhibition with increasing doses of CBD and CID on colony formation in PDAC cell lines: AsPC1 (A) and HPAFII (B). The results are presented as mean ± SEM of 3 independent experiments; *p<0.05, **p<0.01.*

Figure 5 in the following publication: R Ferro, **A Adamska**, R Lattanzio, I Mavrommati, CE Edling, SA Arifin, CA Fyffe, G Sala, L Sacchetto, G Chiorino, V De Laurenzi, M Piantelli, OJ Sansom, T Maffucci, Marco Falasca *GPR55 signalling promotes proliferation of pancreatic cancer cells and tumour growth in mice, and its inhibition increases effects of gemcitabine*; Oncogene, 2018 (37); doi: 10.1038/s41388-018-0390-1

The similar effects reported with the use of both inhibitors, further confirm specificity of CBD in GPR55 inhibition. These results confirmed the potential of CBD treatment in a more complex environment, in which not only cell proliferation but also their tumorigenic potential and clonal expansion is verified. This *in vitro* validation gave us the rationale for further GPR55 pharmacological testing in *in vivo* models of PDAC.

3.3.4.4 Investigation of the effects of the combination of GPR55 inhibition and chemotherapy

Combination therapies, using targeted therapies with chemotherapy, have attracted attention as a more effective way to counteract cancer progression. Therefore, in order to test the potential of CBD in enhancing the efficacy of chemotherapeutics,

the combination of CBD and gemcitabine, the standard of care for PDAC patients, was evaluated. Both anchorage-dependent and independent cell growth was assessed upon the treatment with different combinations of both drugs. Suboptimal concentration of gemcitabine was chosen for the combination treatments in order to be able to fully elucidate the potential additive effects of drug combinations.

HPAFII cells were treated with 5µM and 10µM CBD and 20 nM gemcitabine separately, as single drugs, and in combination. For the assessment of cell proliferation, cells were manually counted after 72 hours with trypan blue exclusion. A reduction in the number of viable cells was detected following GPR55 inhibition with CBD at both concentrations. 20 nM Gemcitabine, showed a slight impact on cell viability, comparable with CBD treatment (Figure 3.13). However, combination of gemcitabine and CBD significantly improved the inhibitory effects of each drug applied as a single agent, showing almost doubled effectiveness.

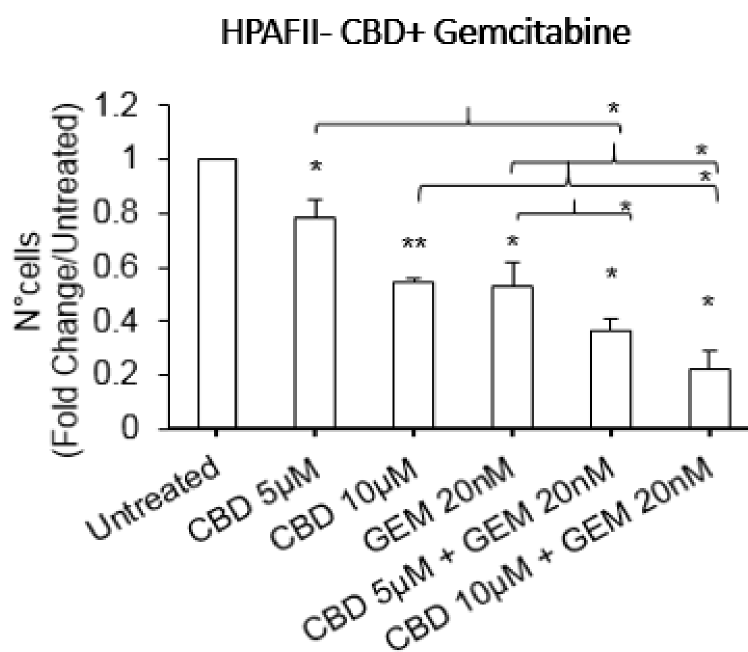


Figure 3.13 ***CBD potentiates the efficacy of chemotherapy in vitro.*** *The effects of the pharmacological inhibition of GPR55 with CBD combined with treatment of HPAFII cell line with chemotherapeutic drug, gemcitabine, on cell number. Results are presented as a mean ± SEM of 3 independent experiments; *p<0.5, **p<0.01. Performed by Dr R.Ferro.* Supplementary Figure *7* in the following publication: R Ferro, **A Adamska**, R Lattanzio, I Mavrommati, CE Edling, SA Arifin, CA Fyffe, G Sala, L Sacchetto, G Chiorino, V De Laurenzi, M

Piantelli, OJ Sansom, T Maffucci, Marco Falasca *GPR55 signalling promotes proliferation of pancreatic cancer cells and tumour growth in mice, and its inhibition increases effects of gemcitabine*; Oncogene, 2018 (37); doi: 10.1038/s41388-018-0390-1

The involvement of GPR55 in the activation of the MAPK pathway in PDAC has been previously demonstrated in our group. The silencing of GPR55 and its pharmacological inhibition significantly decreased the activity of MAPK pathway as shown by analysis of phosphorylated ERK1/2 (Ferro et al. (398), Supplementary Figure 3f, and Figure 4e). Therefore, I investigated the potential of the horizontal inhibition of GPR55 and ERK1/2 with CBD and Trametinib- a specific ERK inhibitor. BxPC3 cell line, the only KRAS wild type pancreatic cell line in the panel of tested cell lines, was chosen for the study. Cells were treated with 2.5µM and 5µM CBD and 1nM Trametinib and cell viability was assessed by manual counting. Trametinib alone significantly reduced the number of viable cells, at a similar level than observed for 5µM CBD treatment. Moreover, the results of the drug combination showed a remarkable decrease in cell viability compared to each drug applied as single agent (Figure 3.14).

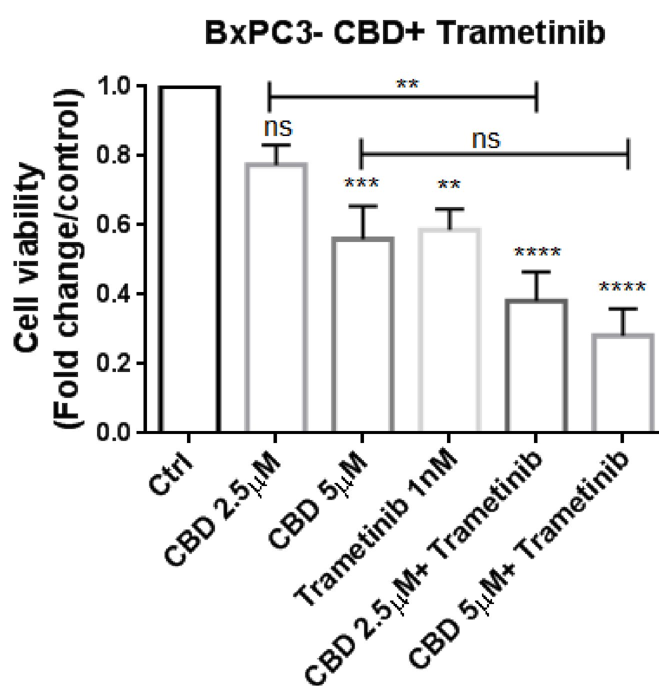


Figure 3.14 ***Vertical inhibition of GPR55-regulated pathways.*** *The effects of dual inhibition of GPR55 and its downstream effector ERK1/2, with CBD and Trametinib respectively, on*

*BxPC-3 cell number. Results are presented as mean ± SEM of 3 independent experiments; **p<0.01, ***p<0.001, ****p<0.0001*

These data show the potential for the horizontal inhibition of GPR55 and the proteins belonging to the same pathway to increase the effectiveness of targeted therapies. Nevertheless, further studies are necessary to explore the potential of this and other combination therapies involving GPR55 inhibition in PDAC therapy.

3.3.4.5 In vivo validation of the effects of pharmacological inhibition of GPR55

Following the confirmation of the importance of GPR55 in PDAC progression in an *in vivo* model and the *in vitro* validation of the pharmacological blockade of GPR55 on PDAC cell growth, the pharmacological potential of GPR55 was sought *in vivo*. A genetically engineered mouse model for pancreatic cancer, the KPC mouse model, which resembles the histopathology and characteristics of human disease, was used for the studies. Dr Riccardo Ferro, at Queen Mary University of London, UK, performed the experiments. KPC mice were treated with vehicle, a synthetic CBD (100mg/kg), Gemcitabine (100 mg/kg) and the combination of the two treatments. All the treatments were administered by intraperitoneal (IP) injection. The animals were euthanized when visible signs of pain and distress were noted. CBD applied as a single agent, slightly prolonged survival of the mice, compared to the mice treated with vehicle. Interestingly, the efficacy of CBD was comparable with gemcitabine, standard-of-care chemotherapy, in terms of the increase in mice survival. However, none of the drugs significantly prolonged mice lifespan. These results mirror the effects obtained in human therapy, in which gemcitabine only marginally improves the overall survival. Importantly, combination of chemotherapy (gemcitabine) with CBD injections resulted in the significant and outstanding increase in the survival, of almost three times compared to untreated mice (Ferro R. Adamska A. et al (398), Figure 5c). These promising data gave basis for further exploration of CBD-based therapies for PDAC treatment and commencement of phase I clinical trials.

3.3.5 Downregulation and pharmacological inhibition of GPR55 induces autophagy in PDAC cells

Pancreatic cancer cells are characterized by elevated growth rates and often depend on the host for the supply of their increased proliferation needs. Cancer cells are frequently exposed to hypoxic conditions and need to adapt their metabolism to obtain energy and survive in the oxygen and glucose-depleted environment. Macroautophagy, generally called autophagy, is a cellular mechanism that relies on self-degradation induced by nutrient deprivation (400).

Autophagy is a highly conserved, multi-step process that consists on engulfing of the cytoplasmic content, formation of the autophagosome, fusion of the autophagosome with lysosome and degradation of the autolysosome content by lysosomal hydrolases (401). LC3 (microtubule-associated protein 1 light chain 3) is a key component in autophagy and is considered as one of the main markers of autophagy. During autophagy, a soluble cytosolic LC3 (LC3-I) is converted to LC3-II (lipidated, membrane-bound form of LC3), which localized on autophagosome is degraded together with the cytoplasmic content. Thus lysosomal turnover of LC3-II, LC3-phosphatidylethanolamine conjugate recruited to autophagosomal membranes, reflects induced autophagic activity (402). Importantly, correlation between LC3 expression and poor outcome and short disease-free period has been demonstrated for PDAC patients. On the other hand, abundant expression of another protein, p62, delays the delivery of substrate to proteasome, influencing autophagic degradation. Therefore, increased levels of p62 lead to decreased levels of autophagy, while the reduced p62 expression supports autophagy induction.

It is known that in PDAC basal levels of autophagy are increased in order to aid quickly proliferating PDAC cells to cope with their increased energetic needs. However, a conflicting impact of autophagy in PDAC has been argued having both: tumour-promoting and suppressing roles. The majority of the studies demonstrated that genetic or pharmacological inhibition of autophagy in PDAC leads to considerable reduction in PDAC cell growth and tumour regression in mouse models, mediated mainly by changes in cell metabolism and decrease in oxidative phosphorylation

(403). On the other hand, it has been proposed that excessive levels of autophagy may promote cell death, designated as type II programmed cell death (404). Therefore, it has been suggested that, depending on its levels, autophagy exhibits anti-tumour effects either through cell death (low or high levels) or through cell death-independent tumour suppressing mechanisms. In addition, PDAC cells might activate autophagy in response to chemotherapy, mitigating cellular damage, which may lead to the slow-down of carcinogenesis. Therefore, both autophagy-promoting and autophagy-limiting strategies have been considered for targeted PDAC therapies. Therapy with autophagy inhibitor, hydroxychloroquine, was explored as a potential anti-tumorigenic agent and its clinical application is evaluated in several clinical studies; however, minimal activity has been observed so far when applied as monotherapy (405, 406).

In recent years, cannabinoid-based therapies have attracted attention as potential anti-tumorigenic agents. Interestingly, it was shown that different ligands of cannabinoid receptors CB1 and CB2 inhibit cell growth and induce autophagy in PDAC cell lines through ROS production, leading to autophagic cell death (407). Interestingly, similar mechanism was shown for gemcitabine and its combination with cannabinoid compounds potentiated the observed effects synergistically increasing autophagy levels and reducing PDAC cell proliferation and, more importantly, significantly decreasing tumour growth in the xenograft mouse model of PDAC (407). However, no studies so far explored the potential involvement of GPR55 and its inhibition in the regulation of autophagy in pancreatic cancer. Therefore, I aimed to investigate, whether observed increase in the survival of the KPC mice treated with cannabidiol (CBD) and gemcitabine combination might be due to induction of autophagy.

3.3.5.1 Transient knockdown of GPR55 alters expression of LC3II and p62 proteins in HPAFII PDAC cell line

To test the involvement of GPR55 in autophagy in PDAC the receptor was either transiently silenced with siRNA or pharmacologically blocked with CBD. After 72h of treatments, cells were collected and the expression of p62 and LC3 was analysed by

Western blotting. A clear and statistically significant decrease in expression of p62 following the transient knockdown of GPR55 in HPAFII cell line could be observed, suggesting increased levels of autophagy (siGPR55-1 p=0.0016; siGPR55-2 p=0.0038). At the same time, a distinctive increase in LC3II activity was observed (siGPR55-1 p=0.0408; siGPR55-2 p=0.0076) (Figure 3.15). Importantly, the detected changes in p62 and LC3II expression were consistent after GPR55 knockdown with both sequences.

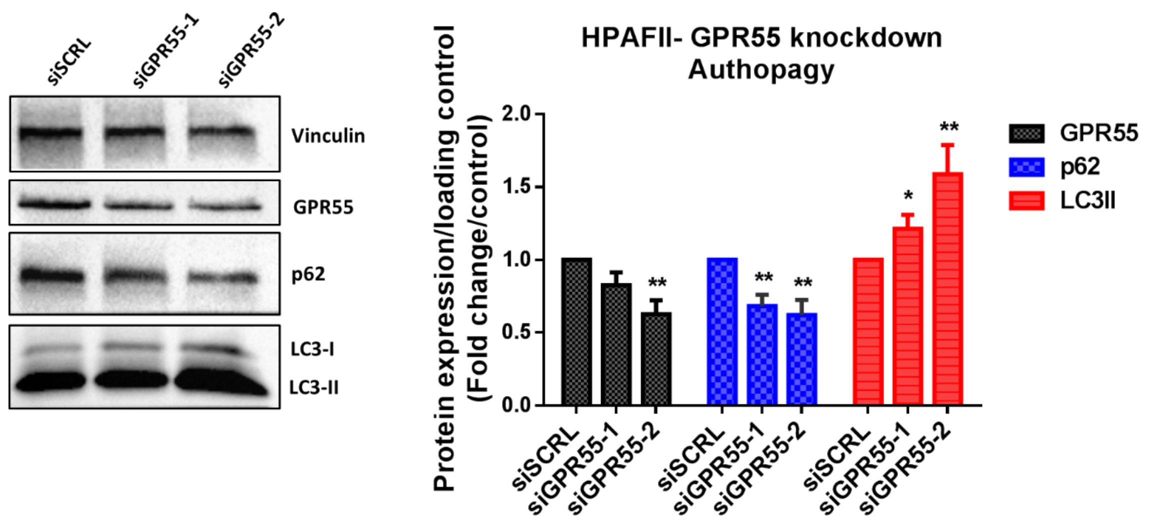


Figure 3.15 ***GPR55 silencing induces autophagy in PDAC cells.*** *Effects of GPR55 silencing in HPAFII cell line on the expression of the markers of autophagy p62 and LC3. The quantitative analysis is presented as mean± SEM of 6 independent experiments; *p<0.05, **p<0.01*

3.3.5.2 Reintroduction of miR-34b alters expression of LC3II and p62 proteins in AsPC1 and HPAFII PDAC cell lines

It has been demonstrated that PDAC cell lines are characterized by decreased levels of microRNAs belonging to miR-34 family. In chapter 3.3.3.2 I have shown that the functional form of miR-34b represses GPR55 expression. I demonstrated that the reintroduction of miR-34b into HPAFII and AsPC1 cell lines resulted in the impairment of GPR55 expression.

Consequently with the results obtained after GPR55 knockdown, the reintroduction of miR-34b into HPAFII (Figure 3.16-A) and AsPC1 (Figure 3.16-B) cell lines, which was shown to downregulate GPR55 expression, resulted in the induction of autophagy as shown by the altered expression of both p62 (decreased) and LC3-II (increased). These results demonstrate that the silencing of GPR55, either directly, using transient siRNA transfection, or indirectly, caused by miR-34b- mediated blockage of GPR55 expression, results in the increase in autophagy levels. We may speculate that silencing of GPR55 expression causes a cellular stress, which in turn activates autophagy in the knockdown cells as a survival mechanism in the stress environment.

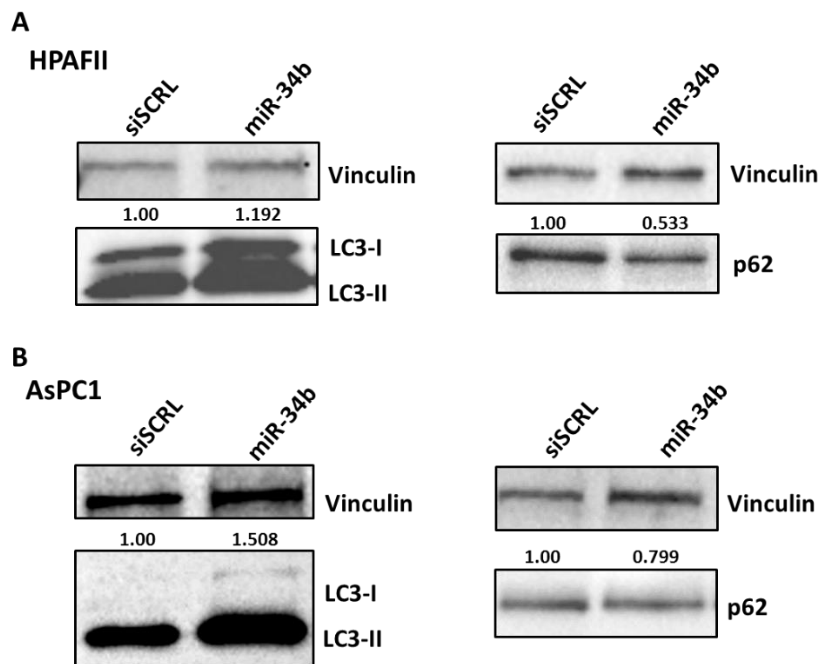


Figure 3.16 ***Indirect GPR55 silencing induces autophagy in PDAC cells.*** *Effects of the indirect downregulation of GPR55 through reintroduction of miR34b on the expression of autophagy markers LC3II and p62 in HPAFII (A) and AsPC1 (B) cells. The quantitative analysis is presented as mean ± SEM of 3 (LC3) and 8 (p62) independent experiments for HPAFII cell line and 2 independent experiments for AsPC1 cell line; *p<0.05, **p<0.01, ****p<0.0001*

3.3.5.3 Pharmacological inhibition of GPR55 induces autophagy in PDAC cell lines

Importantly, similar effects could be detected in PDAC cells following pharmacological inhibition of GPR55 activity with CBD. Treatment of several pancreatic cancer cell lines (AsPC1, CFPAC-1 and HPAFII) with CBD remarkably increased LC3II expression, indicating induction of autophagy (Figure 3.17).

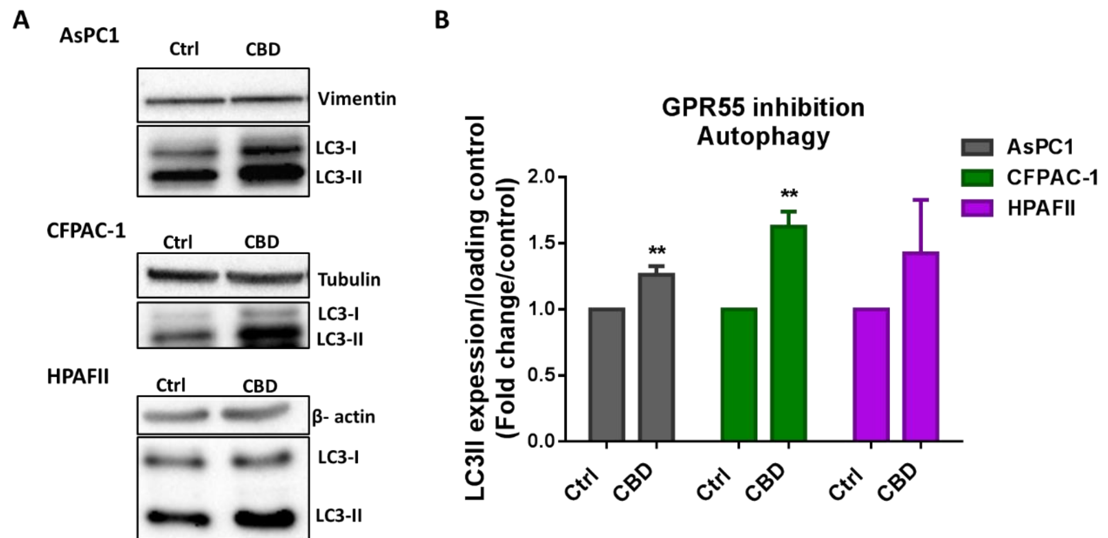


Figure 3.17 ***GPR55 inhibition induces autophagy in PDAC cells.*** *Representative Western blot image (A) and quantitative analysis (B) of the effect of the pharmacological inhibition of GPR55 with CBD on the expression of autophagy marker LC3II in AsPC1, CFPAC-1 and HPAFII cell lines. The quantitative analysis is presented as mean± SEM of 4 (AsPC1, CFPAC-1) or 3 (HPAFII) independent experiments; *p<0.05, **p<0.01*

Taken together, these data suggest that the blockage of GPR55 activity in PDAC cell lines increases the cellular stress and induces autophagy in PDAC cells.

3.3.6 Pharmacological inhibition of GPR55 may block Epithelial to Mesenchymal Transition

One of the main reasons for the high mortality of pancreatic cancer patients is the fast and early metastatic spread of the disease to distant organs, mainly liver, spleen and lungs. One of the events necessary for the tumour cell dissemination is the epithelial to mesenchymal transition (EMT), which allows the cells to change their phenotype to mesenchymal, which is characterized by higher mobility, migratory capacity and invasiveness. Therefore, the cells can migrate from the epithelial tissue from which they originated to distant metastatic sites. Thus, management of the EMT process in PDAC and development of therapies blocking activation of EMT regulators should be investigated. It has been previously demonstrated that in some cancer types, CBD treatment inhibited the expression of EMT transcription factors (348), suggesting its possible anti-metastatic effects. Since high efficacy of CBD treatment in prolonging survival of the KPC mice was demonstrated, I investigated the potential effects of CBD in EMT regulation in PDAC.

To verify the possible involvement of GPR55 in EMT of pancreatic cancer cells, the effects of pharmacological inhibition of the receptor with CBD on the regulation of mesenchymal proteins in PDAC cells were verified. In parallel, the effects of chemotherapy (gemcitabine treatment) on EMT regulation was also investigated. A panel of pancreatic cancer cell lines (AsPC1, HPAFII, CFPAC-1 and BxPC3) was used for this experiment. Cells were seeded in a 6 well plate at a density of 2.5×10^5 cells/well and treated with 5µM, 10µM CBD and 20nM gemcitabine. Cells were collected 48 hours post treatment and the expression of mesenchymal markers (Snail, Slug) was verified by Western blotting.

The preliminary data showed the CBD-mediated changes in the expression of both tested markers. However, no consistent results could be obtained across the tested cell lines in terms of changes in the expression of EMT markers (Figure 3.18). No considerable effects could be detected in the AsPC1 cell line, following CBD treatment. In fact, a slight increase in the expression of both markers could be observed. Similarly, treatment of AsPC1 cell line with 20nM gemcitabine did not

cause substantial or consistent changes in Snail or Slug expression. On the other hand, treatment of HPAFII and CFPAC-1 cell lines with both concentrations of CBD remarkably lowered expression of Snail and Slug proteins, while no effect on protein expression could be observed in the gemcitabine-treated samples. Similarly, 5µM CBD treatment decreased the levels of both proteins in BxPC3 cell line. However, no effects were apparent for 10µM CBD nor Gemcitabine.

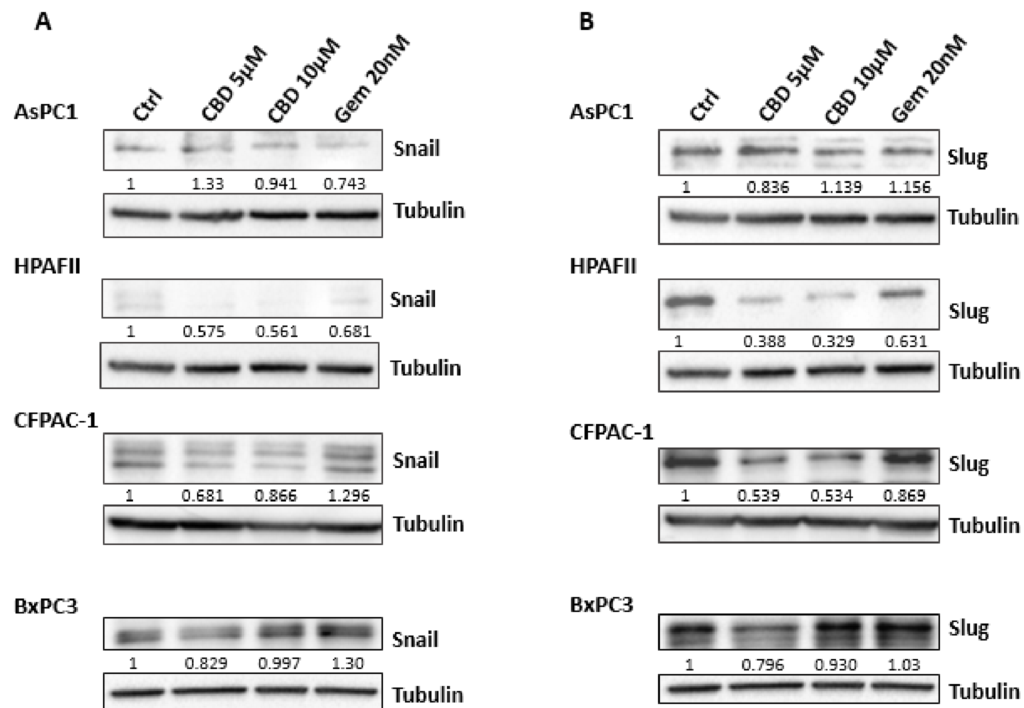


Figure 3.18 ***GPR55 inhibition influences the expression of mesenchymal markers in PDAC cells.*** *The representative Western blot images showing the effects of treatment of AsPC1, HPAFII, CFPAC-1 and BxPC3 cell line with 5µM CBD, 10µM CBD and 20nM gemcitabine on the expression of two mesenchymal markers: Snail (A) and Slug (B). Quantification of N=2 independent experiments is presented.*

Although preliminary and not entirely conclusive, the presented data suggest the downregulation of expression of mesenchymal markers Snail and Slug following pharmaceutical inhibition of GPR55 with Cannabidiol. The difference in the response of AsPC1 cell line to the CBD treatment may be explained by the high metastatic potential and chemoresistance of this cell line, compared to other tested cell lines. Thus, mechanisms may exist in AsPC1 cells that prevent the downregulation of EMT,

causing their more aggressive metastatic phenotype. Further studies are required for a full elucidation of the effects of CBD treatment on EMT in pancreatic cancer.

3.4 Discussion and future aims

In this project, I validated the G protein coupled receptor GPR55 as a novel pharmacological target in pancreatic cancer.

The involvement of GPR55 in the progression and aggressiveness of several human cancer types has been previously documented. In this study, I showed that GPR55 is overexpressed in a panel of human and murine PDAC samples compared to non-malignant pancreatic cells and tissues. Subsequently, the key role played by GPR55 in the progression of PDAC was shown. The impact of the presence of a fully active receptor on the proliferation and clonal expansion of PDAC cells was shown *in vitro*. More importantly, the influence of GPR55 expression on survival of a very aggressive transgenic mouse model of pancreatic cancer (KPC mouse model) was demonstrated, underlining the importance of GPR55 in PDAC progression. In addition, I demonstrated the regulation of GPR55 expression by mutated *TP53*, one of the main tumour suppressors altered in pancreatic cancer. Importantly, defined mechanism provides a basis for the selection of cohort of patients that could benefit from GPR55 targeted therapies.

Consequently, pharmacological potential of GPR55 in PDAC was explored and the specificity of the non-psychoactive cannabinoid, cannabidiol (CBD), was demonstrated towards GPR55. CBD has been proven as a potent anti-carcinogenic agent in several cancer types. The efficiency of CBD in slowing down cell proliferation and tumour growth has been previously proven in breast cancer. Interestingly, a decrease in invasion and metastasis of breast cancer cells has been also demonstrated. Moreover, the effectiveness of CBD was demonstrated in glioblastoma, lung and colon cancer, although in none of these studies the mechanism of action of CBD was shown. We showed that inhibition of GPR55 by CBD significantly reduced PDAC cell growth and clonal expansion, similarly to the effects obtained with its silencing. Importantly, the specificity of CBD in PDAC cells was indicated, as no significant effects could be noted in non-malignant pancreatic cell lines.

In vivo testing of the effectiveness of CBD in counteracting PDAC progression was performed in the transgenic KPC model of pancreatic cancer. In this model, driven by *KRAS* and *TP53* mutations under pancreatic specific Cre promoter, tumour development occurs spontaneously, with a pathophysiology closely mimicking the human disease. Thus, the KPC model represents an ideal platform for the validation of potential anticancer therapies. We could demonstrate that treatment of KPC mice with CBD slightly, although not statistically improved the survival of the mice, similarly to chemotherapy treatment with FDA-approved gemcitabine. This data stays in agreement with the marginal effects of gemcitabine reported in PDAC patients' therapy. Strikingly, the combination treatment of the mice with both drugs, increased mice survival almost three times compared to untreated mice. Therefore, the pharmacological potential of GPR55 targeting in combination with chemotherapy represents a promising therapeutic approach in PDAC treatment that should be explored clinically. Based on my data, I might speculate on the potential mechanisms standing behind observed efficacy of CBD/gemcitabine combination therapy in PDAC.

Primarily, upregulation of autophagy following GPR55 downregulation was proposed. Several studies documented the dependence of autophagy on the status of two main genetic alterations occurring in PDAC, oncogenic *KRAS* and tumour suppressor *TP53*. Elevated autophagy levels were correlated with accumulating mutations in TP53 and different functions of autophagy were defined depending on TP53 status (408). Upregulation of autophagy has been also observed in KRAS-mutated PDAC (409). Therefore, therapies targeting KRAS-induced pathways have been one of the main priorities in PDAC research. Interestingly, a recent study has shown that blocking of KRAS signalling through inhibition of ERK induced autophagy in PDAC cells (410). The study also indicated the impairment of metabolic processes including glycolysis or mitochondrial function upon ERK inhibition, suggesting that these cells might show increased dependence on autophagy and higher sensitivity of the cells to autophagy inhibition (410). Therefore, a strategy of combining KRAS and autophagy inhibition might be a novel and potent strategy in slowing-down PDAC progression (411). Our data validated that inhibition of GPR55 with cannabidiol (CBD) considerably increased autophagy in several PDAC cell lines. We have also previously shown that GPR55

regulates the activity of MAPK signalling in PDAC. Both, silencing of GPR55 and its pharmacological inhibition resulted in significant reduction in pERK1/2 levels both *in vitro* and in the transgenic mouse model of PDAC. Therefore, our data stand in agreement with reported induction of autophagy upon KRAS pathway disruption. Interestingly, I showed that the vertical inhibition of KRAS signalling with the combination of CBD and ERK inhibitor, Trametinib, potentiated the effectiveness of each drug alone, highly inhibiting proliferation of BXPC3 cell line (Figure 3.15). We may speculate that the vertical inhibition of KRAS pathway may increase the levels of autophagic death in these cells.

In addition, in this project, we demonstrated that GPR55-mediated reduction of PDAC cell proliferation and disease progression is not dependent on apoptosis. No increase in activity of caspase 3 or Annexin V could be detected in the samples with GPR55 knockdown or inhibition with CBD. Thus, we hypothesize that the observed effects might be due to the increased autophagy leading to induction of type II cell death. In agreement with that, previous studies have shown that cannabinoids, when combined with gemcitabine, prevented chemotherapy- induced apoptosis (407). It was speculated that elevated levels of autophagy, induced by the cannabinoids treatment, inhibited development of apoptosis (412). On the other hand, inhibition of autophagy was shown to promote apoptosis (413).

It has been also previously demonstrated in pancreatic cancer that antagonists of CB_1 (ACPA) and CB_2 (GW405833) increase chemosensitivity of the cells by induction of autophagy (407). My data, confirming the induction of autophagy in CBD treated samples, combined with our results showing striking efficacy of CBD/GEM combination in KPC transgenic model of PDAC might support these findings. I might speculate that the substantial increase in the survival of KPC mice treated with a combination of Cannabidiol and gemcitabine may be partially explained by the CBD-mediated sensitization of the tumours to gemcitabine therapy. In addition, the effects of CBD in reduction of chemoresistance has been shown in several cancers, supporting this hypothesis.

All these data support the hypothesis that treatment of PDAC cells and mouse models with GPR55 inhibitor, CBD, reduces PDAC cell proliferation and disease progression

through induction of autophagy. Moreover, I showed for the first time that the mechanisms of autophagic death induced in PDAC cells via cannabinoids treatment might involve inhibition of GPR55. It is still to be determined whether CBD treatment reduces resistance of PDAC cells to gemcitabine treatment or potentiates gemcitabine-induced autophagic death in these cells. In addition, ROS activity should be investigated in PDAC cells to determine if CBD-induced autophagy is mediated by increase of ROS, as reported in other studies. Interestingly, it has been suggested that combination of ERK and autophagy inhibition may remarkably decrease growth of the tumours in the xenograft mouse models. We therefore propose that combination of CBD treatment with autophagy inhibition could potentiate the efficacy of CBD alone. Additionally, the effects of the combination of CBD and gemcitabine, which we showed to be a potent therapeutic strategy in the transgenic model of PDAC, could be additionally enhanced by combining with hydroxychloroquine.

Although only preliminary, the altered expression of the Epithelial to Mesenchymal (EMT) markers following CBD treatment was also shown in several PDAC cell lines. In particular, decreased levels of Snail and Slug proteins, markers for mesenchymal cell phenotype, was reported in HPAFII and CFPAC-1 cell lines, while AsPC1 cells, characterized by high metastatic potential, did not seem to be responsive to GPR55 inhibition in terms of EMT alteration. The reduction in metastatic spread following CBD treatment has been demonstrated in few cancer types. My results may indicate that inhibition of GPR55 may contribute to decreased metastatic spread of the PDAC cells by blocking the cells in the epithelial, less invasive phenotype. This, in consequence, could contribute to prolonged survival of the KPC mice treated with CBD and gemcitabine combination. I might speculate that the observed in vivo effects could be due to reduction of primary tumours growth caused by gemcitabine treatment and slow-down in metastatic spread attributed to CBD. However, more in depth analysis of other mesenchymal markers, as well as potential increase in epithelial markers should be carried out. Migration and invasion assays should be also performed to verify the metastatic potential of the PDAC cells with or without active GPR55.

3.5 Whole publication

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ARTICLE

GPR55 signalling promotes proliferation of pancreatic cancer cells and tumour growth in mice, and its inhibition increases effects of gemcitabine

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Abstract

The life expectancy for pancreatic cancer patients has seen no substantial changes in the last 40 years as very few and mostly just palliative treatments are available. As the five years survival rate remains around 5%, the identification of novel pharmacological targets and development of new therapeutic strategies are urgently needed. Here we demonstrate that inhibition of the G protein-coupled receptor GPR55, using genetic and pharmacological approaches, reduces pancreatic cancer cell growth in vitro and in vivo and we propose that this may represent a novel strategy to inhibit pancreatic ductal adenocarcinoma (PDAC) progression. Specifically, we show that genetic ablation of *Gpr55* in the $KRAS^{WT/G12D}/TP53^{WT/R172H}/Pdx1\text{-}Cre^{+/+}$ (KPC) mouse model of PDAC significantly prolonged survival. Importantly, KPC mice treated with a combination of the GPR55 antagonist Cannabidiol (CBD) and gemcitabine (GEM, one of the most used drugs to treat PDAC), survived nearly three times longer compared to mice treated with vehicle or GEM alone. Mechanistically, knockdown or pharmacologic inhibition of GPR55 reduced anchorage-dependent and independent growth, cell cycle progression, activation of mitogen-activated protein kinase (MAPK) signalling and protein levels of ribonucleotide reductases in PDAC cells. Consistent with this, genetic ablation of *Gpr55* reduced proliferation of tumour cells, MAPK signalling and ribonucleotide reductase M1 levels in KPC mice. Combination of CBD and GEM inhibited tumour cell proliferation in KPC mice and it opposed mechanisms involved in development of resistance to GEM in vitro and in vivo. Finally, we demonstrate that the tumour suppressor p53 regulates GPR55 protein expression through modulation of the microRNA miR34b-3p. Our results demonstrate the important role played by GPR55 downstream of p53 in PDAC progression. Moreover our data indicate that combination of CBD and GEM, both currently approved for medical use, might be tested in clinical trials as a novel promising treatment to improve PDAC patients' outcome.

Introduction

The progression from normal duct epithelium to infiltrating pancreatic ductal adenocarcinoma (PDAC) involves development of a characteristic pattern of precursors named pancreatic intraepithelial neoplasias (PanIN), histologically classified into distinct stages (PanIN 1a, PanIN 1b, PanIN 2,

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and PanIN 3) which eventually develop into PDAC, further classified in five distinct stages [1]. The genetic alterations associated with this process have been extensively characterised and they involve activating mutations of oncogenes, inactivating mutations of tumour suppressors as well as increased copy numbers of receptors [2]. For instance it is now well established that activating mutations of the oncogene *KRas* occur early during PanINs development and are detected in 85–90% of PDAC tumours [3]. On the other hand inactivating mutations of the tumour suppressor *TP53* occur in 70% of the latest stages of PanIN progression [4]. Transgenic mouse models have shed much light into the role of these specific mutations during PDAC development. The $KRAS^{WT/G12D}$/Pdx1-$Cre^{+/+}$ (KC) model which expresses a constitutively active KRas selectively in the pancreas is able to reproduce the PanIN lineage with a 100% penetrance but only few mice actually develop PDAC [5]. On the contrary, the transgenic $KRAS^{WT/G12D}$/$TP53^{WT/R172H}$/Pdx1-$Cre^{+/+}$ (KPC) mice which additionally bear the *TP53* inactivating mutation develop the full PanIN range and PDAC with pathology very similar to human PDAC [6]. Therefore these transgenic models point to a key role for activated KRas in the early stage of neoplasias/cancer development and a central role for loss/inactivation of p53 in driving progression from the final PanINs stages to full PDAC.

In the last years our understanding of the genetic causes of PDAC has greatly increased but sadly this has not resulted in significant improvement of treatment options for patients. Surgical resection can lead to long-term survival and provides effective palliation but it is only applicable to patients with stage I and II PDAC. Chemotherapy and radiation therapy following the resection reduce metastatic development but these treatments result in little improvement of patient survival. Until very recently, Gemcitabine (GEM) was the only FDA-approved treatment for primary PDAC, but in most cases it can only prolong survival by several weeks [7]. Some combinations of drugs have proven slightly more successful although they still effectively increase patients' survival by merely 2–4 months compared to GEM treatment [8, 9]. Several clinical trials are ongoing but currently PDAC remains one of the most aggressive cancers with a one year survival rate of 19% and five years survival rate of 5% [10]. Identification of novel pharmacological targets and development of new therapeutic strategies are urgently needed [11].

Here we investigated the therapeutic potential of targeting the G protein-coupled receptor GPR55 in PDAC. GPR55 was identified as the receptor for the phospholipid lysophosphatidylinositol (LPI) [12]. Increasing evidence now suggests that GPR55 plays an important role in many cancer types [11]. Whether targeting the receptor could ultimately result in improvement of survival and whether this strategy could represent a genuine novel therapeutic approach remains to be determined. Indeed, no study so far has investigated whether inhibition of GPR55 could improve survival of transgenic models that closely mirror the human disease.

Using genetic and pharmacological approaches we demonstrate that GPR55 has a central role in PDAC progression driven by *TP53* mutations. Furthermore, we show that inhibition of this receptor, especially in combination with GEM, reduces cancer progression and significantly improves survival in a transgenic mouse model of PDAC. These data provide the first evidence that inhibition of GPR55 represents a novel therapeutic strategy which can counteract PDAC progression and improve survival rate.

Results

Genetic disruption of Gpr55 inhibits pancreatic cancer proliferation in vivo and it improves survival in a PDAC mouse model

Immunohistochemistry (IHC) analysis of human normal pancreatic and PDAC specimens showed that GPR55 immunoreactivity was confined to the islets of Langerhans (Fig. 1a) in normal pancreatic tissues, as previously reported [13], while acinar cells and ducts were consistently negative (Fig. 1a). On the other hand, GPR55 was expressed in 14 out of 54 human PDAC specimens (25.9%), indicating an accumulation of GPR55 in cancer tissues. Consistently, GPR55 was detected in PDAC specimens derived from implantation of patient-derived pancreatic cancer cells (patient-derived xenografts, PDX, Fig. 1a) and in a panel of PDAC cell lines (Supplementary Figure 1a).

To determine the role of GPR55 in PDAC, KPC mice were crossed with mice harbouring homozygous deletion of *Gpr55* ($GPR55^{-/-}$) [14] to obtain the "KPCG" strain. Consistent with results from human tissues, IHC analysis indicated that GPR55 was specifically expressed by cells of the islets of Langerhans in Pdx1-$Cre^{+/+}$ and KPC mice, but not in KPCG mice (Supplementary Figure 1b). Moreover, expression of GPR55 was detected in PDAC cells from KPC but not KPCG mice (Supplementary Figure 1b), confirming the specificity of the anti-GPR55 antibody. Strikingly, genetic disruption of *Gpr55* significantly improved survival (Fig. 1b). Specifically, the median survival was 32.5 days longer in KPCG mice ($n = 18$) than in KPC mice ($n = 21$). IHC analysis of corresponding dissected tumours indicated that GPR55 disruption reduced expression of the proliferative index Ki67 in the epithelial cells, specifically during the PanIN 2 and PanIN 3 progression stages (Fig. 1c), indicating a role for GPR55 in pancreatic cancer cell proliferation.

These data demonstrate that GPR55 is crucial for PDAC development and/or progression in vivo.

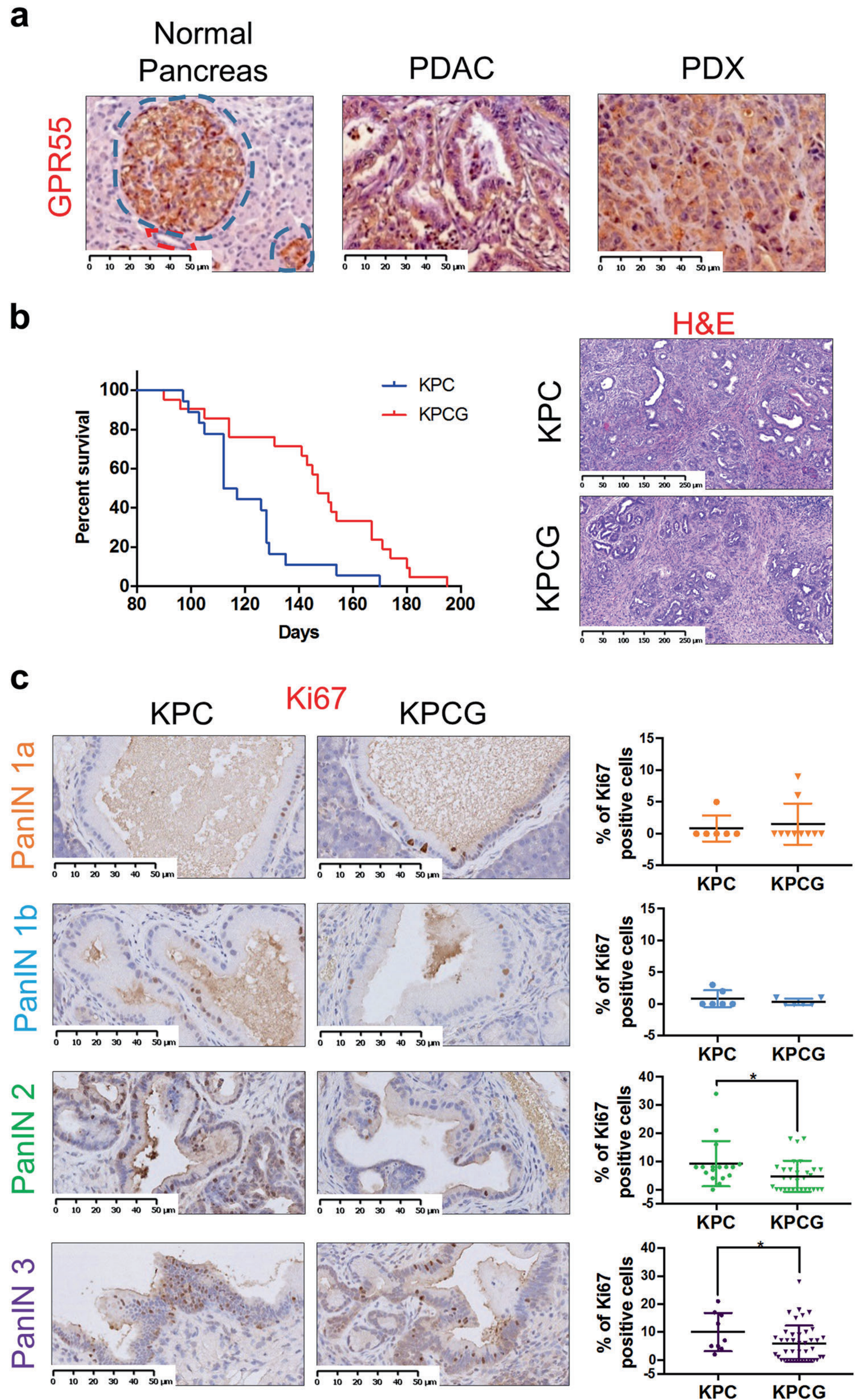


Fig. 1 Genetic disruption of *Gpr55* reduces PDAC growth in vivo. **a** Representative images of GPR55 protein expression in human normal pancreas, PDAC, and PDX tissues assessed by IHC. Scale bar: 50 µm. Blue dotted lines indicate Islets of Langerhans, red dotted line indicates normal pancreatic duct. **b** Kaplan–Meier survival curves of KPC ($n = 18$) and KPCG ($n = 21$) mice. Logrank (Mantel-Cox) test $p = 0.0013$, Gehan-Breslow-Wilcoxon test $p = 0.0032$. Representative H&E staining of tissues from KPC and KPCG mice confirms presence of PDAC. Scale bar: 250 µm. **c** Representative images of Ki67 protein expression in tissues from KPC and KPCG at the indicated stages of PanIN progression. Scale bar: 50 µm. Graphs indicate the percentage of PanIN cells showing Ki67 staining. $^{*}p < 0.05$

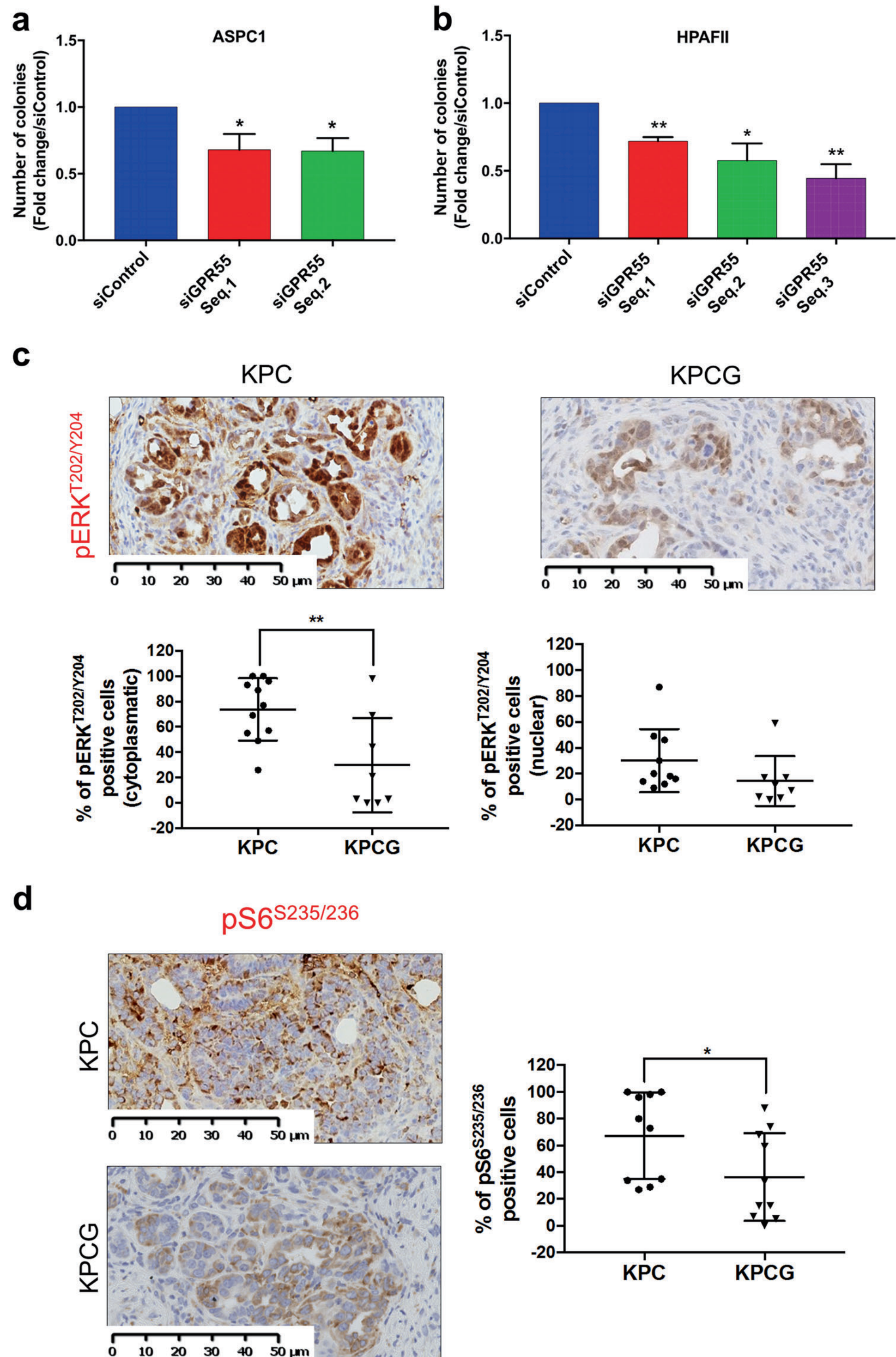


Fig. 2 In vitro and in vivo effects of GPR55 downregulation. **a, b** The indicated PDAC cell lines were transfected with a non-targeting siRNA (siControl) or siRNAs specifically targeting GPR55 and plated on soft agar as described in the Materials and Methods. Colonies were allowed to grow for 3–4 weeks. Data are means ± s.e.m. of $n = 3$ independent experiments performed in duplicate. $*p < 0.05$, $**p < 0.01$ vs siControl. **c, d** Representative images of PDAC specimens from KPC and KPCG mice stained with antibodies recognising phosphorylated $ERK^{T202/Y204}$ (**c**) and $S6^{S235/236}$ (**d**). Scale bar: 50 µm. Graphs indicate the percentage of PDAC cells showing positive staining for each antibody. $*p < 0.05$

GPR55 regulates cell cycle progression and MAPK signalling pathway

Consistent with the in vivo data, siRNAs-mediated downregulation of GPR55 in PDAC cell lines significantly reduced cell proliferation (Supplementary Figures 2a and c and e) and anchorage-independent growth (Fig. 2a, b). Efficient downregulation of GPR55 was confirmed by RT-qPCR (Supplementary Figures 2b and d and f). The inhibition of cell growth was mainly due to an effect on cell cycle progression as GPR55 downregulation significantly blocked the cell cycle at the G1/S transition phase (Supplementary Figures 3a and b) and reduced the mRNA levels of cyclins involved in regulation of the G1/S transition

phase (including cyclin D1 and cyclin D2) without affecting mRNA levels of cyclin B1, which is involved in the G2/M transition (Supplementary Figure 3c). No increase in apoptosis was detected in PDAC cells upon GPR55 downregulation as assessed by Caspase 3 activity (Supplementary Figure 3d) or Annexin V/FACS (Supplementary Figure 3e) assays. These data demonstrate that GPR55 plays a specific role in PDAC cell proliferation/growth.

To further investigate the mechanism involved in cell growth and cell cycle regulation, the signalling pathways downstream of GPR55 were investigated in PDAC cells. As shown in Supplementary Figure 3f, phosphorylation of ERK1/2 at residues Threonine 202 and Tyrosine 204 was reduced in HPAFII cells transiently transfected with specific siRNAs targeting GPR55 compared to cells transfected with a non-targeting siRNA ("siControl") or incubated with transfection reagent alone ("untreated"). GPR55 downregulation further inhibited phosphorylation of S6 at its residues Serine 235/236 (Supplementary Figure 3f), which can be regulated downstream of the MAPK/ERK signalling pathway [15]. Efficient downregulation of GPR55 was confirmed by Western blot (Supplementary Figure 3f). No effect on the total levels of ERK and S6 was detected upon downregulation of GPR55 (Supplementary Figure 3f). Consistently, IHC analysis revealed a decrease in both ERK1/2 and S6 phosphorylation in tumour specimens from KPCG mice compared to KPC mice (Fig. 2c, d).

These data indicate that one of the mechanisms by which GPR55 controls pancreatic cancer cell growth may be through regulation of the MAPK/ERK signalling pathway.

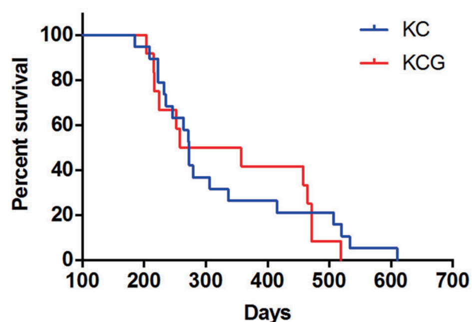
p53 regulates GPR55 protein expression through modulation of miR34b-3p levels

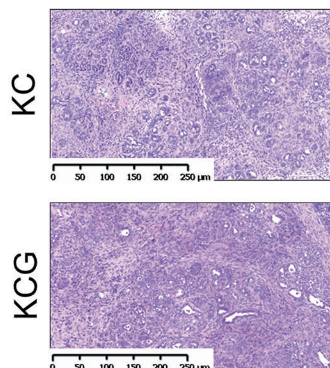
To assess further the specific role of GPR55 during PDAC development/progression we crossed GPR55$^{-/-}$ mice with KC mice, which do not harbor the *TP53* mutation. No statistical differences were found in the survival of KC ($n = 19$) compared to GPR55$^{-/-}$/KRAS$^{WT/G12D}$/Pdx1-Cre$^{+/+}$ (KCG, $n = 12$) mice, suggesting a role for the tumour suppressor p53 in the regulation of GPR55. To investigate this hypothesis, GPR55 protein expression was analysed in murine PDAC cell lines established from different transgenic mouse models. Results in Supplementary Figure 4a suggest that GPR55 protein expression is negatively associated with TP53 status, as the protein appears to be less expressed in the presence of wild type TP53 (PZR1 cells, derived from the KC model), whereas it is more expressed when TP53 is mutated (PZPR1 cells, derived from the KPC model) or deleted (PZPflR cells). Furthermore, overexpression of wild type p53 in ASPC1 cells (that harbour a *TP53* mutation) reduced the expression levels of GPR55 compared to cells transfected with the empty vector (Supplementary Figure 4b). On the other hand downregulation of p53 with two specific siRNAs strongly increased the expression levels of GPR55 in pancreatic cancer cells SW1990 that express wild type p53 (Fig. 3b) and in HEK293T cells (Supplementary Figure 4c). These data indicate that wild type p53 negatively regulates GPR55 protein levels.

To gain further insight into the mechanisms of the p53-dependent regulation of GPR55, we performed luciferase assays using a plasmid containing the luciferase gene under the control of the 3′-untranslated region (3′-UTR) of *GPR55* ("GPR55" in Fig. 3c). A plasmid encoding the luciferase gene but lacking a regulatory region was used as a control ("Control" in Fig. 3c). ASPC1 cells were co-transfected with each luciferase plasmid in combination with either an empty vector (pcDNA) or plasmids encoding wild type p53 or mutants p53 (harbouring mutations at positions 143^{Ala} or 175^{His}). Wild type and p53 mutants were expressed to similar levels in these experimental conditions (Supplementary Figure 4d). Results showed that the luciferase activity driven by 3′-UTR GPR55 was significantly decreased in cells expressing wild type p53 but not in cells expressing the mutant p53 (Fig. 3c). These data demonstrate that wild type p53, but not its mutated forms, negatively affects GPR55 protein expression by specifically regulating its 3′-UTR and influencing GPR55 mRNA degradation or translation. We next investigated whether the p53-dependent regulation of 3′-UTR GPR55 occurred directly or indirectly, possibly through regulation of microRNAs (miRs). More than one algorithm predicted GPR55 as a target of several miRs belonging to the miR34 family (Supplementary Table 1), which is known to be regulated by p53 [16], to be downregulated in PDAC and to have a key role in PDAC progression [17]. Specifically, we observed that miR34b-3p was the only miR within this family with a binding site on 3′-UTR GPR55 as predicted by MicroCosm (Supplementary Figure 4e), strongly suggesting that this specific miR could be involved in the p53-mediated regulation of GPR55 in PDAC cells. Consistent with this, we observed that miR34b-3p was downregulated in ASPC1 and HPAFII cells compared to the immortalised pancreatic cell line HPDE (Supplementary Figure 4f). Re-introduction of wild type p53 in ASPC1 cells increased miR34b-3p levels (Supplementary Figure 4g) while re-introduction of miR34b-3p in HPAFII and ASPC1 cells decreased GPR55 protein expression (Fig. 3d).

These data indicate that wild type p53 downregulates GPR55 protein expression by modulating the levels of miR34b-3p (Fig. 3e) and suggest a mechanism by which *TP53* mutations might promote cell growth through impaired regulation of miR34b-3p levels, which in turn results in increased expression of GPR55 and amplification of proliferative signals (Fig. 3e).

a





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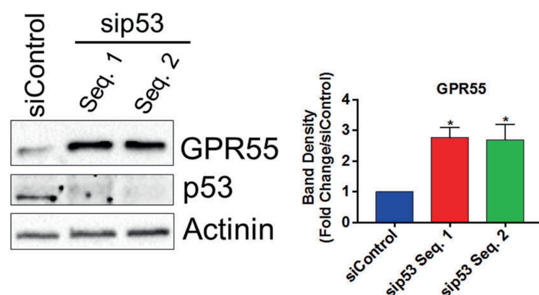

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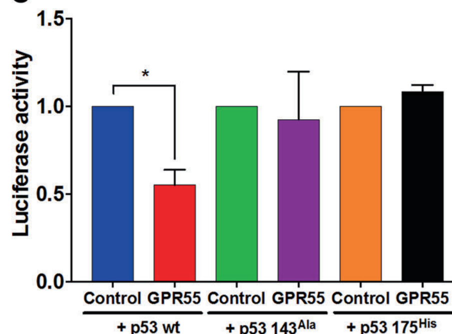

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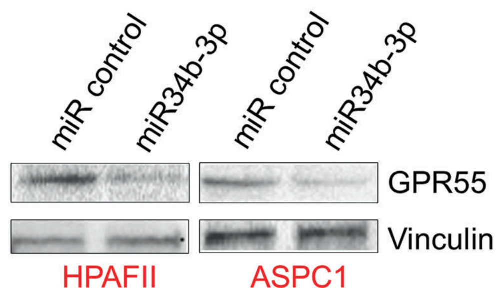

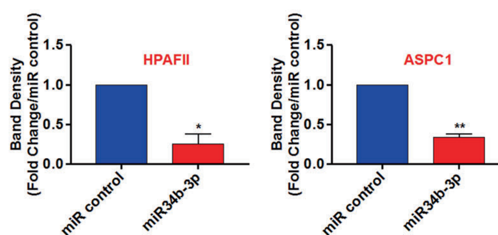

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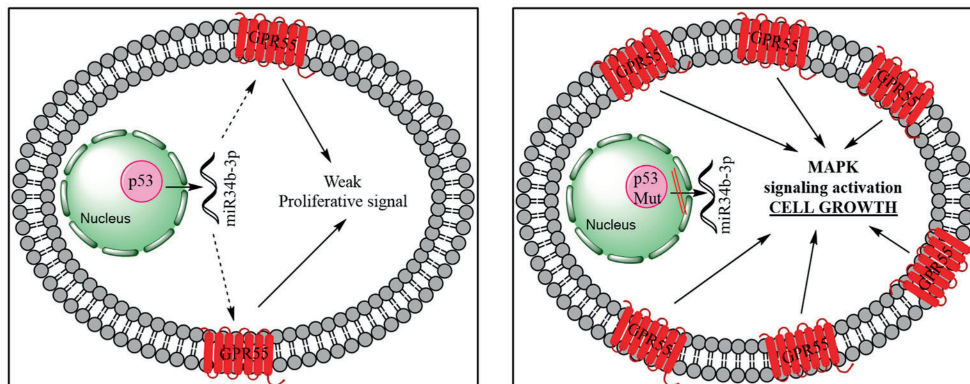


◀ **Fig. 3** p53 regulates GPR55 protein levels in PDAC. **a** Kaplan–Meier survival curves of KC ($n = 19$) and KCG ($n = 12$) mice. Log-rank (Mantel-Cox) test $p = 0.17198$, Gehan-Breslow-Wilcoxon test $p = 0.9677$. Representative images of H&E staining confirm presence of tumours. Scale bar: 250 µm. **b** Pancreatic cancer cells SW1990, expressing wild type p53, were transfected with siRNAs targeting p53 or siControl and lysed after 48 h. Representative Western blot and results from densitometry analysis are shown. Actinin was used as loading control. Data are means ± s.e.m. of $n = 3$ independent experiments and are expressed as fold change of normalised GPR55 levels in cells transfected with siControl. $^{*}p < 0.05$. **c** Luciferase activity assays were performed in ASPC1 co-transfected with the indicated plasmids as specified in the Materials and Methods. Results are means ± s.e.m. of $n = 5$ independent experiments. $^{*}p < 0.05$. **d** The effect of re-introduction of miR34b-3p in ASPC1 and HPAFII cells on GPR55 protein expression was assessed by Western blot. Vinculin was used as loading control. Data from densitometry analysis are expressed as fold change of normalised GPR55 levels in cells transfected with miR control and are means ± s.e.m. of $n = 3$ independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$. **e** Proposed model of p53/GPR55 signalling. Wild type, active p53 negatively regulates GPR55 protein expression by increasing miR34b-3p levels. The reduced GPR55 protein levels result in a weak proliferative signal. Mutated p53 is not able to regulate miR34b-3p levels and therefore GPR55 protein expression is not affected. High GPR55 protein expression results in a strong proliferative signal. Dotted arrows indicate inhibition; solid arrows indicate activation

Pharmacological inhibition of GPR55 reduces PDAC cell growth, cell cycle progression and MAPK signalling in vitro

Our data so far demonstrated that pancreatic cancer cell proliferation in vitro and, importantly, PDAC progression in vivo could be inhibited by genetic *Gpr55* disruption. To validate the possibility of targeting GPR55 as a novel potential strategy in PDAC, we next investigated the effect of its pharmacological inhibition in vitro and in vivo. The GPR55 antagonist cannabidiol (CBD) efficiently inhibited anchorage-dependent growth of ASPC1, HPAFII, BXPC3 and PANC1 cells (Supplementary Figure 5a-d). Similar results were obtained using the GPR55 antagonist CID16020046 (CID) in ASPC1 and HPAFII cells (Supplementary Figure 5a and b). Treatment of HPAFII (Fig. 4a) and PANC1 cells (Fig. 4b) with CBD blocked cell cycle at the G1/S transition phase in a dose-dependent manner and it reduced DNA synthesis/entry in the S phase, as assessed by EdU incorporation (Fig. 4c). Consistent with this, CBD reduced expression of cyclin D1 and activation of the tumour suppressor retinoblastoma (RB) without affecting the total levels of RB (Fig. 4d). Inhibition of MEK/ERK and ERK-dependent pathways was also observed in cells treated with CBD (Fig. 4e). On the other hand, no effect was detected on the total levels of any of the analysed proteins (Fig. 4e). To investigate further the effect of CBD on different cell signalling pathways, we performed a human phospho-kinase array assay on lysates from untreated and CBD-treated HPAFII cells. Consistent with our previous data, results from the array confirmed a specific inhibition of ERK1/2 phosphorylation (25% threshold) upon treatment with CBD (Supplementary Figure 6). Importantly, Stat5a was the only other kinase whose phosphorylation appeared to be reduced by CBD treatment (25% threshold), although further investigation of additional, independent lysates did not confirm the Stat5a phosphorylation inhibition. Overall data from the array ruled out the possibility that CBD, at the concentrations used in this study, had many off target inhibitory effects on additional signalling pathways involved in regulation of cell growth and cell cycle progression. Finally, we observed that both CBD and CID inhibited anchorage-independent growth of ASPC1 and HPAFII cells (Fig. 5a, b).

These data indicate that pharmacological inhibition of GPR55 reduces PDAC cell cycle progression and cell growth, suggesting that GPR55 may represent a novel target to counteract PDAC progression.

Pharmacological inhibition of GPR55 potentiates the effect of gemcitabine (GEM) in vivo and in vitro

We then investigated the effect of CBD on PDAC progression in vivo either alone or in combination with GEM. KPC mice were given CBD (100 mg/kg), GEM (100 mg/kg) or a combination of the two drugs, and survival curves were determined (Fig. 5c). Lifespan of mice given CBD (mean 25.4 days, median 22 days) was very similar to survival of mice given GEM (mean 27.8 days, median 23.5 days). Survival of mice given the vehicle was: mean 18.6 days, median 20 days. Strikingly, a remarkable and statistically significant increase in survival was observed when CBD was used in combination with GEM, with a nearly three-fold extension of mice survival compared to mice given the vehicle (mean 52.7 vs 18.6 days, median 56 vs 20 days). To determine the mechanism(s) underlying the pronounced effect of the drug combination on PDAC growth, we next analysed tumour specimens from the four groups of mice. IHC analysis indicated that combination of the two drugs strongly reduced the percentage of proliferative cells, as assessed by Ki67 staining (Fig. 5d). Combination of CBD and GEM reduced the number of HPAFII (Supplementary Figure 7a) and PANC1 (Supplementary Figure 7b) cells more efficiently than each compound alone, as further confirmed by analysis of combination (CI) and dose reduction (DRI) indexes [18] using CompuSyn software (Supplementary Table 2).

These data indicate that combination of CBD and GEM strongly inhibits PDAC growth in vitro and in vivo.

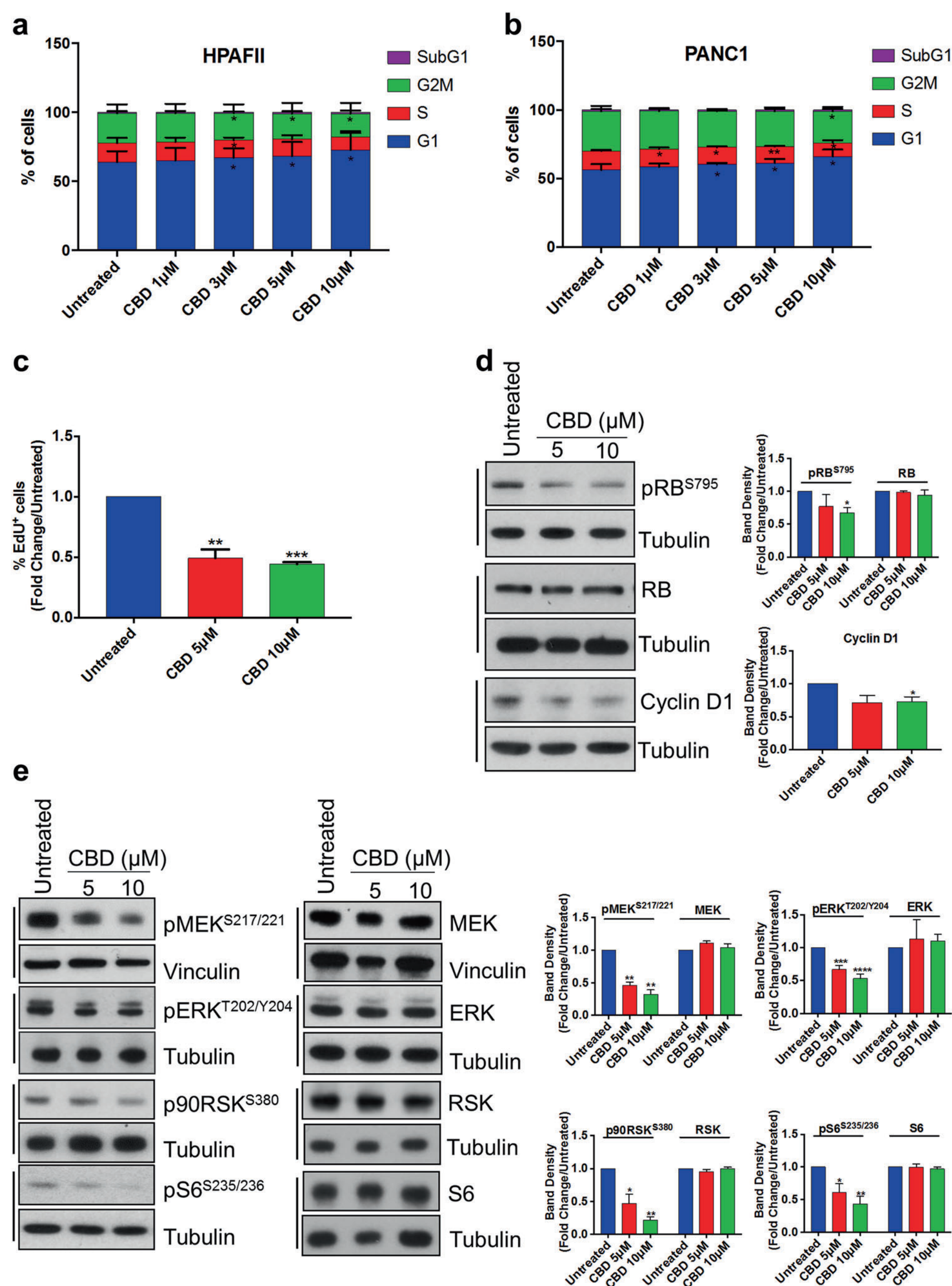


Fig. 4 Pharmacological inhibition of GPR55 inhibits cell cycle progression and MAPK signalling pathways. HPAFII (**a**) and PANC1 (**b**) cells were treated for 72 h with the indicated concentrations of CBD or vehicle alone ("untreated") in DMEM containing 10% FBS. Cell cycle analysis was performed by FACS. Results are expressed as percentage of cells in each phase of the cycle and are means ± s.e.m. of $n = 3$ independent experiments. *$p < 0.05$, **$p < 0.01$. **c** HPAFII cells were treated with the indicated concentrations of CBD for 70 h before incubation with 10 μM EdU for further 2 h. Graph indicates the percentage of cells that had incorporated EdU. Data are expressed as fold change of cells treated with vehicle ("untreated") and are means ± s.e.m. of $n = 3$ independent experiments performed in duplicate. **$p < 0.01$, ***$p < 0.001$. **d** Effect of CBD treatment on the cell cycle regulators Cyclin D1 and pRB^{S795}. Total levels of RB were also assessed. Tubulin was used as loading control. **e** Effect of CBD treatment on activation and total levels of the indicated members of the MAPK/ERK signalling pathway. Tubulin or vinculin were used as loading controls. Results from densitometry analysis are expressed as fold change of normalised results from cells incubated with vehicle ("untreated") and are means ± s.e.m. of $n = 3$ independent experiments apart from: $pERK^{T202/Y204}$ ($n = 7$-9), $pS6^{S235/236}$ ($n = 6$), pRB^{S795}, and RB ($n = 4$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Pharmacological inhibition of GPR55 affects signalling pathways involved in acquired resistance to GEM

IHC investigation revealed reduced ERK phosphorylation in tumours from mice given CBD (Fig. 6a). A trend towards inhibition of S6 phosphorylation was also observed, although data did not reach statistical significance (Fig. 6b). We detected increased ERK activation in tumours from mice given GEM compared to mice given the vehicle (Fig. 6a). Increased ERK activation was previously reported upon GEM treatment and it was proposed as one of the mechanisms of acquired resistance to GEM treatment [19]. Importantly, IHC analysis of tumours from mice given a combination of CBD and GEM showed that CBD was able to counteract the effect of GEM on ERK and ultimately to reduce the GEM-dependent ERK phosphorylation (Fig. 6a). Similarly, we observed that GEM increased ERK activation in HPAFII cells and this was opposed by CBD when the two drugs were used in combination (Fig. 6c). No effects were observed on ERK expression levels (Fig. 6c). In the same experiments GEM induced phosphorylation of the histone variant H2AX (Fig. 6c), a well-known marker of DNA damage.

Next, we investigated the effect of CBD on additional proposed mechanisms of GEM resistance. It was demonstrated that GEM can act by inhibiting the enzyme ribonucleotide reductase 1 (RRM1), leading to imbalance in the deoxyribonucleotides pool. Moreover, it was shown that cancer cell resistance can be associated with increased RRM1 and RRM2 expression [20, 21]. We observed that GPR55 downregulation reduced RRM1 protein expression in HPAFII cells (Fig. 7a). Similarly, treatment with CBD reduced the levels of both RRM1 and RRM2 (Fig. 7b). Moreover, reduced expression of RRM1 was detected in tumour specimens from KPCG compared to KPC mice (Fig. 7c). Reduced levels of RRM1 mRNA were also observed in HPAFII cells upon treatment with CBD (Supplementary Figure 8a) and in KPCG compared to KPC mice (Supplementary Figure 8b). These data led us to hypothesise that CBD could counteract potential resistance mechanisms associated with upregulation of ribonucleotide reductases. Supporting this hypothesis, we detected increased expression of RRM1 in tumours from KPC mice given GEM (Fig. 7d). While CBD alone did not seem to affect RRM1 levels, it was able to oppose the increase of RRM1 expression induced by GEM when the two drugs were used in combination (Fig. 7d).

Discussion

GPR55 has recently emerged as a key player in many cellular functions associated with cancer progression [11]. This role was initially suggested by the demonstration that GPR55 is the specific receptor for LPI [12, 22] whose role in cancer has been extensively described [11, 23–25]. From our original studies reporting the mitogenic properties of LPI [24, 26], data in literature have increasingly documented the involvement of LPI in several cellular processes required for cancer progression, including cancer cell proliferation, migration and angiogenesis [22, 27, 28]. Studies also demonstrated that Ras-transformed epithelial thyroid cells and fibroblasts [24, 26] and different cancer cell lines [22, 28] are able to release LPI and increased levels of this phospholipid were found in ovarian cancer [29] and colon cancer [30] patients. Data indicating a specific requirement for GPR55 in modulation of most of the detected LPI-dependent functions provided the first indication that the receptor might be involved in cancer progression. Subsequent evidence supported this conclusion, including data demonstrating that GPR55 itself is overexpressed in many cancer cells [22, 27, 31, 32] and that GPR55 mRNA levels increase in human skin, larynx and oral squamous cell carcinoma compared to healthy tissues [32]. Increased levels of GPR55 mRNA were also detected in highly aggressive breast tumours [31] and high expression of GPR55 was recently associated with basal/triple-negative breast cancer subtype [33].

A previous study reported increased levels of GPR55 mRNA in PanIN 2/3 compared to PanIN 1b [31]. GPR55 mRNA was also detected in the PDAC cell lines Mia PaCa-2 [31] and PANC1 [34], the latter cells also expressing the receptor at the protein level [34]. Apart from these preliminary observations, no study has investigated whether accumulation of GPR55 occurs in PDAC and whether the receptor plays a role during PDAC development and progression. In this study, we show for the first time that GPR55 accumulates in human PDAC specimens compared to corresponding ductal areas in normal pancreatic tissue and it is detectable at the protein levels in a panel of PDAC cell lines. We demonstrate that the tumour suppressor p53 negatively regulates GPR55 protein expression in a mechanism involving regulation of miR34b-3p. As miR34b-3p itself was previously reported to be downregulated in PDAC and to have a key role in PDAC progression [35] our data identify a novel p53/miR34b-3p/GPR55 axis in this process.

We further show that downregulation and pharmacological inhibition of GPR55 reduced anchorage-dependent and independent growth of PDAC cells, consistent with data previously indicating that GPR55 is an important regulator of cancer cell proliferation [11, 22]. More importantly we report that genetic disruption of *Gpr55* in KPC mice significantly reduced cancer cell proliferation in vivo, providing the first evidence that this receptor is important for pancreatic cancer proliferation in this

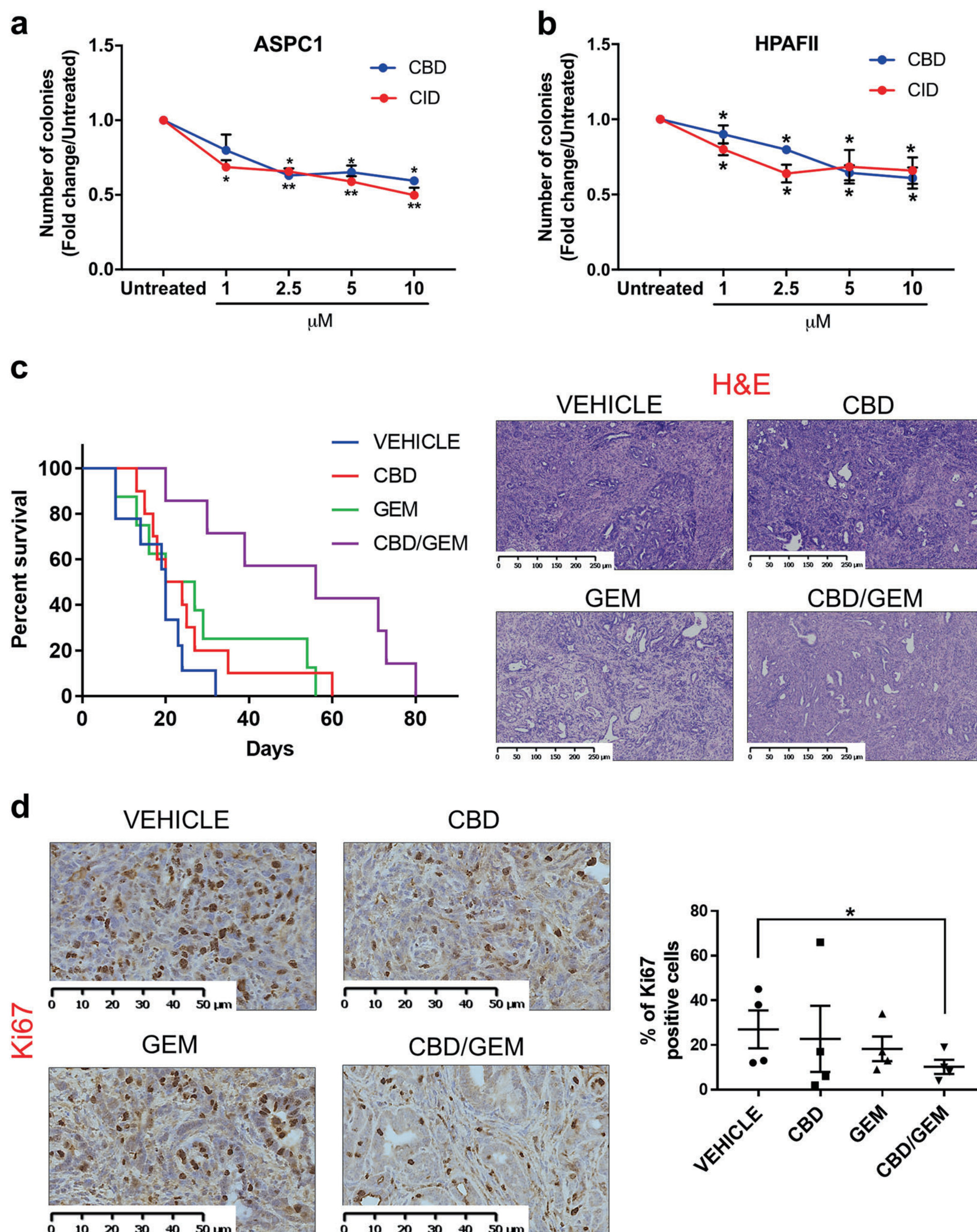


Fig. 5 Pharmacological inhibition of GPR55 inhibits anchorage-independent growth of PDAC cells and potentiates the effect of GEM in vivo. **a,b** Effect of CDB and CID on anchorage-independent growth of ASPC1 (**a**) and HPAFII (**b**) cells. Colonies were allowed to grow for 3–4 weeks. Control cells were incubated with the corresponding amount of vehicle ("untreated"). Data are means ± s.e.m. of $n = 3$ independent experiments performed in duplicate. *$p < 0.05$, **$p < 0.01$ vs control cells. **c** Kaplan–Meier survival curves of KPC mice given the vehicle ($n = 9$), CBD ($n = 10$), GEM ($n = 8$) and CBD plus GEM ($n = 7$). Curves indicate days after start of each treatment. Logrank (Mantel–Cox) test $p = 0.0059$, Logrank test for trend $p = 0.0007$, Gehan-Breslow-Wilcoxon test $p = 0.0268$. Images show representative H&E staining confirming the presence of tumours. Scale bar: 250 μm. **d** Representative images of Ki67 protein expression in PDAC specimens from each group of mice. Scale bar: 50 μm. Graph indicates the percentage of PDAC cells showing Ki67 staining. *$p < 0.05$

established PDAC mouse model. Extensive in vitro characterisation further demonstrated that growth inhibition was due to inhibition of cell cycle progression without increased apoptosis and it involved regulation of MAPK signalling pathways. Indication of a direct role of GPR55 in cancer progression was previously provided by the observation that GPR55$^{-/-}$ mice were more resistant to skin cancer development compared to wild type mice [32]. Similarly,

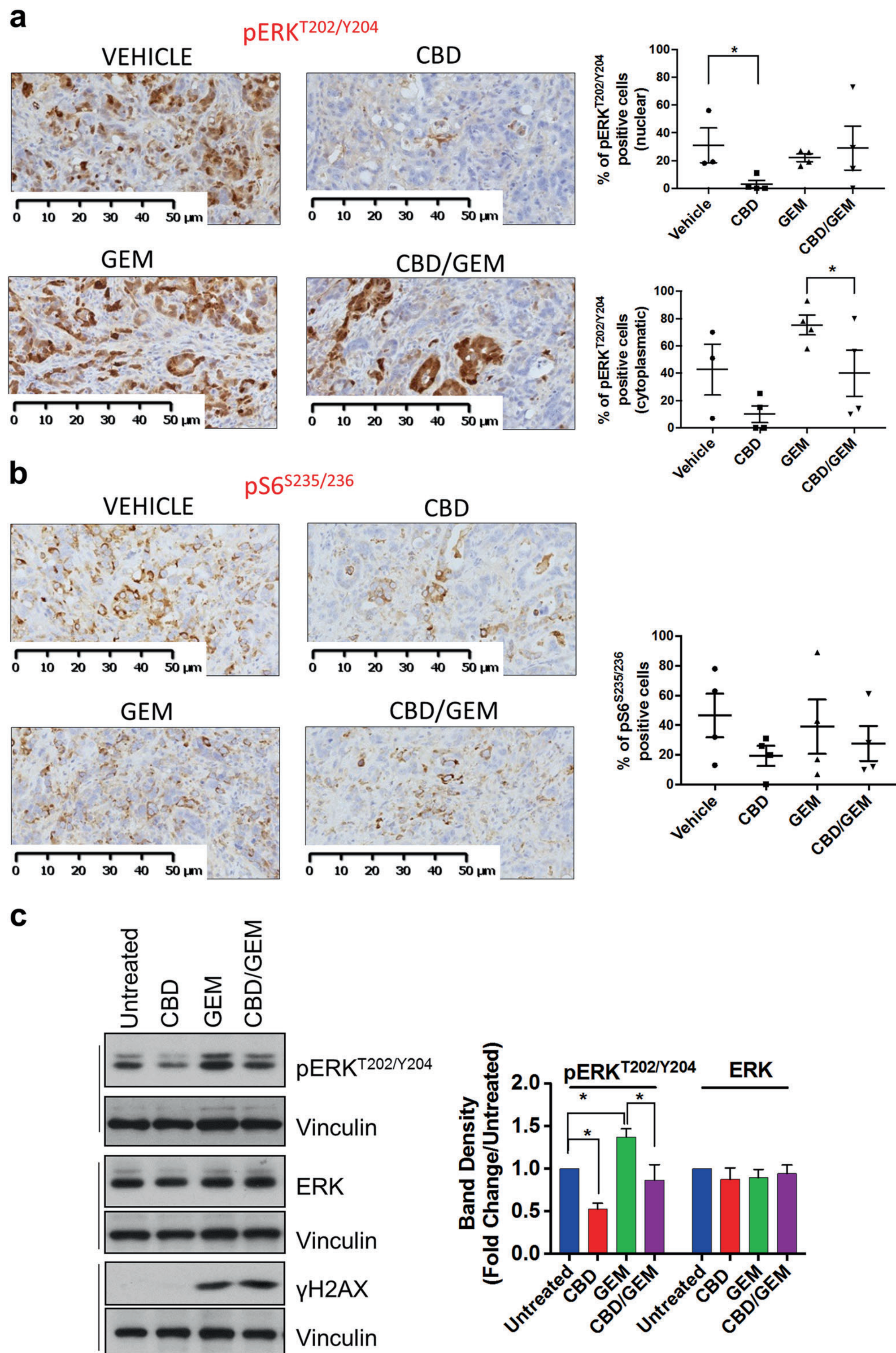


Fig. 6 Effect of drugs combination on MAPK signalling pathway in vitro and in vivo. **a, b** Representative images of PDAC specimens from KPC mice given the indicated drugs, stained with antibodies recognising pERK$^{T202/Y204}$ (**a**) and pS6$^{S235/236}$ (**b**). Scale bar: 50 μm. Graphs indicate the percentage of PDAC cells showing positive staining for each antibody. $*p < 0.05$. **c** HPAFII cells were incubated in DMEM containing 10% FBS supplemented with 10 μM CBD or 20 nM GEM alone or in combination for 48 h. Phosphorylation of ERK$^{T202/Y204}$ and H2AX and levels of ERK were assessed by Western blotting analysis. Vinculin was used as loading control. Data from densitometry analysis are expressed as fold change of normalised results from cells incubated with vehicle ("untreated") and are means ± s.e.m. of $n = 3$ independent experiments. $*p < 0.05$

delivery of siRNA targeting GPR55 in xenografts of T98G glioma cells reduced tumour growth in vivo [31]. Inhibition of metastasis formation was detected in mice injected with human colon cancer cells and treated with pharmacological inhibitors of GPR55 [30] and in mice injected with human breast cancer cells lacking GPR55 [33]. Adding to these data, our study provides the first demonstration that disruption of *Gpr55* can directly affect proliferation of the highly aggressive PDAC in the closest genetic model that is currently available to mimic the human disease and it can significantly extend the lifespan of KPC mice, providing the first evidence that targeting GPR55 can result in improvement of survival.

Although previous studies had provided preliminary indication that targeting GPR55 could potentially represent a novel therapeutic strategy in cancer [31, 32] no study so far had investigated whether pharmacological inhibition of GPR55 could directly improve survival in a model of PDAC. As we observed that GPR55 downregulation inhibited proliferation without inducing apoptosis, we decided to determine the effect of the GPR55 antagonist CBD alone or in combination with GEM, a cytotoxic drug currently used for PDAC treatment. It is worth mentioning that, although CBD has been confirmed to be a GPR55 antagonist, we could not completely rule out the possibility of additional effects of the drug, independent of GPR55 inhibition. However, the observation that similar data were obtained in vitro upon treatment with CBD or with the specific GPR55 antagonist CID as well as upon downregulation of GPR55 strongly supported the conclusion that the reduced cell growth/cell cycle progression detected in PDAC cells upon treatment with CBD was mainly due to inhibition of GPR55. Furthermore, results from the phospho-kinase array assay indicated that CBD did not inhibit activation of many signalling pathways, ruling out the possibility that the compound, at the concentrations used in our study, had many off target effects. Finally, we decided to use CBD in the in vivo experiments as this drug is already approved for medical use therefore results from our study could have an immediate potential translational value. Here we report that KPC mice given a combination of CBD and GEM survived nearly three times longer compared to KPC mice given the vehicle (mean 52.7 vs 18.6 days, median 56 vs 20 days) and also longer than mice given GEM alone (mean 52.7 vs 27.8 days, median 56 vs 23.5). Our data further indicate that the remarkable increase in survival is likely due to the ability of the drugs combination to inhibit cancer cell proliferation and to overcome mechanisms involved in development of resistance to GEM treatment. To the best of our knowledge our study is the first demonstration that inhibition of GPR55 not only reduces cancer progression in a well-established transgenic model but it also represents a therapeutically valid strategy. In this respect, this study provides the first validation of GPR55 as a novel target for cancer treatment likely to be able to improve patients' outcome significantly.

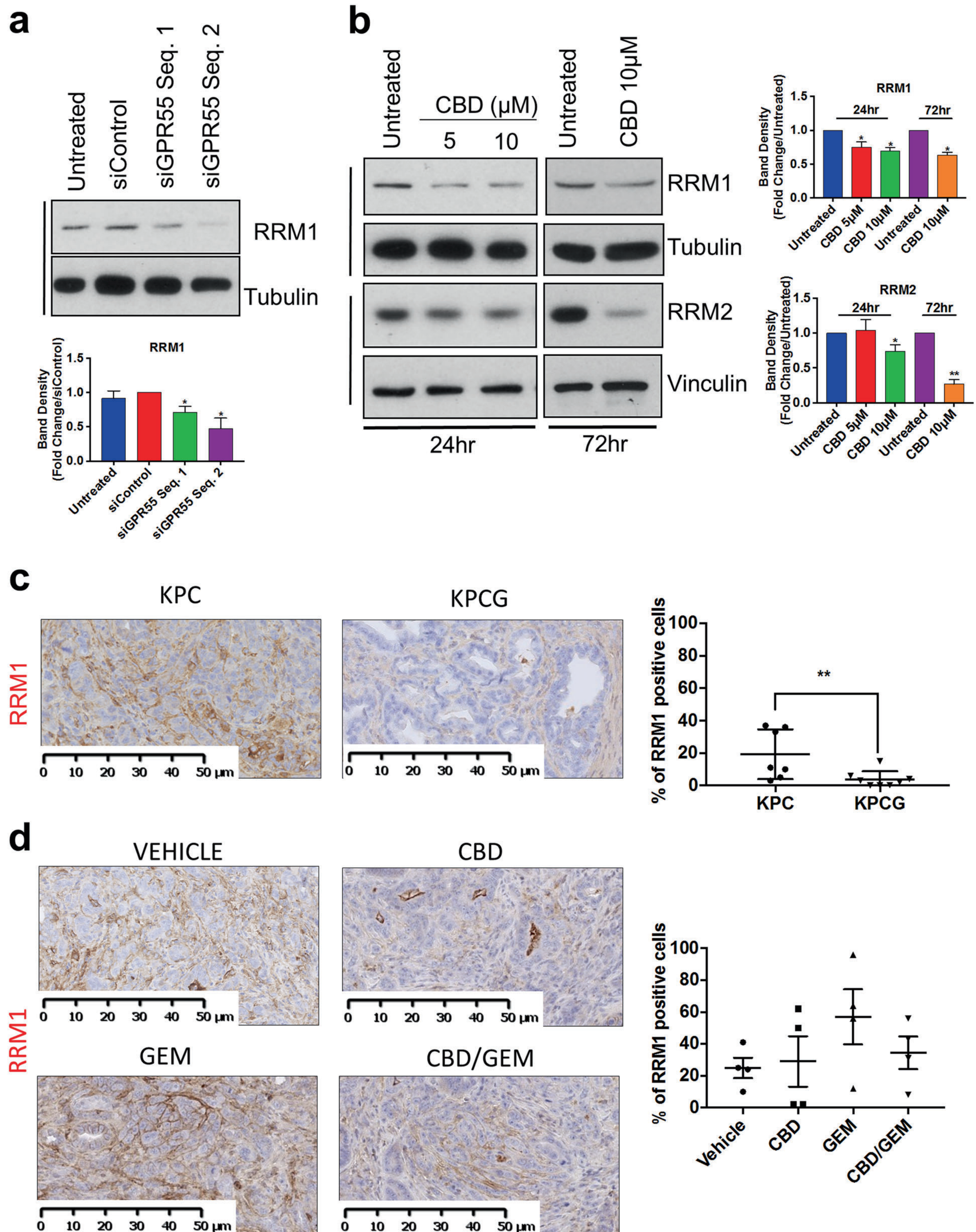
The importance and clinical relevance of these results are further highlighted by the observation that they were obtained in a model of PDAC, one of the deadliest cancer types and in urgent need of novel treatment options. The very few therapeutic options currently available for advanced PDAC solely increase survival by few months leaving the five years survival rate at a mere 5%. Development of drug resistance is one of the main reasons for such an abysmal prognosis. Our demonstration that combination of CBD and GEM can oppose mechanisms associated with drug resistance and increase survival of KPC mice is very important considering that both drugs are already approved for medical use and therefore this combination can be quickly tested in clinical trials.

In conclusion, our study identified GPR55 as a novel critical mediator of PDAC development and progression. The demonstration that GPR55 is negatively regulated by p53 and it controls cell cycle progression and growth of pancreatic cancer cells provides novel information into the mechanisms by which *TP53* mutations can lead to PDAC development. Moreover, our study provides the first evidence that GPR55 is a therapeutically valid target whose inhibition, in particular in combination with GEM, results in improved survival in the transgenic model closest to the human disease currently available. These results represent a huge step forward towards the identification of a novel treatment regime that could highly benefit PDAC patients.

Materials and methods

Mouse strains

All animal experiments were conducted in compliance with institutional and national guidelines. Mice were housed in ventilated cages under standardised conditions (21 °C, 60% humidity, 12 h light/12 h dark cycle, 20 changes air/h), palpated daily and culled by carbon dioxide-mediated asphyxiation or cervical dislocation. $KRAS^{WT/G12D}$/$TP53^{WT/R172H}$/Pdx1-$Cre^{+/+}$ (KPC), $KRAS^{WT/G12D}$/Pdx1-Cre $^{+/+}$ (KC) and Pdx1-$Cre^{+/+}$ mice were kindly provided by Prof. David A. Tuveson (Cancer Center at Cold Spring Harbor Laboratory). $GPR55^{-/-}$ mice were kindly provided by Prof. David Baker (Queen Mary University of London). $GPR55^{-/-}$ mice and KPC control mice were maintained on a GPR55 background. Mice were ear-marked and specimen-genotyped via DNA extraction and PCR (out sourced to Transnetyx Inc.). KPC mice used for drug treatments were maintained on a mixed C57BL/6,129Sv1 background. KPC mice were treated with CBD (GW



Pharmaceuticals, 100 mg/kg), GEM (100 mg/kg) and with a combination of the two treatments. Mice enrolment was based on tumour size, measured by palpation. Specifically, mice underwent palpation every 24 h once they reached 80 days of age (predicted age when tumours should start to develop). Mice were assigned to the four arms (vehicle, CBD, GEM, CBD + GEM) by simple randomisation using a shuffled deck of cards as described [36]. Vehicle and CBD

◀ **Fig. 7** Effect of GPR55 disruption and pharmacological treatment on ribonucleotide reductase levels. **a** HPAFII cells were transfected with the indicated siRNAs or transfection reagent ("untreated") and lysed after 72 h. Levels of RRM1 were assessed by Western blotting. Data from densitometry analysis are expressed as fold change of normalised results from cells transfected with siControl and are means ± s.e.m. of $n = 3$ independent experiments. $*p < 0.05$. **b** HPAFII cells were treated with the indicated concentrations of CBD for 24 h or 72 h and levels of RRM1 and RRM2 were assessed by Western blotting. Tubulin or vinculin were used as loading controls. Data from densitometry analysis are expressed as fold change of normalised results from cells incubated with vehicle ("untreated") and are means ± s.e.m. of $n = 3$ independent experiments apart from RRM2 at 24 h ($n = 4$). $*p < 0.05$, $**p < 0.01$. **c, d** Representative images of PDAC specimens from KPC and KPCG mice (**c**) or KPC mice given CBD and GEM (**d**) stained with anti-RRM1 antibody. Scale bar: 50 μm. Graph indicates the percentage of PDAC cells showing positive staining

were administered by daily intraperitoneal injection while GEM was administered by intraperitoneal injection every 3 days. Mice were checked daily and left until death or culled when pre-assigned end points were reached. The pre-assigned end points included mice displaying one of the following: development of abdominal ascites, severe cachexia, significant weight loss (approaching 20% of initial weight), extreme weakness, inactivity, discomfort, or pain. No major side/adverse effects and no weight loss were observed in mice treated with CBD.

Immunohistochemistry (IHC)

Mouse pancreatic tissues were placed in 10% neutral-buffered formalin immediately after sacrifice and incubated for at least 24 h. After embedding and sectioning procedures, tissues were stained with Hematoxylin and Eosin (H&E) to confirm the presence of tumours. Antibody staining was performed on 5 μm-thick sections with the following antibodies and dilutions: GPR55 (1 M urea buffer; dilution 1:100, 1:800; Novus Biologicals); pERK$^{T202/Y204}$ (pH 6.0; dilution 1:75; Cell Signalling Technology); pS6$^{S235/236}$ (pH 9.0; dilution 1:100; Cell Signalling Technology); RRM1 (pH 6.0; dilution 1:250; Abcam); Ki67 (pH 6.0; dilution 1:75; eBioscience). Representative images of antibody optimisation are shown in Supplementary Table 3. IHC slides were scored independently by two pathologists (RL and MP) blind to molecular data. The normal pancreatic tissue presented in Fig. 1a was obtained from a patient without any findings of pancreatic cancer.

Tissue microarray (TMA)

Archived formalin-fixed, paraffin-embedded blocks from 54 patients diagnosed with primary PDAC were retrieved at the "Regina Elena" National Cancer Institute (Rome, Italy). TMA were constructed by removing 2 mm diameter cores of histologically confirmed tumour areas. TMA sections were then incubated with anti-GPR55 rabbit polyclonal antibody (dilution 1:100, incubation overnight, Novus Biologicals) after antigen retrieval by microwave treatment at 750 W for 10 min in 1 M urea buffer. Anti-rabbit EnVision kit (K4003, Dako, Glostrup, Denmark) was used for signal amplification. In control sections, the primary antibody was replaced with isotype-matched immunoglobulins. The expression of markers was quantified as percent of immunoreactive cells.

Statistics

All sample sizes were chosen based upon prior studies performed in our laboratories and appropriate power calculations performed by expert biostatisticians of the School of Public Health at Curtin University. Unless otherwise specified Student's *t*-test (one-sided) was used to determine statistical significance. In each case $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

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Author contributions RF and MF designed, coordinated, and carried out the bulk of the experiments. RF, AA, IM, TM and MF performed in vitro experiments. RF, TM, and MF designed and supervised in vitro experiments. RF, GS, and MF designed and supervised in vivo experiments. RF performed in vivo experiments. SAA, CAF, CEE, and VDL contributed to in vivo experiments. RF and RL performed IHC assay. RL and MP performed IHC analysis. LS and GC performed in silico analysis. OJS provided key reagents. RF, TM, and MF wrote the manuscript. MF conceived the project, led and supervised the study.

Conflict of interest The authors declare that they have no conflict of interest.

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4. ABCC3 is a novel drug target in PDAC

4.1 Introduction

Parts of this introductory subchapter are adapted from the following publication:

Adamska A., Falasca M. *ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward?* World J Gastroenterol; 2018, 7; 24(29): 3222-3238. doi: 10.3748/wjg.v24.i29.3222

Whole review article is attached at the end of the thesis, with adapted fragments highlighted in the publication. The author contribution form stating my contribution to the publication is attached at the end of the thesis.

In the last years, ABC transporters have attracted remarkable attention of researchers from different scientific areas. The role of ABC transporters in different physiological and pathological conditions, including cancer, has been widely reported, increasing the interest in the development of their specific inhibitors.
While the role of ABC transporters in mediating chemoresistance is well established, little is known about their direct, drug-efflux independent contribution to pancreatic cancer progression. Nevertheless, intensive studies in recent years suggest that beyond their role in drug resistance, the biological functions of ABC transporters are more complex. It has been proposed that tumour-promoting functions of ABC transporters are based on their ability to export active signalling molecules and hormones, which by autocrine or paracrine regulation activate cancer cells as well as tumour environment. Increasing interest in this area has demonstrated the significant impact of these proteins on invasion, migration and differentiation of malignant cells (414). In addition, ABC transporters-released molecules may induce changes in metabolism as well as redox status, characteristics pivotal in PDAC tumorigenesis. Looking at the wide variety of substrates transported by ABC

transporters, together with their increased expression in cancer cells and especially cancer stem cells, the role of these proteins in the transport of signalling molecules, which activity promotes cancer progression, has become an area of interest. High impact of bioactive lipids, including phospholipids, sphingolipids or cholesterol on PDAC tumorigenesis and an emerging role of ABC transporters in their release presents a novel opportunity for targeting the disease. It has been previously demonstrated that one of the hallmarks of PDAC is lipid-dependence and that the decrease of the lipids levels may reduce cancer progression. Accordingly, aiming to block specific ABC transporters responsible for their extrusion, mainly members of ABCA and ABCC subfamilies, and depriving cancer cells of the necessary fuel, may highly contribute to slowing down PDAC development. In fact, it has been demonstrated in several cancers that targeting of ABC transporters involved in lipid transport (e.g. ABCC1 in prostate or ovarian cancer or ABCC4 in neuroblastoma) showed significant improvement in in vitro and in vivo models (414), slowing down cancer progression. Additionally, tumour environment and its engagement in cancer progression and metastatic spread has emerged as key player in PDAC carcinogenesis. Recently, expression of several of ABC transporters in PDAC stroma has been reported. One of the main stromal components- macrophages- have been demonstrated to express several of the drug transporters, inter alia ABCC1 and ABCC3, contributing to both chemoresistance and tumour progression (415).

There have been very limited studies on the role and expression of ABC transporters in pancreatic cancer; however, a strong correlation between few of their members and PDAC has been recently suggested. A recent study by Mohelnikova-Duchonova et al. showed an upregulation in transcript levels of several ABC transporters in PDAC compared to non-neoplastic tissues. Particularly, upregulation of two members of ABCA family, ABCA1 and ABCA7 involved in cholesterol export, together with expression of ABCG1 transporting phosphatidylserine, phosphatidylcholine, sphingomyelin, suggests their involvement in cellular cholesterol imbalance in the disease (268). A study by the same group revealed the existence of ABC transporters expression signatures in PDAC. On the basis on mRNA analysis, the expression of ABCC1, ABCC3, ABCC4, ABCC5 and ABCG2 in both pancreatic cancer samples and in

healthy pancreas has been demonstrated (416, 417) and was correlated with cell resistance to commonly applied chemotherapeutics (418). Furthermore, a more in-depth analysis showed that while ABCG2, ABCC1 and ABCC4 levels did not differ significantly between tumour and healthy tissues, ABCC3 and ABCC5 were found to be remarkably overexpressed in PDAC specimens. Moreover, although their expression could not be coupled with cancer stage, the differentiation status and tumour grading were related with increased ABCC3 levels and correlated with poor survival, whereas no such correlation could be found for ABCC5.

Although the investigation on the role of ABC transporters in PDAC is still in its outset, the initial analysis suggests their probable contribution to PDAC development and points at potential beneficial clinical consequences. Database analysis showed that the high importance and the potential of ABC transporters as pharmacological targets in PDAC is reflected in the association of their expression with patients' survival (204). Notable correlation between observed 5-year survival and expression of several ABC transporters has been observed. In particular, strong correlation between expression of ABCC3 transporter and survival probability of PDAC patients was indicated by the GEPIA database analysis (Figure 4.1).

ABCC3 transporter is involved in transporting of bile salts and organic ions (419, 420). It has been also implicated in mediation of drug resistance, e.g. to vincristine, methotrexate or etoposide; compounds used in clinical studies for PDAC treatments, which demonstrated only marginal effects (421). Analysis of Oncomine data base showed considerable upregulation of ABCC3 in PDAC specimens compared to healthy pancreas (Figure 4.2). Moreover, its expression levels have been correlated with survival of patients after resection, suggesting possible predictive aspect of ABCC3 expression in PDAC.

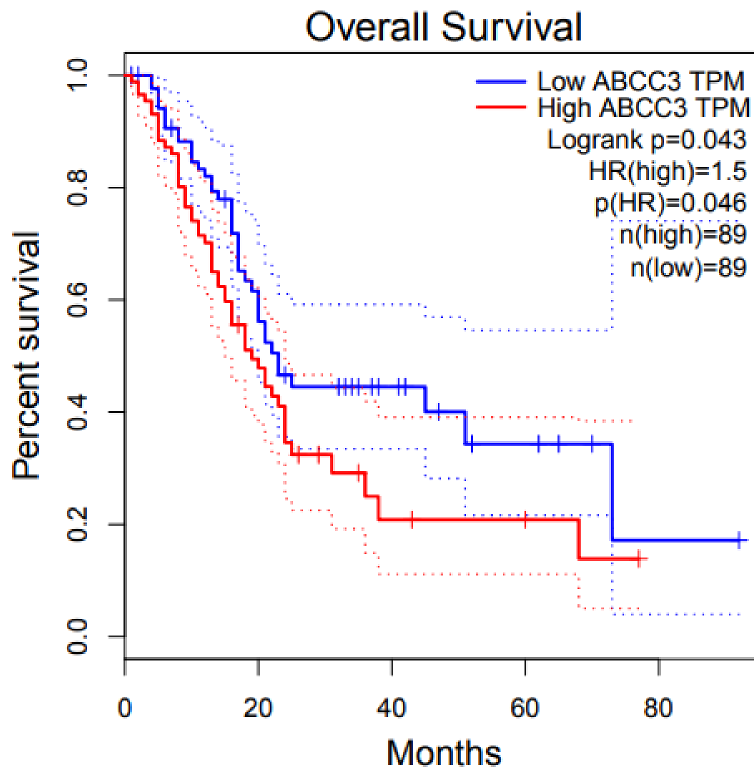

Figure 4.1 ***Overexpression of ABCC3 correlates with poor survival of PDAC patients.*** *Comparison of the survival probability of the patients with high (red) and low (blue) expression of ABCC3; p<0.05.*

Taken from: http://gepia.cancer-pku.cn/detail.php?gene=ABCC3

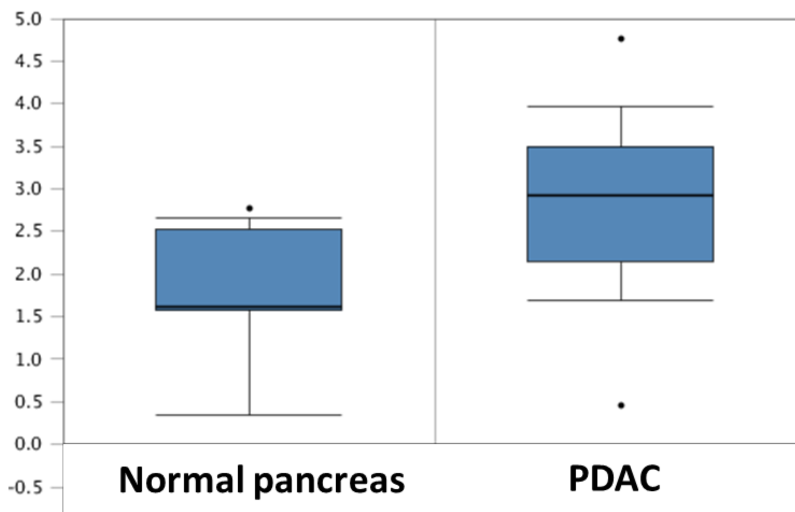

Figure 4.2 ***ABCC3 is overexpressed in PDAC.*** *Comparison of ABCC3 expression between healthy pancreatic tissues and PDAC specimens. Adapted from: oncomine.org*

Importantly, a study by Morse et al. comparing 2177 cell-surface genes from 28 pancreatic tumour specimens and 4 normal pancreas tissues validated ABCC3 as one of two specific cellular markers that could be used for detection of pancreatic cancer (422).

All these data suggest that ABCC3 is as a novel player in PDAC development and progression, which role and pharmacological potential has not been explored yet.

4.2 Aims of the project

Based on the recent evidence of a more active role of ABC transporters in PDAC progression and on the *in-silico* analysis showing the upregulation and high correlation of ABCC3 with patients' survival in PDAC, we aimed to investigate the role potential of ABCC3 as a novel pharmacological target in PDAC therapy.

In this project, we aimed to:

- Investigate the expression of ABCC3 in pancreatic cancer specimens, including cell lines and tumour tissues
- Investigate the role of ABCC3 transporter in PDAC *in vitro* and *in vivo*
- Investigate the cancer-specific ABCC3 functions
- Investigate the mechanisms upregulating ABCC3 activity in PDAC

4.3 Results and Discussion

This chapter is presented in the form of the published research article:

Adamska A, Ferro R, Lattanzio R, Capone E, Domenichini A, Damiani V, Chiorino G, Akkaya BG, Linton KJ, De Laurenzi V, Sala G, Falasca M. ABCC3 is a novel target for the treatment of pancreatic cancer. Adv Biol Regul 2019 Apr 24. pii: S2212-4926(19)30036-3. doi: 10.1016/j.jbior.2019.04.004.

The majority of the data presented in the publication is my own work. The mRNA analysis was performed by Dr Riccardo Ferro, QMUL; The IHC analysis was done by Dr Rossano Lattanzio, University of Chieti; LPI transport experiments were performed by Prof Kenneth Linton, QMUL; the xenograft data was provided by Dr Emily Capone, Dr Gianluca Sala and Prof Vincenzo de Laurenzi, University of Chieti.

The last version prior to submission is presented. Published version is attached at the end of the thesis. References 433-468 in the bibliography chapter correspond to the references presented in the published publication.

ABCC3 is a novel target for the treatment of pancreatic cancer

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Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) is a very aggressive disease, lacking effective therapeutic approaches and leaving PDAC patients with a poor prognosis. The life expectancy of PDAC patients has not experienced a significant change in the last few decades with a five-year survival rate of only 8%. To address this unmet need, novel pharmacological targets must to be identified for clinical intervention. ATP Binding Cassette (ABC) transporters are frequently overexpressed in different cancer types and represent one of the major mechanisms responsible for chemoresistance. However, a more direct role for ABC transporters in tumorigenesis has not been widely investigated. Here, we show that ABCC3 (ABC Subfamily C Member 3; previously known as MRP3) is overexpressed in PDAC cell lines and also in clinical samples. We demonstrate that ABCC3 expression is regulated by mutant p53 via miR-34 and that the transporter drives PDAC progression via transport of the bioactive lipid, lysophosphatidylinositol (LPI). Disruption of ABCC3 function either by genetic knockdown or pharmacological inhibition reduces pancreatic cancer cell growth *in vitro* and *in vivo*. Mechanistically, we demonstrate that knockdown and pharmacological inhibition of ABCC3 reduces cell proliferation by inhibition of STAT3 and HIF1α signalling pathways previously been shown to be key regulators of PDAC progression. Collectively, our results identify ABCC3 as a novel and promising target in PDAC therapy.

Keywords: Pancreatic Ductal Adenocarcinoma; ABC transporters; ABCC3; PDAC therapy; STAT3; TP53.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related deaths in the Western world (423). Lack of distinctive symptoms leading to late diagnosis, early metastatic spread and huge genetic and phenotypical heterogeneity of PDAC contribute to its aggressive nature and high chemoresistance, making most therapies ineffective (424-426). Surgical resection represents a therapeutic option only for 15-20% of PDAC patients presenting with local or locally advanced disease (427), the majority of whom unfortunately relapse. Radiation therapy and chemotherapy remain the only options for advanced and metastatic patients. However, this approach only marginally extends the overall survival (428). Up until recently, gemcitabine was the main available, FDA-approved chemotherapeutic; however, it prolonged patient survival by only a few weeks (429). Currently, Abraxane (albumin-bound paclitaxel) and FOLFIRINOX are additionally applied as a standard-of-care therapy, providing modest improvement in survival rates but accompanied by a higher incidence of adverse effects (185, 194). The high mutational heterogeneity and plasticity of PDAC limit the options for the development of targeted therapies (425, 430, 431). There is a need therefore to identify novel pharmacological targets and develop more effective and safe therapeutic options for PDAC patients. ABC transporters have previously been linked with poor outcome in cancer and this has generally been attributed to chemoresistance (432, 433). ABC transporters, particularly ABCB1 (P-glycoprotein), ABCG2 (BCRP) and ABCC1 (MRP1) are capable of effluxing a wide variety of substrates, including drugs, across the plasma membrane, lowering their intracellular concentration. The majority of studies have therefore focused on the role of ABC transporters in drug resistance and on its reversal. However, the ability of ABC transporters to also efflux bioactive molecules that play essential roles in cancer progression, suggests a more direct, active contribution of ABC transporters to carcinogenesis (334, 414, 434, 435). In particular signalling lipids, such as phospholipids, which role in several malignancies, including cancer has been well documented (436), were suggested as ABC transporters ligands. However, this area has been overlooked and the therapeutic potential of ABC transporter inhibition in counteracting PDAC progression has not yet been fully explored. Recently, we described the existence of an autocrine loop in which LPI (lysophosphatidylinositol)-activated GPR55 stimulates proliferation of PDAC cell

lines that harbour p53 mutations (398, 437). We showed that the blockade of LPI receptor-GPR55 significantly reduced disease progression in mouse models of PDAC. Considering their involvement in phospholipid efflux from cells (438), we proposed that ABC transporters may mediate LPI transport in PDAC (437).

In this study we investigate the role and the potential of targeting ABC transporters in PDAC therapy. We showed that a member of the ABCC family, ABCC3, is highly expressed in PDAC tumours and that its expression is dependent on mutation of TP53. We also show ABCC3 is required for LPI-mediated PDAC progression via STAT3 and HIF1α signalling pathways, which have previously been shown to be involved in PDAC onset and progression (439, 440).

Materials and Methods

Cell lines and plasmids

Cell lines were purchased from ATCC (VA, USA) and cultured as per manufacturer's instructions in *Mycoplasma*-free conditions: AsPC1 (CRL-1682™), HPAFII (CRL-1997™), CFPAC-1 (CRL-1918™), BxPC-3 (CRL-1687™), Capan-1 (HTB-79™), Capan-2 (HTB-80™), hTERT-HPNE (CRL-4023™). Mouse primary cell lines (PZR1, PZPR1, PZPflR) were kindly provided by Owen Sansom (Beatson Institute, Glasgow, UK). Cells were authenticated and regularly tested for *Mycoplasma*. Wild-type ABCC3 cDNA encoded by recombinant pcDNA3.1 plasmid (pcDNA3-ABCC3) was a kind gift from Prof. Susan Cole (395).

RNA interference

For transient ABCC3 knockdown, four ABCC3-targeting siRNA sequences (siABCC3-1 (Hs_ABCC3_6), siABCC3-2 (Hs_ABCC3_15) QIAGEN; siABCC3-3 (J-007312-05), siABCC3-4 (J-007312-06) (Dharmacon®) and control siRNA (siSCR) were used at a working concentration of 75nM. Cells were collected at 24h (CFPAC-1) or 48h (AsPC1, HPAFII) after transfection. Western blotting was used to verify knockdown efficiency.

The siABCC3-3 sequence was used to generate pSuper retro–based vectors that express short hairpin RNA (shRNA). Control vector pSuper 4Mut contains a four-point mutated sequence unable to target the human ABCC3. Retroviral stocks were generated as previously described (441) and infected CFPAC-1 cells were selected with 1µg/ml of puromycin. Knockdown efficiency was determined by Western blotting. For proliferation

analysis, stably transfected CFPAC-1 cells (shABCC3 and 4Mut) were seeded at a density of 10,000 cells/well in a 12-well plate in the presence of 1 µg/ml puromycin and incubated for 6 days. Cells were counted daily in duplicate with trypan blue exclusion.

Cell viability and colony formation assays

PDAC cell lines seeded at a density of $5x10^4$ cells/well in 12-well or $2x10^4$ cells/well in 24-well cell culture plates were treated in duplicate, DMSO was used as a negative control. After 72 hours cells were counted with trypan blue exclusion.

To validate the effects of therapies on the ability of cancer cells to form colonies in anchorage-independent condition, soft agar colony formation assay was performed (442). Colonies were grown for 4 weeks and then fixed in 10% Acetone/Methanol, stained with a 0.05% crystal violet solution and counted.

RT-qPCR

Total RNA was extracted using GeneJET TNA Purification (Thermo Scientific, # K0732) according to the manufacturer's instructions, followed by cDNA synthesis (Thermo Scientific, # EP0742). RT-qPCR was performed according to the manufacturer's instructions (Fermentas, #K0222) using an ABI 7500 RT-QPCR system. As a control, QARS cDNA was also amplified. Changes in gene expression, relative to control, was calculated using relative $\Delta\Delta C_T$ analysis.

Protein analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer and sonicated. Proteins were separated by SDS-PAGE and detected by Western blotting according to standard procedures using the following antibodies at 1:1000 dilution: ABCC3 (Invitrogen, #PA5-23653), HIF1α (Novus Biologicals, #NB100-479), pSTAT3 Tyr705 (CST, #9131), GAPDH (CST, #5174), β-actin (CST, #4970), α-actinin (CST, #3134), α/β tubulin (CST, #2148). Anti-rabbit secondary antibody (CST, #7074) was used at 1:20000 dilution. Immunoblots were quantified using ImageJ and Image Lab 5.2.1.

Caspase 3/7 activity

Following ABCC3 silencing or pharmacological inhibition, PDAC cells were incubated with Caspase 3/7 reagent (1:1000 dilution) (Essen Bioscience) accordingly to manufacturer's instruction and monitored for up to 72h using IncuCyte Life Cell Analysis Imaging System (Sartorius).

LPI stimulation and release analysis

PDAC cells were serum-starved overnight before LPI stimulation. Cells were incubated with 1µM LPI (MerckMillipore, cat# 440153) for 8 min (acute stimulation) or 0.5 µM LPI for 72h (long-term stimulation). Cell viability and protein analysis were performed as described above.

HPAFII cells were transfected with siRNAs targeting ABCC3 and cPLA2. Twenty-four hours post-transfection, cells were labelled with [^{3}H]myo-Inositol for 48 hours. Cells were then incubated with or without EGF (20ng/ml) for 1h. Lipids were extracted from cell supernatants by phase separation and radioactivity was assessed by scintillation counting.

Radiolabelled LPI preparation

HEK293T cells were fed tritiated myo-inositol to convert into ^{3}H-LPI. The ^{3}H-LPI released by the cells was separated by thin layer chromatography and recovered.

3H-LPI transport assay

Membrane vesicles were prepared as described previously (443) from HEK293T (untransfected cells or cells transiently-transfected with pcDNA3-ABCC3). Vesicles containing 60 µg of membrane protein were incubated at 37°C for 15 minutes in 150 µl total volume containing 50mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM ATP, 10 mM $MgCl_2$, 100 µg/ml creatine kinase (Roche, UK), 10 µM creatine phosphate (Roche, UK) and 2 µM LPI (cold-LPI (Sigma-Aldrich) spiked with 0.5 nCi ^{3}H-LPI prepared as described above) in the presence or absence of 100 µM vanadate (Sigma-Aldrich). The reaction was stopped by adding ice-cold buffer (50mM Tris-HCl, 250 mM sucrose, pH 7.5). The vesicles were recovered by rapid filtration through cellulose nitrate discs (0.2µm pore size, 25mm diameter Whatman; Fisher, UK) using a 1225 Sampling Manifold (Millipore) and washed four times with of ice-cold transport buffer. The ^{3}H-LPI accumulated in the vesicles was measured in a 1049 DSA scintillation counter (Wallac).

Mouse xenograft model

All animal experiments were performed accordingly to standards of national and institutional guidelines. Xenograft work was approved by the Italian Ministry of Health (N.484/2016-PR). All animals were kept at 21°C in ventilated cages, with 12h light/ 12h dark cycle. Cages were changed twice weekly. 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* and the animals' health status was monitored daily. Stably silenced for ABCC3 expression CFPAC-1 cells were produced as described in the materials and methods (RNA interference). Athymic CD-1 nude mice (n=20) were injected subcutaneously with 3x 10^6 of CFPAC-1 sh4Mut or CFPAC-1 shABCC3 cells. Tumours of the different xenografts were monitored every week using a calliper and volumes were calculated using the formula: tumour volume = (length * width^2)/2.

Immunohistochemistry

Immunohistochemical analysis with the anti-ABCC3 antibody was performed on tissue microarrays (TMA) constructed by removing 2-mm diameter cores of histologically confirmed ductal pancreatic cancer areas from 60 invasive primary tumours. After antigen retrieval (microwave treatment at 750 W for 35 min in Tris-EDTA buffer, pH 9.0), TMA sections were incubated overnight (+4C°) with the anti-ABCC3 (sc-5776, S.Cruz, CA) goat polyclonal antibody at 1:100 dilution. The LSAB kit (K0690, Dako, Glostrup, Denmark) was used for signal amplification. In control sections, the specific primary antibody was replaced with non-immune goat serum. Tissues were counterstained with Haematoxylin. The normal pancreatic tissue presented in Figure 1C was obtained from the peritumoral tissue. The expression of ABCC3 was quantitatively assessed according to the percentage of positive tumour cells.

Statistics

The sample size for each experiment was assessed based on previous work. Results are represented as a mean ± SEM and the statistical analysis was performed for at least three independent experiments using GraphPad PRISM® V6.0 software (GraphPad Software, CA, USA). Unpaired, one-sided *t*-test (Western blot and IHC quantification), one-way ANOVA (cell growth) and two-way ANOVA (tumour growth) were used assuming independent samples and normal distributions. A 95% confidence interval was used for statistics and $P < 0.05$ was considered significant.

Results

ABCC3 is overexpressed in pancreatic cancer and correlates with poor prognosis

Enhanced expression of ABCC transporters in PDAC was suggested by previous studies (416). More detailed screening for ABCC1, ABCC3 and ABCC5 revealed significant upregulation of ABCC3 in PDAC cell lines both at the mRNA and protein levels (Figure 4.1-A, 4.1-B), suggesting that ABCC3 may be an important, but as yet unexplored player in PDAC progression. Consistent with this, our recent proteomic analysis of AsPC1 PDAC cell line showed increased expression of ABCC3 compared to undetectable levels in normal pancreatic cell lines (HPDE and hTERT-HPNE) (444). Immunohistochemical (IHC) analysis of human tissues from normal pancreas (peritumoral tissue) and PDAC samples (Figure 4.1-C) confirmed high levels of ABCC3 protein in cancer specimens. Importantly, analysis of publicly available datasets (http://www.proteinatlas.org/ENSG00000108846-ABCC3/pathology/tissue/pancreatic+cancer) showed that ABCC3 is an unfavourable prognostic marker in pancreatic cancer patients in whom high expression significantly correlates with lower survival rates (Figure 4.1-D).

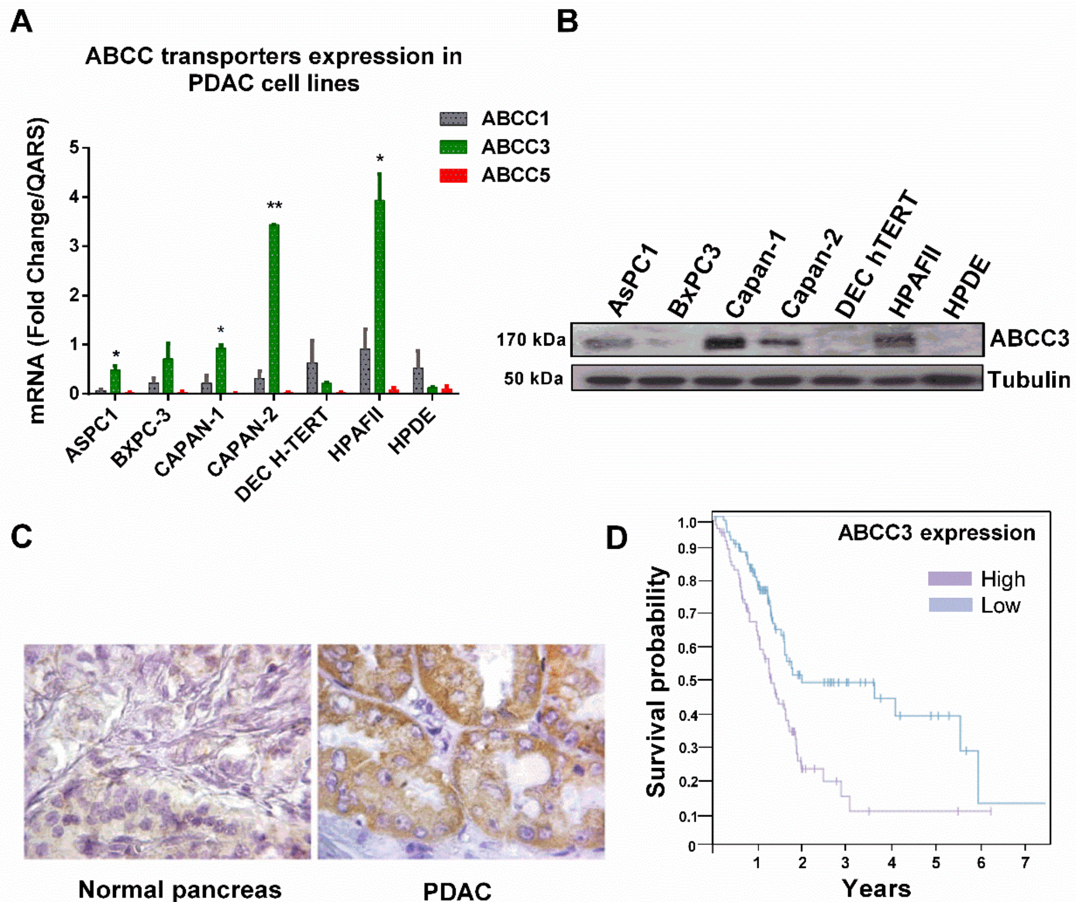

Figure 4.1. ABCC3 is overexpressed in PDAC and regulates its progression and survival

*Expression analysis of ABCC1, ABCC3 and ABCC5 in PDAC cell lines compared to non-neoplastic pancreatic cell lines (HPDE, DEC-hTERT) at (A) mRNA and (B) protein level *p<0.05, **p<0.01; (C) Immunohistochemistry (IHC) analysis of ABCC3 expression in human pancreatic tissues confirming overexpression of ABCC3 in tumour specimens (right) compared to healthy pancreas (left) (Magnification: 63x); (D) Correlation of levels of ABCC3 expression (high or low) and survival rates of the patients (http://www.proteinatlas.org/ENSG00000108846ABCC3/pathology/tissue/pancreatic+cancer).* (Figure 1 in the presented publication)

ABCC3 is an important player in PDAC growth

To investigate the role of ABCC3 in PDAC growth and progression, we stably silenced ABCC3 in CFPAC-1 pancreatic cancer cells with the use of short hairpin RNA (shRNA) (Figure 4.2-A)

and measured their growth rate *in vitro* and *in vivo* following xenograft implantation in mice. Decreased ABCC3 expression resulted in significant reduction of CFPAC-1 growth *in vitro* (Figure 4.2-B). More importantly, implantation of cells with ABCC3 stable knockdown in immunocompromised mice remarkably reduced tumour growth in the mouse xenograft model (Figure 4.2-C), suggesting that ABCC3 expression is important for PDAC progression. We confirmed the impact of ABCC3 expression on PDAC anchorage-dependent and independent growth by transient knockdown of ABCC3 in three pancreatic cancer cell lines: AsPC1, CFPAC-1 and HPAFII. Two different short interfering RNAs (siRNAs) were used to allow direct comparison of the effects of ABCC3 knockdown in different cell lines. Decreased ABCC3 expression, confirmed by Western blotting (as shown in Figure 4.4-A), correlated with a significant reduction of PDAC anchorage-dependent (Figure 4.2-D) and anchorage-independent growth (Figure 4.2-E) in all three PDAC cell lines with at least two different siRNAs. These data highlight the important role of ABCC3 in PDAC growth.

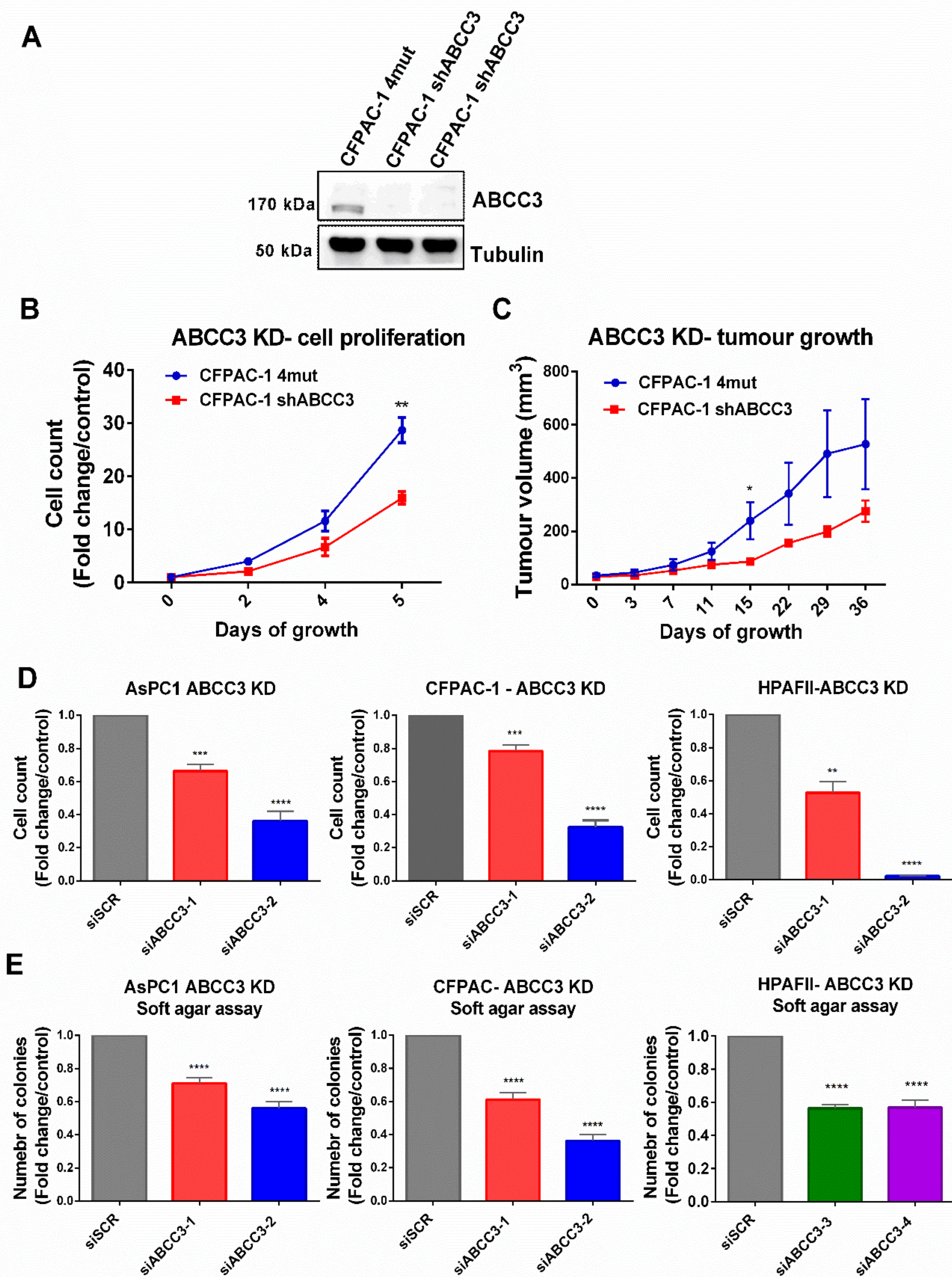

Figure 4.2. ABCC3 as an important player in PDAC progression

*(A) Stable silencing of ABCC3 expression with short hairpin RNA (shRNA) confirmed by Western blotting of two independent samples. The reduction in the in vitro (B) cell growth and in vivo (C) tumour growth induced by ABCC3 knockdown in PDAC cells *p<0.05, **p<0.01. The data is the mean ± SEM of 3 independent experiments. Blue line indicates control group, red line indicates*

*ABCC3-silenced group; The effect of knockdown of ABCC3 expression with specific siRNAs targeting ABCC3 (siABCC3-1, si-ABCC3-2, siABCC3-3, siABCC3-4) or non-targeting control siRNA (siSCR) on anchorage dependent cell proliferation (D) and anchorage-independent soft agar colony formation (E); The experiments was performed in 5 (AsPC1), 4 (HPAFII) and 7 (CFPAC-1) independent repetitions measured as mean ± SEM, **p<0.01, ***p<0.001, ****p<0.0001.*

(Figure 2 in presented publication)

ABCC3-mediated LPI release regulates progression of PDAC through STAT3 activation and induction of apoptosis

In order to investigate the role of ABCC3 in promoting PDAC progression at the molecular level, we transiently-expressed the transporter in naïve HEK293T cells and generated inside-out vesicles to test the hypothesis that ABCC3 was indeed the efflux transporter for the GPR55 ligand LPI. A seven-fold increase in accumulation of LPI was measured for the ABCC3-containing inside-out vesicles compared to naïve vesicles (Figure 4.3-A). Accumulation was dependent on added extravesicular ATP and inhibited by vanadate (Vi), as would be expected for primary-active transport by ABCC3. siRNA gene silencing experiments confirmed that EGF-dependent LPI release was both ABCC3- and PLA2-mediated, suggesting that the phospholipase is responsible for LPI production and the transporter for its release (Figure 4.3-B, 4.3-C). Furthermore, we confirmed that the addition of exogenous LPI in serum-free conditions stimulates PDAC cell line proliferation (Figure 4.3-D), which we have previously shown to be dependent on GPR55 (398). These results confirmed the involvement of LPI in the mechanisms regulating the proliferation and growth of PDAC cells. It also suggests that the inhibition of LPI synthesis or release from the cells could substantially reduce PDAC cell proliferation and decrease disease progression.

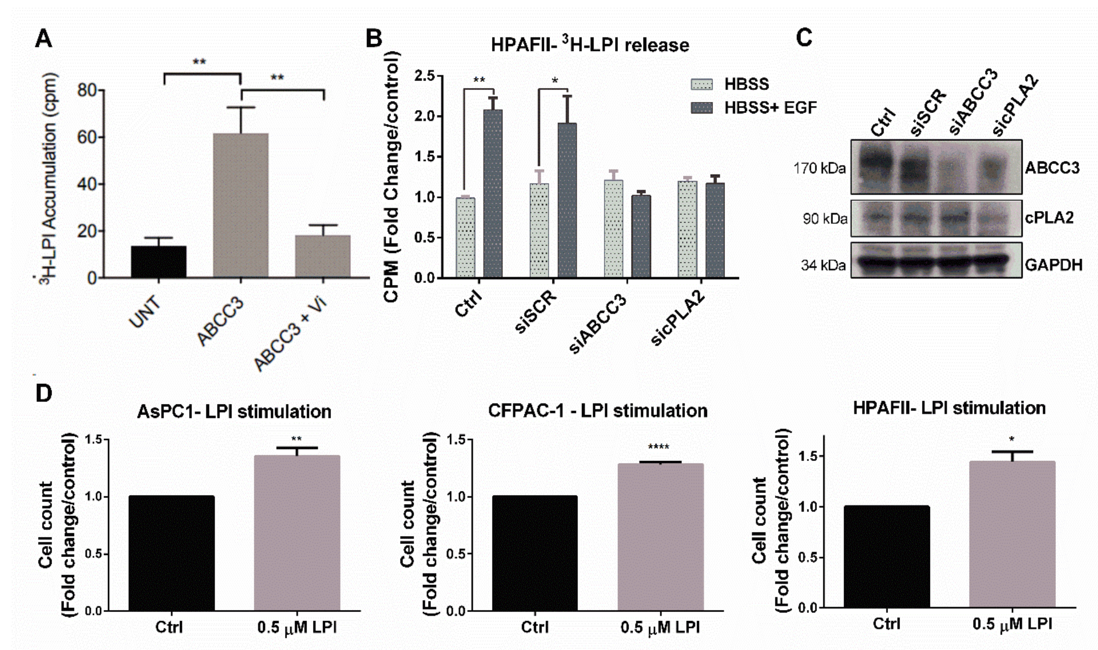

Figure 4.3 ABCC3- mediated LPI release regulates progression of PDAC

*(A) Comparison of ^{3}H LPI accumulation in HEK293T inside-out vesicles prepared from untransfected cells and cells transiently expressing ABCC3 in the presence and absence of the ATPase inhibitor vanadate (Vi) **p<0.01 (B) The effect of genetic knockdown of ABCC3 and cPLA$_2$ expression in HPAFII PDAC cell line on the [^{3}H]myo-Inositol efflux induced by EGF. Results are expressed as fold change of radioactivity detected in supernatants from untreated cells *p<0.05, **p<0.01; (C) Representative Western blot image showing the effects of the knockdown of ABCC3 and cPLA2 on the expression of the two proteins; (D) The effect of long-term stimulation with exogenous LPI (0.5 µM) of serum-starved PDAC cells (AsPC1, HPAFII and CFPAC-1) on the proliferation of cells measured after 72h. The results are presented as mean ± SEM of 4 (CFPAC-1) and 3 (AsPC1, HPAFII) independent experiments, *p<0.05, **p<0.01, ****p<0.0001.*

(Figure 3 in the presented publication)

To gain further insight into the mechanisms of ABCC3-mediated regulation of PDAC cell proliferation, signalling pathways regulated by the transporter were investigated. Expression levels of relevant signalling molecules with a proven role in PDAC tumorigenesis were studied following transient knockdown of ABCC3. Both pSTAT3 Y705 and HIF1α were shown to play a key role in both PDAC carcinogenesis and cancer stroma signalling (129,

439, 440). Western blot analysis of all three PDAC cell lines revealed suppression of phosphorylated STAT3 (pSTAT3 Y705) levels, together with a reduction in HIF1α protein levels following ABCC3 siRNA knockdown (Figure 4.4-A). Conversely, stimulation of PDAC cell lines with 1 µM LPI significantly enhanced the phosphorylation of STAT3 at tyrosine 705 (Figure 4.4-B), strongly suggesting that LPI is the mediator of ABCC3-dependent signalling.

In addition, we hypothesized that ABCC3-mediated regulation of tumour cell proliferation and growth might be partly due to the involvement of ABCC3 in apoptosis. Increased activity of caspase 3/7 was observed in cells after knockdown of ABCC3, as indicated by caspase 3/7 probe activity (Figure 4.4-C). These observations demonstrate the role of ABCC3 in PDAC progression through regulation of cell proliferation and apoptosis.

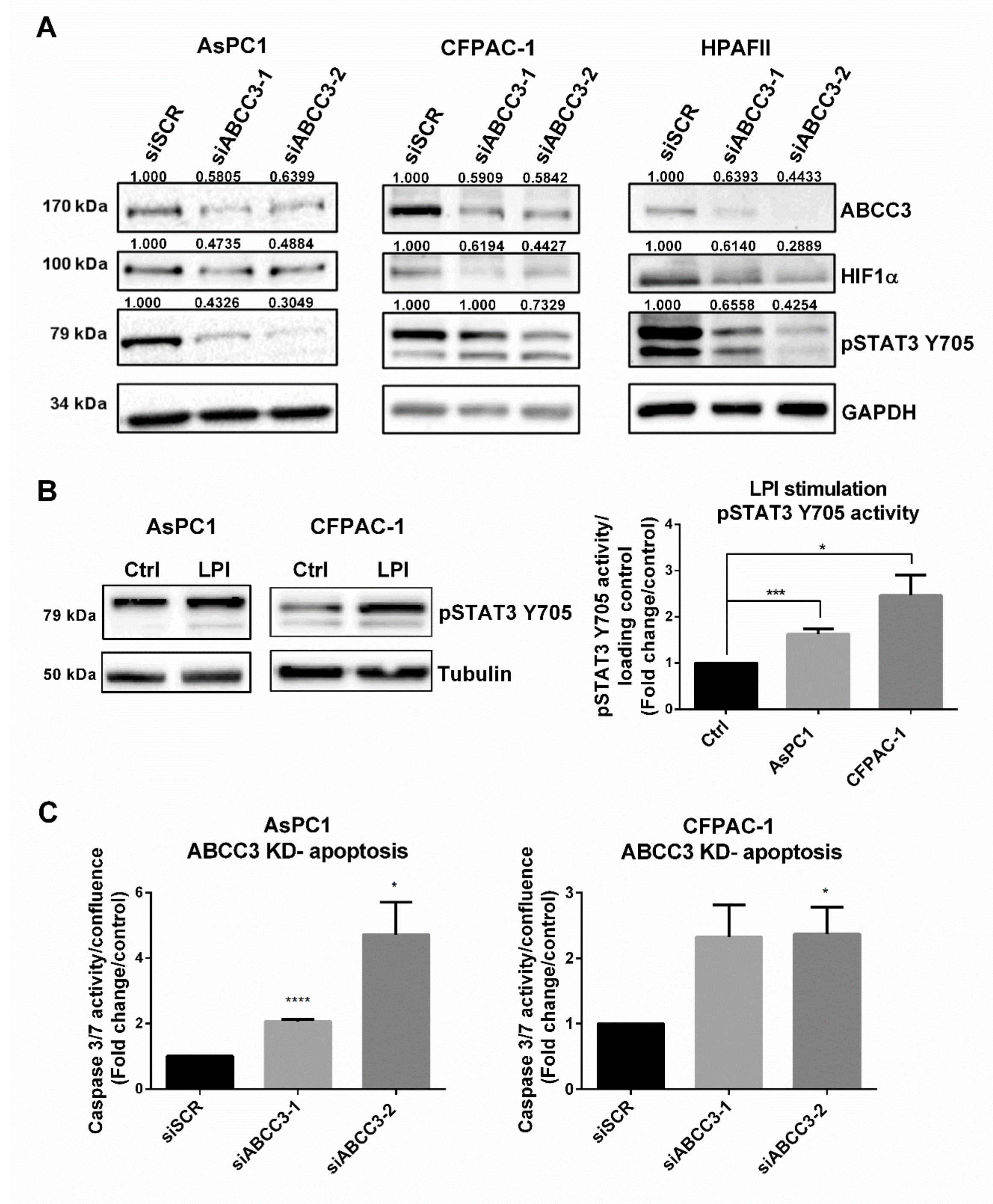

Figure 4.4 ABCC3- mediated LPI release regulates progression of PDAC through STAT3

*(A) Representative Western blot images showing the effects of transient knockdown of ABCC3 with 2 specific siRNAs (siABCC3-1, siABCC3-2) on the expression of pSTAT3 Y705 and HIF1α, quantitative analysis, normalised to control, is presented as a mean of 3 independent experiments; (B) Representative Western blot images of the stimulation of AsPC1 and CFPAC-1 cells with 1µM LPI (8 min) on the phosphorylation of STAT3 at tyrosine 705, quantitative analysis, normalised to control, is presented as mean ± SEM of 3 independent experiments, *p<0.05, ***p<0.001 (C) The effects*

*of knockdown of ABCC3 with 2 specific siRNAs (siABCC3-1, siABCC3-2) and treatment with 10µM S3 on the Caspase 3/7 activity (72h post-treatment) measured with Caspase 3/7 probe Each experiment was performed in triplicate and the results are presented as the mean ± SEM, *p<0.05, ****p<0.0001. Quantitative analysis of Western blots was performed with the use of ImageJ and Image Lab software.*

(Figure 4 in presented publication)

p53 regulates ABCC3 expression through miR-34C

Defining the genetic determinants of ABCC3 upregulation could identify a cohort of patients that might benefit from ABCC3 targeted therapy. We therefore investigated a possible mechanistic link between ABCC3 regulation and mutation of key genes in PDAC. Mutations in *TP53* are present in 50-70% of PDAC cases and are known to play an important role in pancreatic cancer progression (423). A negative correlation between ABCC3 and WT p53 expression was observed by Western blot in a panel of mouse cell lines. High expression of ABCC3 was detected in cell lines with mutated ($p53^{R172H/+}$, PZPR1) or deleted ($p53^{fl/+}$, PZPflR) *TP53* gene (Figure 4.5-A). Conversely, lower levels of ABCC3 were observed in a cell line bearing wild-type p53 (p53 WT, PZR1) (Figure 4.5-A). IHC analysis of ABCC3 expression in human PDAC tissues validated these findings, showing a statistically significant increase in ABCC3 expression in specimens with p53 immunostaining, an immunohistochemical surrogate marker of *TP53* mutation (Table 4.1).

	ABCC3 (M) mean ± SE*	P**
p53 wild-type (n = 33)	4.9 ± 2.7	0.042
p53 mutated (n = 27)	17.2 ± 5.6	

Table 4.1 *Expression of membrane ABCC3 in PDAC (n=60) according to p53 IHC status; * Mean percent of positive tumour cells ± Standard Error (SE), ** Independent-sample t-test.*

(Table 1 in the presented publication)

Analysis of an available PDAC dataset (TCGA provisional, from cBioPortal: http://www.cbioportal.org/) also confirmed increased ABCC3 mRNA expression in specimens with *TP53* mutations (Figure 4.5-B). Database analysis also suggested that miR-34C, whose expression is p53-dependent (445, 446) and is typically downregulated in PDAC, might regulate ABCC3 expression (Figure 4.5-C). For instance, Capan-2 cells characterized by low expression of miR-34C (447), show elevated expression of ABCC3 (Figure 4.1-B). Also, microRNA target prediction using miRWalk 2.0 (184) revealed that ABCC3 was a predicted target of miR-34C. Indeed, transient overexpression of miR-34C in PDAC cell lines significantly decreased ABCC3 expression (Figure 4.5-D), thus demonstrating a direct mechanism for p53-dependent regulation of ABCC3 expression. Notably, miR-34C is a tumour suppressor miRNA, and low expression of miR-34C correlates with a reduced survival rate in patients (448, 449).

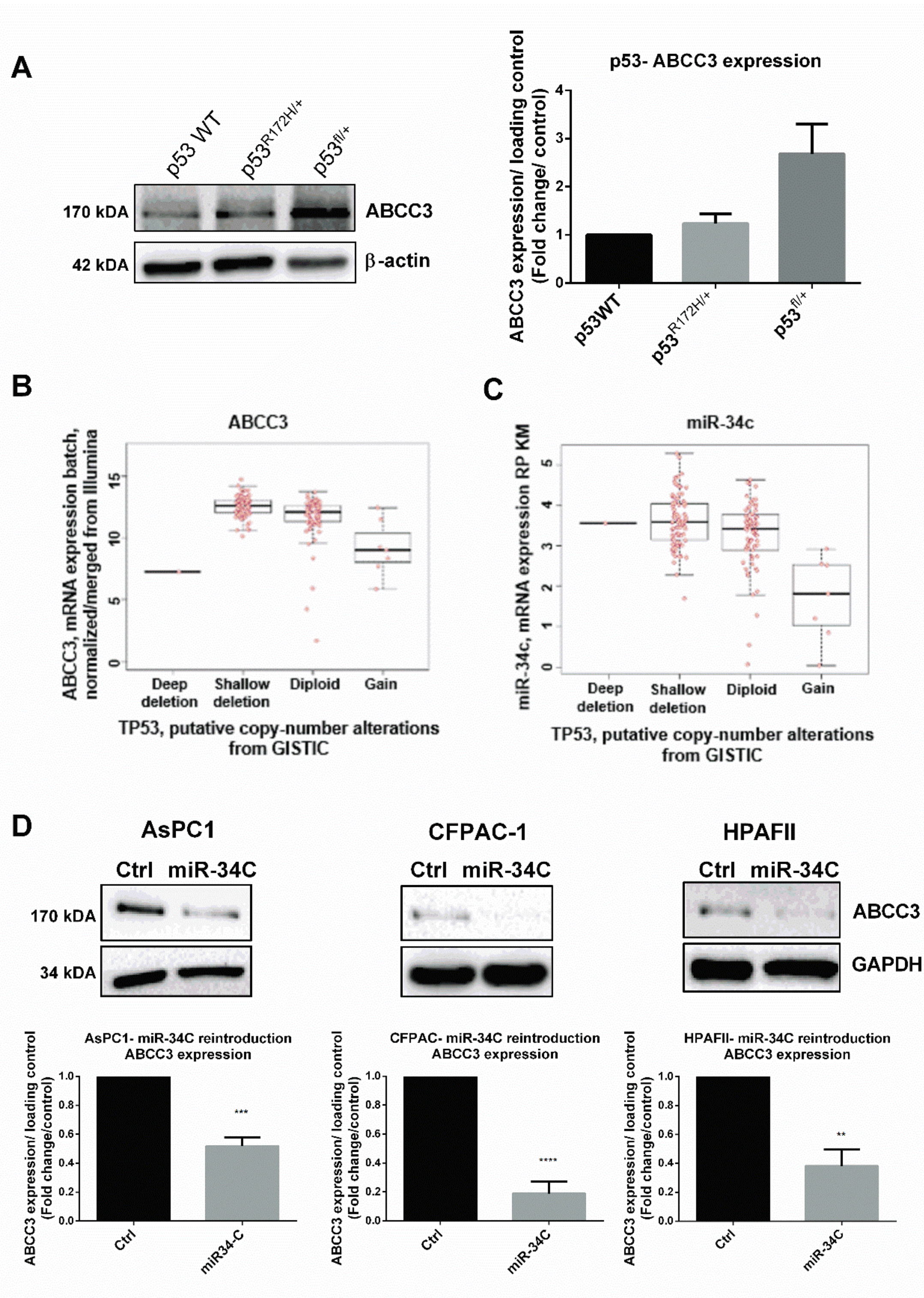

Figure 4.5 ABCC3 expression is p53-dependent

(A) Representative western blot analysis of ABCC3 expression in mice cell lines with different p53 status (p53 WT- PZR1, p53$^{R172H/+}$-PZPR1, p53$^{fl/+}$-PZPflR); Database analysis of TCGA pancreas showing negative correlation between p53 status and (B) ABCC3 expression and (C) miR-34C; (D) Representative western blot images and quantitative analysis of ABCC3 expression

*following reintroduction of miR-34C into 3 different PDAC cell lines. All results are presented as a mean± SEM of 3 independent experiments *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001*

(Figure 5 in the presented publication)

Discussion

PDAC is an aggressive malignancy whose prognosis has not changed in recent decades compared to other cancer types. Late diagnosis of the disease and high chemoresistance of pancreatic tumours greatly restrict available therapeutic options (425), leaving PDAC patients with grim prognosis. Considering marginal effects obtained with the use of standard chemotherapy, novel therapeutic approaches are necessary. Few of the most recent studies showed that non-standard therapeutic strategies may represent a chance for the improvement of the patients' perspectives. As an example, an elegant study by SL Abrams et al demonstrated the natural product berberine and its chemically modified analogues as potent inhibitors of pancreatic cancer cell proliferation (450). Similarly, metformin, a drug commonly prescribed for type II diabetes, was shown to enhance the effectiveness of co-administered therapeutics, increasing sensitivity of pancreatic cancer cells (451). Nevertheless, the identification of new therapeutic targets in PDAC that can be explored pharmacologically is still pivotal. The contribution of ABC transporters to the failure of chemotherapy has been well documented in several cancer types. Due to their ability to transport a wide variety of substrates, including xenobiotics and drugs, ABC transporters associated with cancer have been mostly studied so far for their involvement in chemoresistance (432, 433) and the potential of their inhibition to reverse the resistance and increase the efficacy of applied therapies has been explored (272, 452). Nevertheless, the involvement of ABC transporters in transporting several bioactive molecules involved in cancer progression, including prostaglandins, leukotrienes or phospholipid and their expression in immune cells and cancer stem cells, raises the question of a more direct role for ABC transporters in carcinogenesis, beyond their contribution to chemoresistance (434, 435, 453). There have been limited studies on the role and expression of ABC transporters in pancreatic cancer, most of which focused on ABC transporter-induced resistance (268, 454). Only one study, after

analysing several ABC transporters, indicated the overexpression of two transporters, ABCC3 and ABCC5, in PDAC ducts, which for ABCC3 correlated positively with the tumour grading (416). However, no study has so far reported the involvement of ABC transporters in PDAC development or identified their potential as pharmacological targets in PDAC.

Here, we have demonstrated that the ABC transporter ABCC3 is a novel and key player in PDAC biology, playing an active role in its progression. We showed that ABCC3 is highly expressed in PDAC specimens and the available bioinformatic data concurs that its expression correlates with poor prognosis for patients. We also showed that ABCC3 regulates PDAC cell proliferation *in vitro* and *in vivo* through the release of lysophosphatidylinositol (LPI), whose importance in PDAC progression we recently reported (398, 455). Having identified the essential role of LPI in PDAC progression and the transporter responsible for its secretion, we now have the opportunity to target the transporter and reduce the level of LPI in the tumour environment and interfere with cancer progression.

Initial indications that ABCC3 is a viable therapeutic target in PDAC was evident from genetic knockdown experiments which reduced PDAC cell anchorage-dependent and independent growth. Moreover, we showed that ABCC3 regulates STAT3 and HIF1α signalling pathways, key regulators of PDAC development and progression. It has been reported that constitutive activation of STAT3 signalling negatively affects the survival of PDAC patients (129). It is known that STAT3 signalling is triggered by IL6 activation of gp130 (439, 456). However, a recent study suggested the existence of gp130-independent STAT3 activation in PDAC (129), which is consistent with our findings of ABCC3-mediated STAT3 induction. These results suggest that ABCC3-regulated function of STAT3 and HIF1α may represent the potential mechanism of ABCC3- mediated PDAC progression.

Apart from the high chemoresistance of PDAC tumours the unsuccessful outcome of the majority of clinical trials in PDAC can also be attributed to the lack of proper stratification of patients into cohorts and the failure to target therapies based on the mutational landscape. We show herein that the expression of ABCC3 is dependent on the genetic status of *TP53*, one of the main genes dysregulated in PDAC. Wild type p53 levels negatively correlate with ABCC3 mRNA and protein levels and this relationship appears

to be mediated by miR-34C whose expression is dependent on p53 activity and is therefore usually downregulated in pancreatic cancer (446, 447) (Figure 4.6). It has been previously documented that in PDAC, constitutive activation of both HIF1α and STAT3 pathways is dependent on the *TP53* mutation or deletion (129), which is consistent with our findings. It has also been shown that one of the mechanisms regulating the expression of the miR34 family involves pSTAT3, whose increased expression in *TP53* mutated samples blocks the activity of miR34a (457). Similarly, in colorectal cancer, HIF1α activity in hypoxic conditions also represses miR34a expression and affects STAT3 signalling (458). It is tempting to speculate whether the activity of miR34-C might also be affected by STAT3 and HIF1α signalling in pancreatic cancer. Therefore, STAT3 and HIF1α downregulation through ABCC3 blockade might eliminate their inhibitory effect on miR34-C activity, which in turn would lower ABCC3 expression. This feed-forward loop might provide the mechanism by which pharmacological targeting of ABCC3 could reprogram pancreatic cancer cells and potentially slow down the disease progression and increase patient survival.

Our data, therefore, propose a mechanism by which pancreatic cancer cells might regulate the expression and activity of pro-tumorigenic proteins like ABCC3. It also shows the importance of genetic screening of patients before the selection of patients for clinical trials and the application of the therapy. Our data also provides an explanation for the recently demonstrated correlation between the presence of WT-p53 and the chemosensitivity of PDAC cells (459). It has been additionally demonstrated that whether the use of some drugs, e.g. Nutlin-3a, MDM-2 inhibitor, will be beneficial depends on the TP53 status, as lack of sensitivity to Nutlin-3a was shown for WT53 pancreatic cancer samples (460). Similarly, presence of WT53, which we have shown would decrease the levels of ABCC3 protein, could suggest the lower effectiveness of potential ABCC3-targeting therapies.

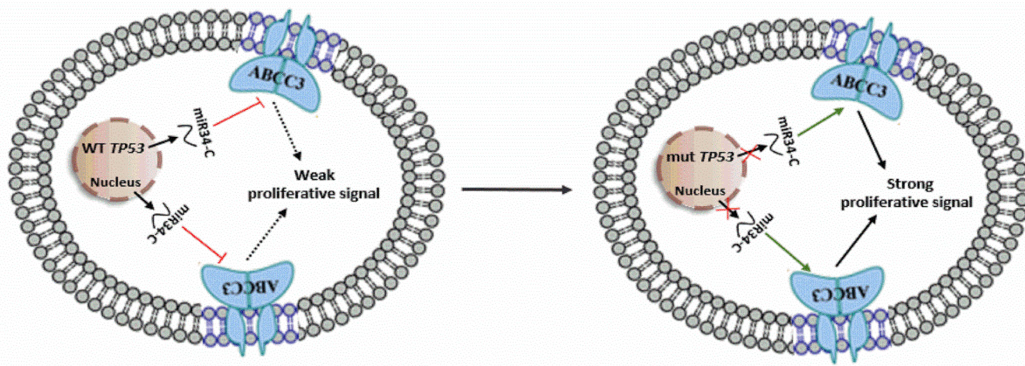

Figure 4.6. ABCC3- mediated regulation of PDAC progression depends on p53 status *The model of regulation of ABCC3 expression and activity in PDAC specimens characterized by TP53 mutations* (Figure 6 in the presented publication)

In conclusion, our data demonstrate for the first time the key role played by ABCC3 in PDAC progression. The involvement of ABCC3 in PDAC cell proliferation *in vitro* and tumour growth *in vivo* xenograft model was demonstrated. The correlation of ABCC3 expression with p53 status as well as LPI-mediated regulation of key signalling pathways in PDAC biology reinforce the importance of ABCC3 in PDAC. Collectively, our data identify ABCC3 as a promising therapeutic target in pancreatic cancer, which potential should be explored clinically. It also suggests a basis for the selection of a cohort of patients that might benefit from ABCC3-targeted therapies.

Conflict of interest

The authors declare that they have no conflicts of interest with the publication of this manuscript.

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5. ABCC3 as a novel pharmacological target in PDAC

5.1. Introduction

Parts of this introductory subchapter are adapted from the following publication:

Adamska A., Falasca M. *ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward?* World J Gastroenterol; 2018, 7; 24(29): 3222-3238. doi: 10.3748/wjg.v24.i29.3222

Whole publication, with adapted fragments highlighted, is attached at the end of the thesis. The author contribution form stating my contribution to the publication is attached at the end of the thesis.

Looking at the key role played by ABC transporters in cancer chemoresistance and the emerging knowledge on their crucial contribution to tumorigenesis, development of targeted therapies, aiming to block or modulate their activity has become a crucial area in cancer research. Inhibition of transporter activity, arrest of the transcription factors regulating their expression or blockade of the transporter-induced signalling pathways present the options for hindering ABC transporters activity (461). Especially, the well-known involvement of ABC transporters in the development of multi-drug resistance (MDR) led to the investigation of the potential of its reversal by blocking ABC transporter activity. Clinical relevance of several ABC transporters in multi-drug resistance reversal has been primarily attributed to P-gp, ABCG2, ABCB4 and 4 members of ABCC subfamily- ABCC1, ABCC2, ABCC3 and ABCC4 (462). Therefore, the main focus so far has been placed on these proteins in terms of their pharmacological potential. However, in spite of the initial enthusiasm regarding ABC transporter inhibitors, their efficacy in clinical settings has failed to provide any improvements, leading to the early closure of the trials (463, 464). Considerably high toxicity caused by lack of specificity and changes in pharmacokinetic of co-applied

chemotherapeutics, decreasing their efficacy were some of the reasons for the disappointing results (465). Therefore, in spite of the enhancement of drug accumulation and reversal of induced chemoresistance demonstrated in vitro, little success has been reported during clinical trials. Also, increased toxicity and insufficient potency observed during clinical trials restrained the majority of tested compounds from the clinical use. Moreover, several of the studies were designed without proper patient stratification for ABC transporters expression. As an example, little success rate in ovarian cancer patients, might be explained by low expression rate of P-gp in this tumour type (232).

Although reversal of the drug resistance was the principal goal of ABC-targeted therapies, considering the increasing awareness of the pivotal role of ABC transporters beyond chemoresistance, their specific inhibition might not only aid to increase the activity of other therapeutics, but directly balk tumour development and progression (466), encouraging their further exploration.

There are several reasons for the lack of success of the ABC transporters inhibition. Increased toxicity caused by off-target action in healthy tissues as well as their high doses were the main reasons for the discontinuation of the trials for first and second generation inhibitors (467). Increasing evidence of substrate similarities between ABC transporters and CYP450, enzyme involved in drug metabolism, suggests interactions of tested compounds with the enzyme, which influences pharmacokinetic properties of co-administrated chemotherapeutics, changing their activity, lowering the efficacy and as a consequence, increasing the toxicity (468). Therefore, single-agent application of ABC transporters inhibitors should be considered in future research. Another reason for high toxicity of these modulators has been attributed to decreased clearance of anticancer agents and natural xenobiotics caused by unspecific blockade of the transporters. As an example, highly increased toxicity in different tissues has been noted after abcb1 gene disruption. ABCB1 inhibition, apart from cancer cells may also result in its blockade in canalicular membrane in healthy liver or kidney, reducing the clearance of chemotherapeutics (467, 469). However, although reversal of the drug resistance was the principal goal of ABC-targeted therapies, current knowledge suggests that developed drugs may

also serve as potent direct inhibitors of cancer development and progression, encouraging for their further exploration.

5.2 Aims of the project

I have demonstrated that ABCC3 is a novel played in PDAC progression. ABCC3-mediated regulation of PDAC progression through STAT3 signalling and apoptosis was demonstrated. Importantly, the importance of the presence of active ABCC3 for PDAC tumour growth was shown in vivo. Therefore, the pharmacological potential of ABCC3 was explored. So far, targeting of ABC transporters was mainly studied in terms of the reversal of multidrug resistance and increase of the activity of co-administered chemotherapeutics. However, no studies verified the effects of inhibition of ABCC3 with single-agent drugs on PDAC progression.

Thus the aim of this study was:

- Development and characterization of specific inhibitor of ABCC3
- Evaluation of the effectiveness of developed drug *in vitro*
- Evaluation of the effectiveness of developed drug *in vivo*
- Determination of the mechanisms of action of developed drug

5.3 Results

Following the demonstration of the essential role that ABCC3 plays in PDAC progression, the pharmacological potential of the transporter was explored. Dr Riccardo Ferro, QMUL, tested several molecules known to target ABCC transporters (e.g. Reversan or MK-571). However, no significant effect of the analysed drugs on the cell proliferation could be observed and high doses of both drugs needed to be used to exert their activity. Therefore, screening of more specific molecules targeting ABCC3 was performed and their anti- proliferative potential was assessed.

5.3.1 S3 is a specific inhibitor of ABCC3

Sulindac is a nonsteroidal anti-inflammatory drug (NAID) that inhibits cyclooxygenases involved in prostaglandin biosynthesis but has also been shown to target ABC transporters (470, 471). Screening of a large library of compounds identified a novel derivative of sulindac coded as MCI-715 and referred to here as S3. The potency of S3 to block ABCC3 activity was investigated and compared to the effects achieved with sulindac treatment. Calcein-AM is a hydrophobic dye that fluoresces in the green spectrum. It is also a transport substrate of ABCC3 allowing the development of a live-cell assay to measure ABCC3 activity and inhibition. ABCC3 was transiently expressed in naive HEK293T cells as shown by Western analysis (Figure 5.1-A). By titration of the concentration of inhibitor, an IC_{50} for S3 was determined and found to inhibit ABCC3 with 2-fold higher potency than the parent compound sulindac sulphide, as shown by measurement of calcein accumulation (Figure 5.1-B). Additionally, plasma levels of orally administered S3 were approximately 10-fold higher relative to sulindac for a sustained period (Appendix, Figure 1). Importantly, relative to sulindac, which inhibited COX-1 and COX-2 with IC_{50} values of 1 and 6.7µM respectively (Figure 5.1-C), S3 did not inhibit COX-1 or COX-2 above 50% at concentrations of 100µM (Figure 5.1-D).

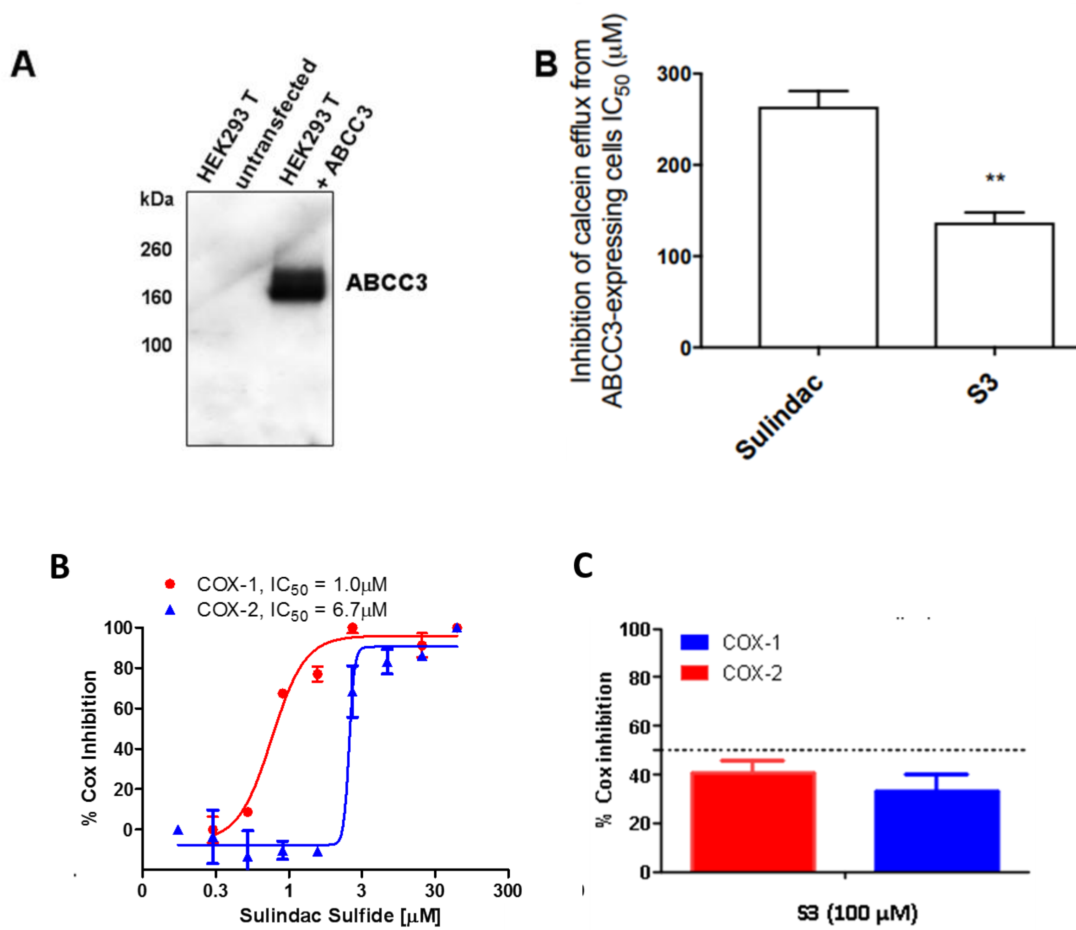


Figure 5.1 ***S3 is a small molecule inhibitor of ABCC3.***

(A) Western blot probed with anti-ABCC3 antibody (C-18; Santa Cruz) confirming the overexpression of ABCC3 in transfected cells. (B) S3 is a more potent inhibitor of calcein-AM efflux by ABCC3 than sulindac sulphide. IC_{50} data are presented as mean ± SEM of 3 independent experiments, statistical analysis by unpaired Student's t-test $p = 0.0033$; Comparison of Sulindac (C) and its derivative S3 (D) in inhibiting COX-1 and COX-2. Results are presented as mean ± SEM of 3 independent experiments. The S3 development and characterization was performed by Dr Gary Piazza (University of South Alabama) and Prof Kenneth Linton (QMUL).

These observations suggest the potential of S3 as an efficient and safe approach, compared to sulindac, as an ABCC3 inhibitor. Importantly, lack of COX-1 or COX-2 inhibition demonstrates that the potential anti-tumorigenic effectiveness of S3 cannot be attributed to the anti-inflammatory activity reported for sulindac.

5.3.2 Pharmacological inhibition of ABCC3 with S3 significantly reduces PDAC cell growth

The potential effectiveness of S3 in blocking PDAC cell proliferation was then tested in four PDAC cell lines (AsPC1, HPAFII, CFPAC-1 and SW1990). At the same time, two other sulindac derivatives, called S3A and SuSu, were also tested to verify the potency and specificity of S3. Briefly, cells were treated with increasing doses of S3, S3A and SuSu (0, 2.5µM, 5µM, 10µM) and cells were counted manually 72 hours post-treatment.

Significant reduction in the number of viable cells was observed after treatment of the PDAC cells with S3 in a dose dependent manner, showing high efficiency of S3 in targeting of ABCC3 and reducing PDAC cell proliferation. Similar trend could be observed in the three analysed cell lines (AsPC1, HPAFII, and CFPAC-1) (Figure 5.2-A, B, C). Interestingly, remarkably reduced effectiveness of S3 was detected in the SW1990 cell line, the only cell line bearing wild type p53 protein, in which low ABCC3 expression was demonstrated (Figure 5.2-D, E). At the same time, no consistent results could be obtained with the use of two other sulindac derivatives, S3A and SuSu, confirming the specificity and efficacy of S3 (Appendix, Figure 2).

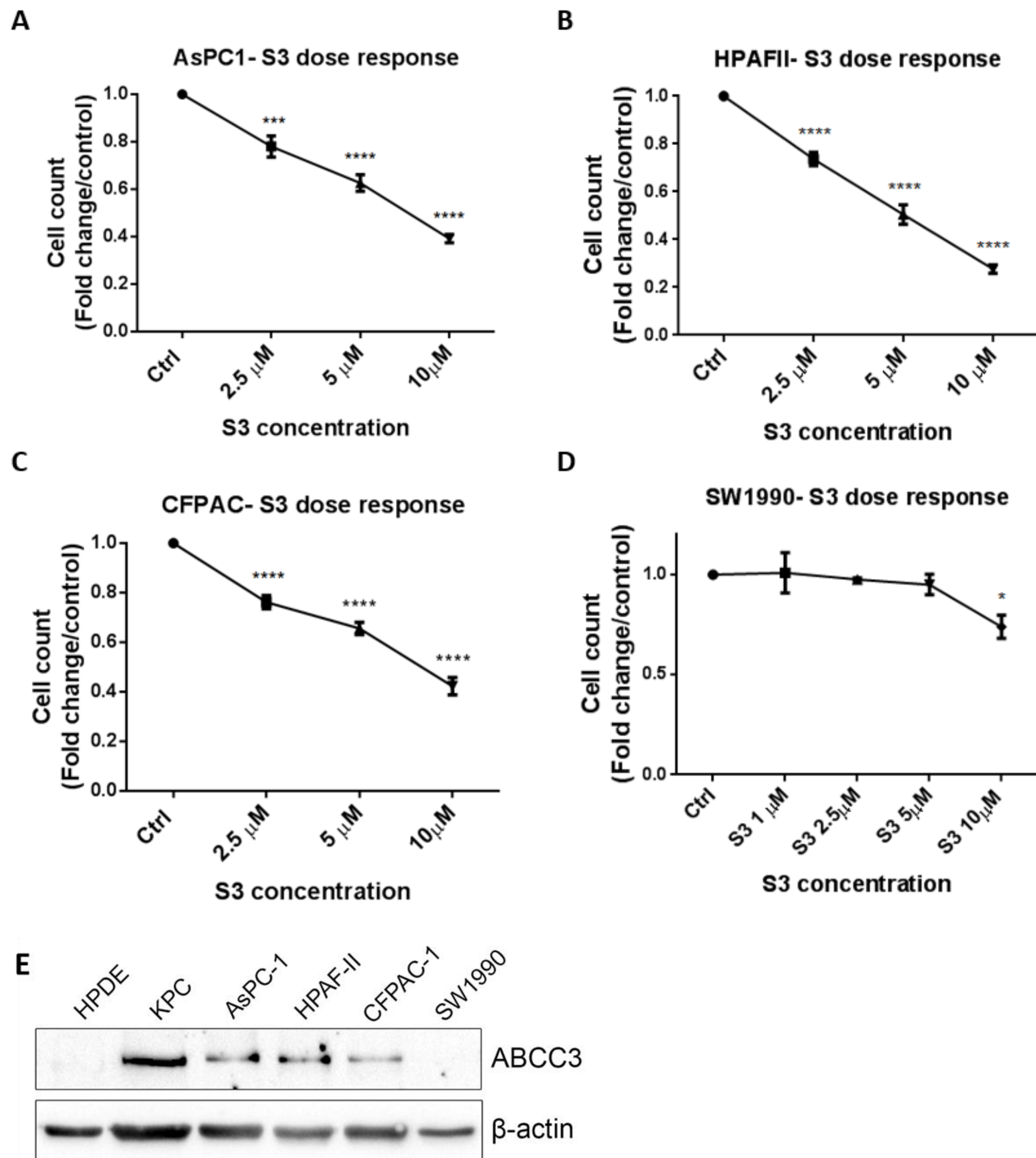


Figure 5.2 ***Pharmacological inhibition of ABCC3 with S3 decreases growth of PDAC cells bearing mutant p53.***

*The effects of S3 treatment on the viability of (A) AsPC1, (B) HPAFII, (C) CFPAC-1 and (D) SW1990 PDAC cells. The results are presented as mean ± SEM of 3 independent experiments, *p<0.05, ***p<0.001, ****p<0.0001; (E) Representative Western Blot image showing the expression of ABCC3 in studied cell lines.*

These data demonstrate S3 as a potent and specific inhibitor of ABCC3 and show its effectiveness in reducing growth of *TP53* mutated PDAC cells.

5.3.2.1 S3 exhibits higher efficiency in serum-free conditions

The tumour microenvironment deprives cancer cells of oxygen and nutrients necessary for their fast-proliferative needs. Therefore, serum-free conditions for the cancer cell growth allow 'mimicking' the harsh condition that tumour cells have to face. In order to verify cell behaviour and response to the S3 treatment in nutrient-deprived conditions, cells were serum-starved for 24h prior to subjection to the treatments. Treatment of three PDAC cell lines (AsPC1, HPAFII, CFPAC-1) with increasing doses of S3 in serum-free media significantly decreased cell growth compared to the control cells (incubated with DMSO). More strikingly, the effect of ABCC3 inhibition was remarkably higher in these conditions, compared to the same treatments performed in complete media, with significantly higher response rates in cells deprived of essential nutrients (Figure 5.3). These data reinforce the effectiveness of S3 and show its potential as a potent therapeutic agent in PDAC therapy.

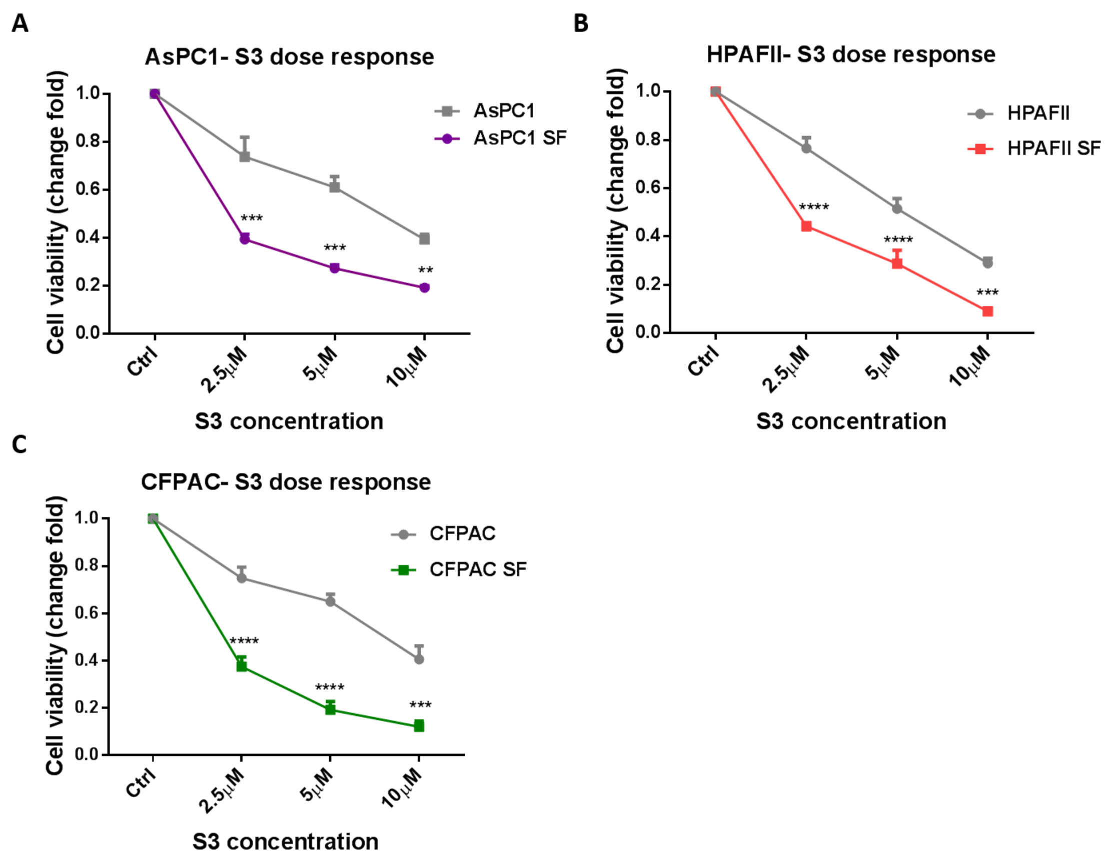


Figure 5.3 ***S3 decreases PDAC cell growth in serum-free conditions****. The comparison of the effects of S3 treatment of (A) AsPC1, (B) HPAFII and (C) CFPAC-1 cells in complete media (grey) and serum-deprived (SF) media on the viability of the cells. The results are presented as mean ± SEM of 3 independent experiments, **p<0.01, ***p<0.001, ****p<0.0001*

5.3.3 Pharmacological inhibition of ABCC3 with S3 significantly reduces PDAC clonal expansion

To further verify the pharmacological potential of ABCC3 inhibition, the *in vitro* anchorage-independent growth of AsPC1, HPFAII and CFPAC-1 cells was tested upon treatment with increasing doses of S3 at the same concentrations used for the cell viability assay. The soft agar assay is used to analyse cancer cell growth in anchorage-independent conditions and enables to investigate other cell characteristics such as *in vitro* clonal expansion. Malignant cells possess the ability to grow and propagate in the three-dimensional (3D) anchorage-independent conditions, characteristic that cannot be attributed to non-neoplastic cells. Thus, soft agar assay may be used to assess the tumorigenic potential of the cells. It is also a valuable tool to verify the pharmacological potential of drug candidates.

Cells were grown in agarose gel in the presence of increasing concentrations of each drug and the number of colonies was assessed after 4 weeks. Consistent with genetic downregulation of ABCC3, expression and similarly to the cell viability assay, a significant reduction in the number of colonies was detected following the inhibition of ABCC3 activity in the tested cell lines (Figure 5.4).

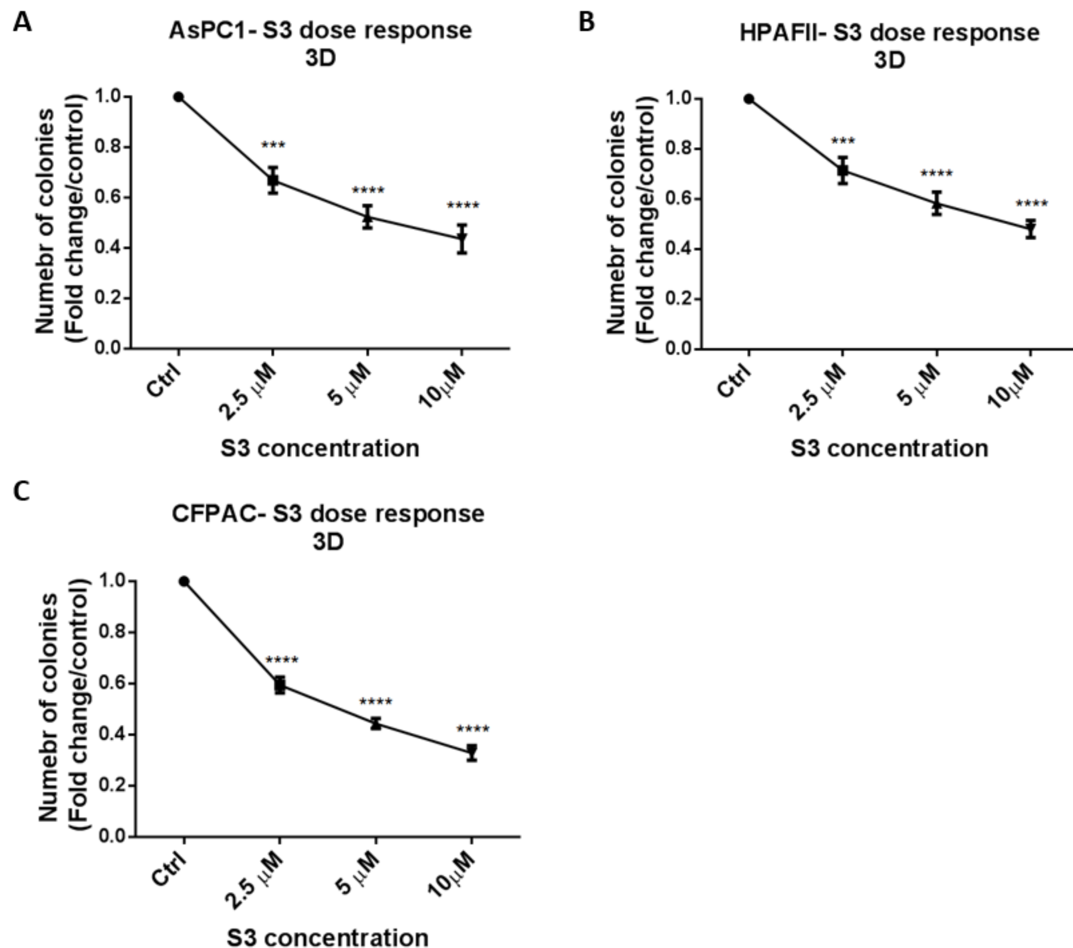


Figure 5.4 Pharmacological inhibition of ABCC3 with S3 significantly reduces PDAC clonal expansion. *The effects of the pharmacological inhibition of ABCC3 with increasing doses of S3 on the colony formation in PDAC cell lines: AsPC1 (A), HPAFII (B) and CFPAC-1 (C). The results are presented as mean ± SEM of 3 independent experiments; ***p<0.001, ****p<0.0001*

The obtained results confirmed the potential of ABCC3 inhibition with S3 for the reduction of PDAC cell growth in the more complex environment, in which not only cell proliferation but also tumorigenic potential and clonal expansion may be assessed. This initial *in vitro* validation also gave the base for further exploration of pharmacological potential of ABCC3 blocking with the developed small molecule inhibitor in counteracting PDAC progression in *in vivo* models.

5.3.4 Investigation of the effects of the combination of ABCC3 inhibition and chemotherapy in vitro

There is a tendency in the current therapeutic regimens and clinical trials to combine analysed targeted therapies with standard chemotherapy in order to increase their efficacy and provide a backup treatment in case of the failure of the experimental targeted treatments. A wide variety of agents targeting main pathways in PDAC tumorigenesis have been analysed in clinical trials, with only few of them showing efficiency in clinical settings (e.g. EGFR-targeting Erlotinib combined with gemcitabine).

Therefore, despite a remarkable and significant potency of S3 in decreasing the proliferative abilities of PDAC cells shown in 2D and 3D settings, the potential enhancement of its efficiency by its combination with several chemotherapeutics used in PDAC therapy was investigated. Gemcitabine, paclitaxel and docetaxel were used for the initial *in vitro* experiments, as the chemotherapeutics most commonly used in PDAC treatment. Initially, a dose response of selected chemotherapeutics was performed on studied PDAC cell lines in order to select the suboptimal drug concentration. Thanks to that, the potential increase in the efficiency of the tested drug combinations could be attributed to their additive/synergistic effects and not to the effectiveness of the single drugs. The selected concentration for each drug was:

Gemcitabine: 2nM

Paclitaxel: 2.5 nM

Docetaxel: 2nM

Each tested PDAC cell line was treated with two different doses of S3, selected concentration of chemotherapeutic and their combination. Cells were counted manually 72 hours post- treatment.

A remarkable reduction in the viability of PDAC cells was demonstrated after the combination of S3 with the analysed chemotherapeutics, compared to each of the drugs used as single agent (Figure 5.5). The effects were more pronounced with the

use of higher doses of S3, which combination with Gemcitabine, Paclitaxel and Docetaxel significantly reduced cell growth.

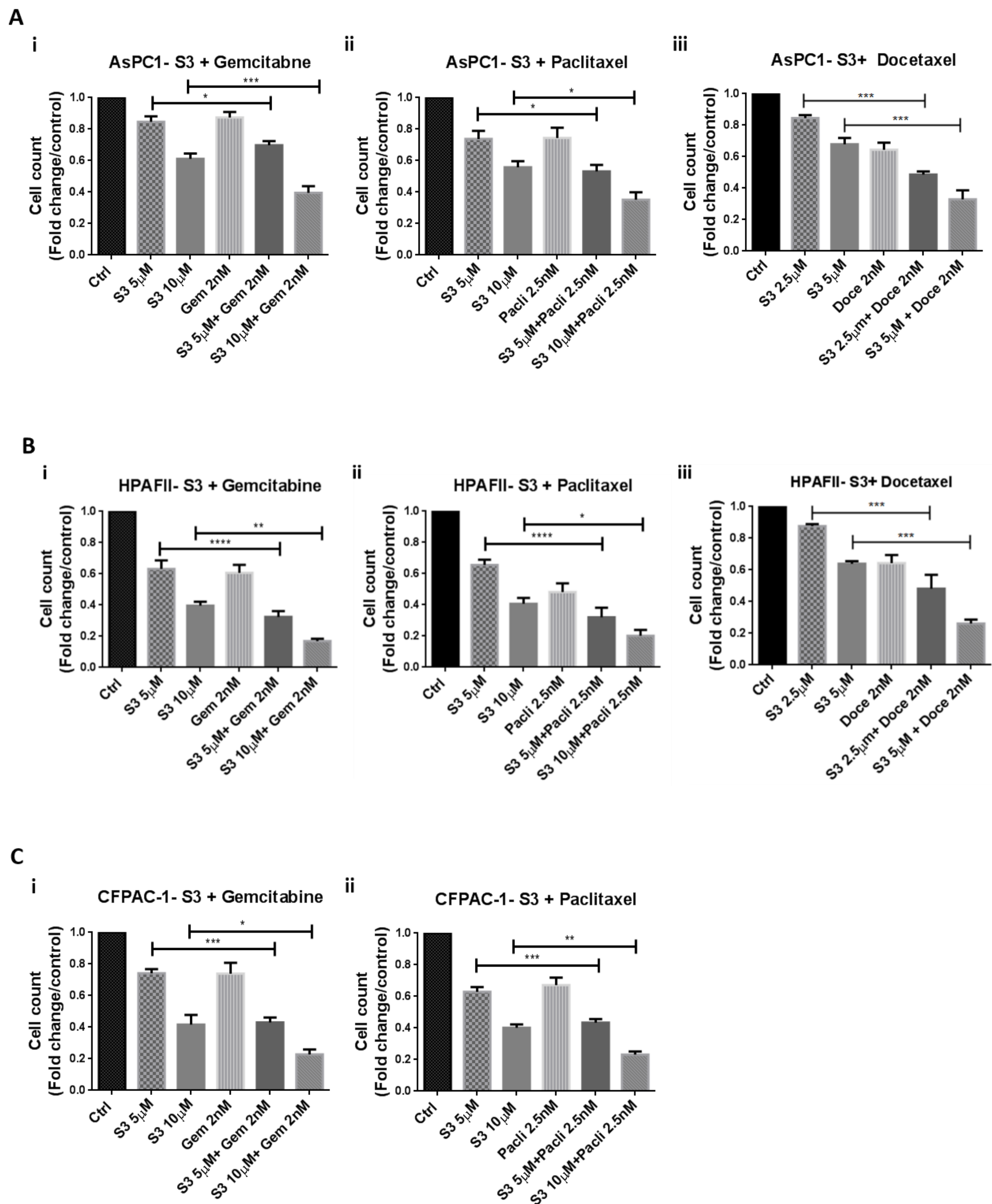


Figure 5.5 Combination of S3 with chemotherapy decreases PDAC cell growth in vitro. *Comparison of the effects of the treatment of (A) AsPC1, (B) HPAFII and (C) CFPAC-1 cells with the combination of S3 and (i) Gemcitabine, (ii) paclitaxel or (iii) docetaxel on the cell number, compared to the effects of S3 alone. The results are presented as mean ± SEM of 3 independent experiments; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

CompuSyn analysis of the tested combinations was then carried out to verify a potential additive/ synergistic effects of the combinations. Based on the cell viability values reported for each of the extract, as well as for the tested combinations, the Combination Index is calculated by the program. CI values lower than one indicate the synergism between tested drugs, whereas values above 1 indicate their antagonism. Values close to 1 are indicative of additive effect. Overall, most of the combinations showed at least additive effects, demonstrating the probability of enhanced effectiveness of proposed drug combinations (Table 5.1).

Of all the tested cell lines, AsPC1 cell line appeared to be the least responsive to S3 and gemcitabine, confirming previous results. However, the combination of both drugs showed a synergistic effect, significantly reducing cell viability, especially when 10 µM S3 was used. A higher synergism could be also demonstrated for S3 and Paclitaxel combinations, which remarkably reduced cell proliferative abilities, significantly decreasing the number of viable cells compared to the single-agent treatments. Similarly, Docetaxel/S3 combination showed a definite synergistic effect in the AsPC1 cell line. Higher responsiveness of the HPAFII cells to both S3 and gemcitabine resulted in an increased synergism of these drugs in reducing the viability of HPAFII cell line. Synergism between S3 and paclitaxel was also demonstrated. However, due to the high responsiveness of HPAFII cells to paclitaxel alone, the combination effect did not show a synergism as high as in case of S3/Gemcitabine combination. Therefore, the significant decrease in cell number observed for this combination might have been mainly due to cell response to paclitaxel. Nevertheless, a synergy of this combination could be also shown. Similarly, S3 and docetaxel treatment showed a high synergism in HPAFII cells, especially when treated with 5µM S3 and docetaxel combination, which reduced cell viability of almost 3 times compared to single drugs. A high synergism between S3 and the analysed chemotherapeutics was also demonstrated in the CFPAC-1 cell line, especially at higher doses of S3.

Drugs combination	AsPC1		HPAFII		CFPAC-1	
S3 concentration	5µM	10µM	5µM	10µM	5µM	10µM
Combination index (CI)						
S3+ 2nM Gemcitabine	1.3587	0.7723	0.7376	0.5383	0.8268	0.5488
S3+ 2.5 nM Paclitaxel	0.8036	0.9766	0.9262	0.7611	0.8804	0.5962
S3 concentration	2.5µM	5µM	2.5µM	5µM	–	
Combination index (CI)						
S3+ 2nM Docetaxel	0.7144	0.4192	1.019	0.6341	—	

Table 5.1 Combination of S3 with chemotherapy shows synergy in inhibiting PDAC cell growth in vitro. *Combination index (CI) values for the treatment with S3 and gemcitabine, paclitaxel and docetaxel combinations in PDAC cell lines. Values <1 indicate synergism, 0.9< CI< 1.1 indicate additive effect*

Overall, based on the preliminary *in vitro* data, I could demonstrate a high synergism of S3-based combination therapies in decreasing the viability of PDAC cell lines, especially at higher concentrations of S3. These results give the basis for the potential enhancement of the effectiveness of current chemotherapies, by their combination with ABCC3-targeted treatments. Therefore, *in vivo* validation of the studied combinations should be considered.

5.3.5 Pharmacological inhibition of ABCC3 with S3 reduces the activity of STAT3 and HIF1α signalling

As demonstrated in the previous chapter, I showed for the first time the novel involvement of ABCC3 in the regulation of two signalling pathways crucial for PDAC tumorigenesis: STAT3 and HIF1α. Having provided the evidence of the high efficiency of S3 in slowing down PDAC cell growth and reducing their tumorigenic potential, the

mechanisms behind these effects were explored. Based on the hypothesized specificity of S3 towards ABCC3, the effects of S3 treatment were compared to the demonstrated ABCC3-regulated processes. Thus the potential effects of S3 treatment on the activity of STAT3 and HIF1α signalling, as well as the induction of apoptosis and cell cycle arrest were verified.

Similar to the knockdown of ABCC3, the optimal time and conditions of the treatment were selected for each of analysed cell lines. Treatment time is an especially important factor for the detection of changes in the activity of phosphorylated proteins like pSTAT3 Y705, which too excessive inhibition leads to the signal upregulation. As an example, 24h treatment of CFPAC-1 cell line with 10 µM S3 was shown to be sufficient to decrease the levels of phosphorylated STAT3 at tyrosine 705, whereas the increase of the treatment time to 48 or 72h resulted in the detection of enhanced levels of that protein compared to the control samples, similarly to the effects observed after ABCC3 knockdown (Appendix, Figure 3-A, B). However, the short treatment time (24h) was not adequate for AsPC1 and HPAFII cell lines, which required 48h treatment to achieve similar effects. For each of the studied cell lines I could show a significant decrease in the phosphorylation of STAT3 at tyrosine 705 following treatment with S3 compared to the control samples (DMSO) (Figure 5.6). At the same time, downregulation of total STAT3 was also observed (Appendix, Figure 4). Similarly, a significant reduction in HIF1α expression was observed for the same samples (Figure 5.6).

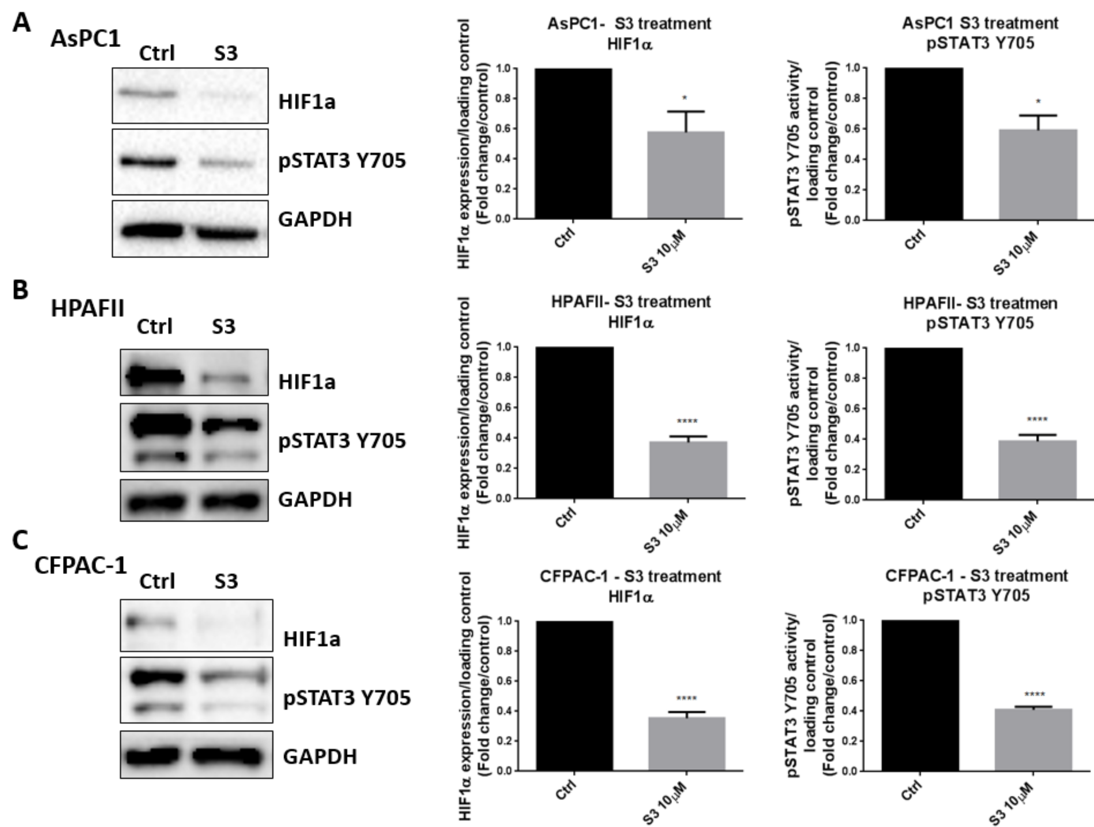


Figure 5.6 Pharmacological inhibition of ABCC3 decreases STAT3 and HIF1α activity. *Representative Western blot images and quantitative analysis of the effects of S3 treatment on the expression of HIF1α and phosphorylation of STAT3 at Tyr 705 in (A) AsPC1, (B) HPAFII and (C) CFPAC-1 cell lines. The results are presented as mean ± SEM of 3 independent experiments, *p<0.05, ****p<0.0001*

These results confirm the role of ABCC3 in the regulation of STAT3 and HIF1α signalling and demonstrate that the pharmacological inhibition of ABCC3 with S3 yields the same results as the ABCC3 knockdown, suggesting specificity of S3 towards ABCC3. In addition, the possibility of indirect targeting and modulation of the STAT3 activity might provide new approach in PDAC targeted therapies.

5.3.6 Pharmacological inhibition of ABCC3 induces apoptosis and blocks cell cycle in PDAC

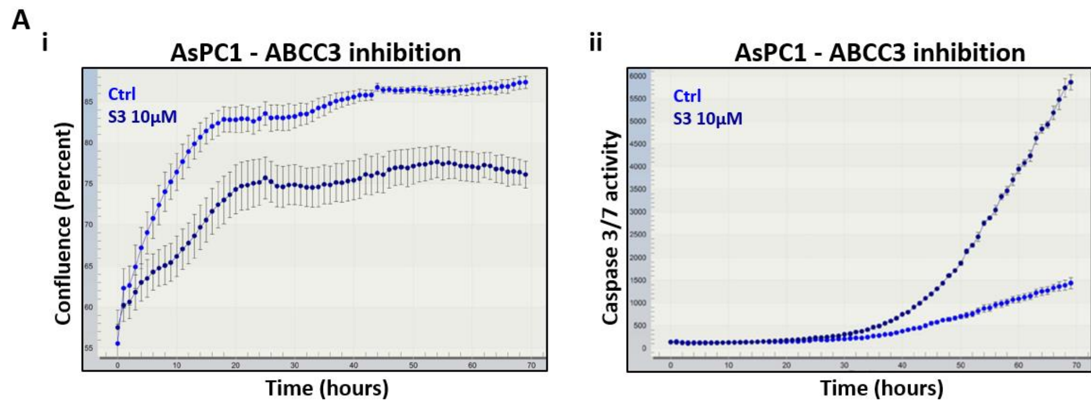
5.3.6.1 Pharmacological inhibition of ABCC3 with S3 induces apoptosis in PDAC cell lines

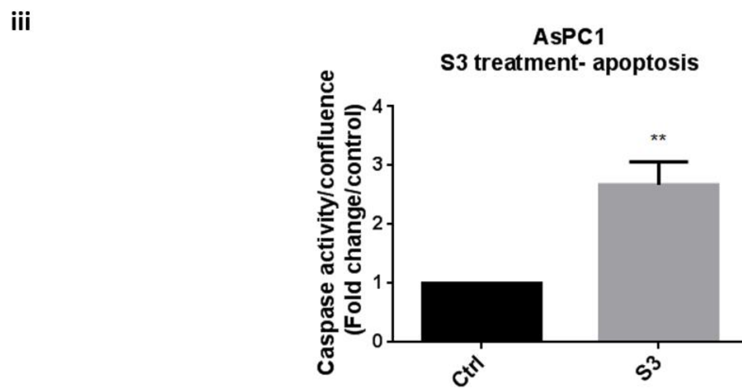
Based on the ability of cancer cells to escape apoptosis stimuli, the mechanisms of action of a great part of anti-cancer therapeutics are based on the induction of apoptosis in cancer cells. In this chapter, I demonstrated that the knockdown of ABCC3 results in the induction of apoptosis in PDAC cells *in vitro*, as shown by the analysis of Caspase 3 activity. To verify if, similarly to ABCC3 knockdown, the inhibition of the transporter with S3 induces the apoptosis in PDAC cells, the activity of caspase 3/7 and expression of cleaved caspase 3 was analysed.

Cells were seeded in 96-well plate at the density of 10.000 cells per well and treated with a media containing 10µM S3 combined with Caspase 3/7 probe. Cells were monitored in the IncuCyte instrument for 72h. The apoptotic index was calculated as a fold change between time 0 and 48 treatment. As a complimentary approach, the expression of the cleaved caspase 3 after S3 treatment was analysed by Western blotting.

Increased activity of Caspase 3/7 was detected in AsPC1 (Figure 5.7-A-i), HPAFII (5.7-B-i) and CFPAC-1 (5.7-C-i) cells after treatment of the cells with 10µM S3. At the same time, a decreased cell confluency was shown in the cells (Figure 5.7-ii) resulting in the increased apoptotic index (Figure 5.7-iii).

A





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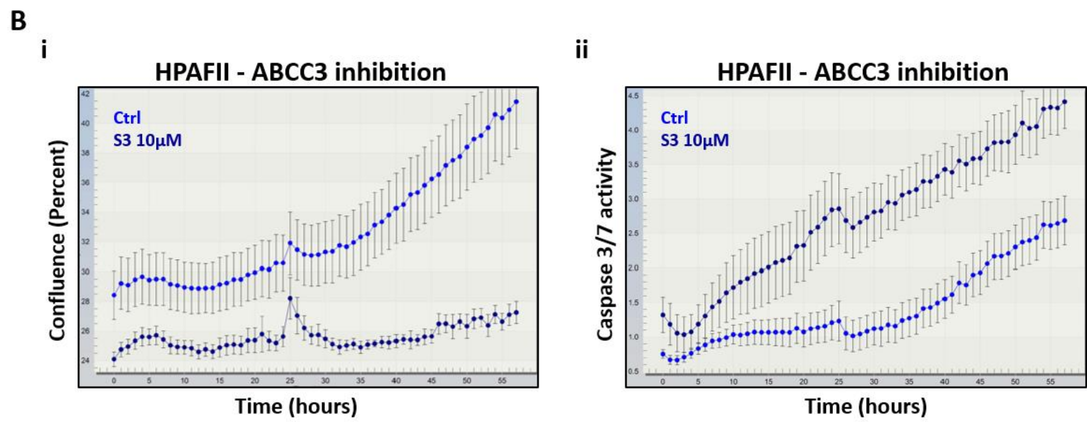
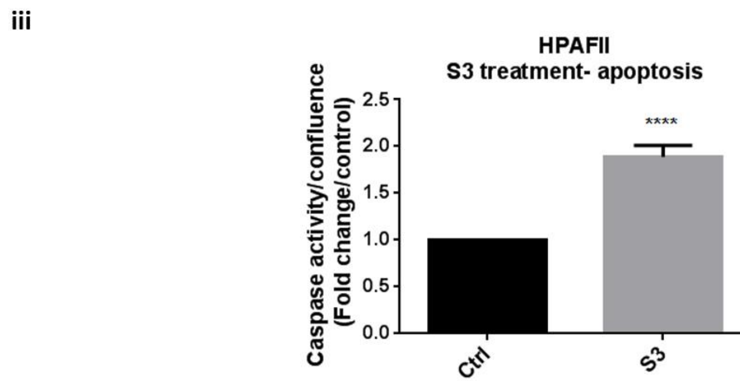




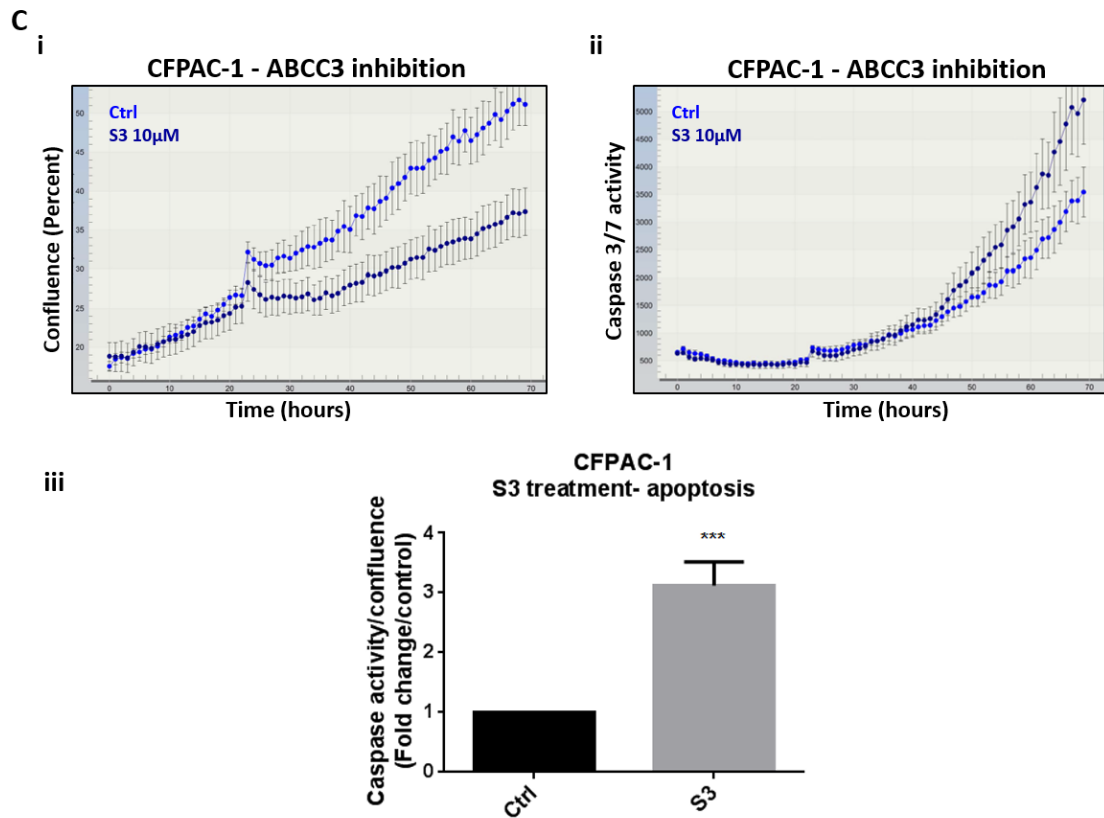


Figure 5.7 Inhibition of S3 induces apoptosis in PDAC cell lines in vitro. *Effects of S3 treatment on the activity of Caspase 3/7 (i) and cell confluency (ii) in AsPC1 (A), HPAFII (B) and CFPAC-1 (C) cell line; (iii) Calculated apoptotic index in AsPC1, HPAFII and CFPAC-1 cell line following ABCC3 inhibition with S3. Results are represented as mean ± SEM of 3 independent experiments, **p<0.15, ***p<0.001, ****p<0.0001*

In addition, elevated levels of cleaved caspase 3 were detected in the same cell lines following ABCC3 inhibition with S3, as demonstrated by Western blot analysis (Figure 5.8). In addition, differences in cell morphology were noted in the cells treated with S3, resulting in cell swelling, suggesting also occurrence of cell senescence; however, these observations need further validation.

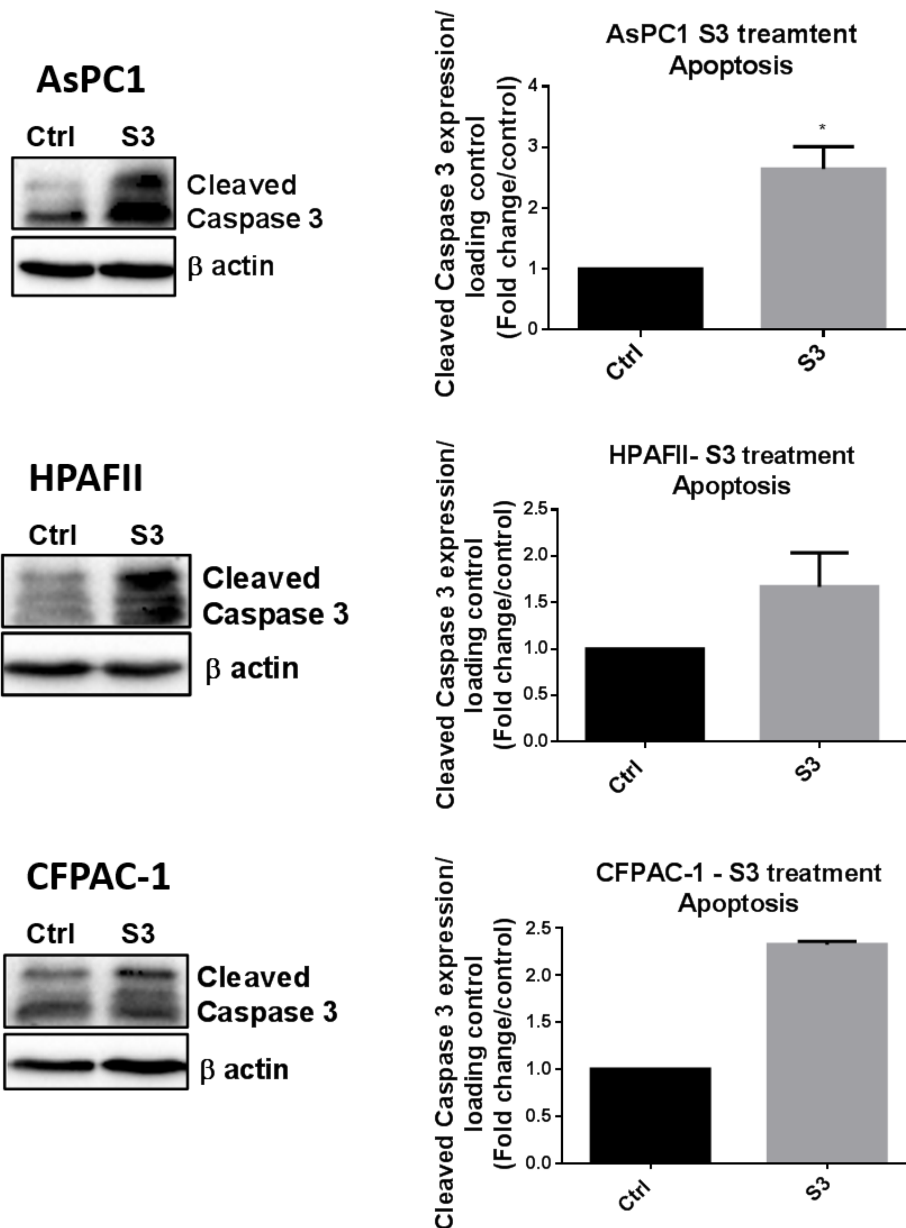


Figure 5.8 Inhibition of S3 induces apoptosis in PDAC cell lines in vitro. *Representative Western blot images and quantitative analysis of the effects of S3 treatment on the levels of cleaved Caspase 3 in AsPC1, HPAFII and CFPAC-1 cell lines. The results are presented as mean ± SEM of 3 independent experiments, *p<0.05*

As a complimentary approach, FACS analysis of Annexin V and PI staining of two PDAC cell lines (AsPC1, CFPAC-1) following S3 treatment was performed. Annexin V is the indicator of cells undergoing apoptosis, as it binds phosphatidylserines, which are translocated from the cytosol to the cell plasma membrane in the apoptotic cells. PI

is a dye that binds late apoptotic and necrotic cells; therefore, the combination of both stains allows selecting for early apoptotic cells. FACS analysis demonstrated a small but statistically significant increase in the number of early apoptotic cells. In addition, late apoptotic cells (stained by both Annexin V and PI) were increased after ABCC3 inhibition (Figure 5.9). The described results could be confirmed in both studied pancreatic cancer cell lines, proving the impact of ABCC3 blocking on the induction of apoptosis in PDAC, resulting in decreased cell proliferation and slowing down of PDAC progression.

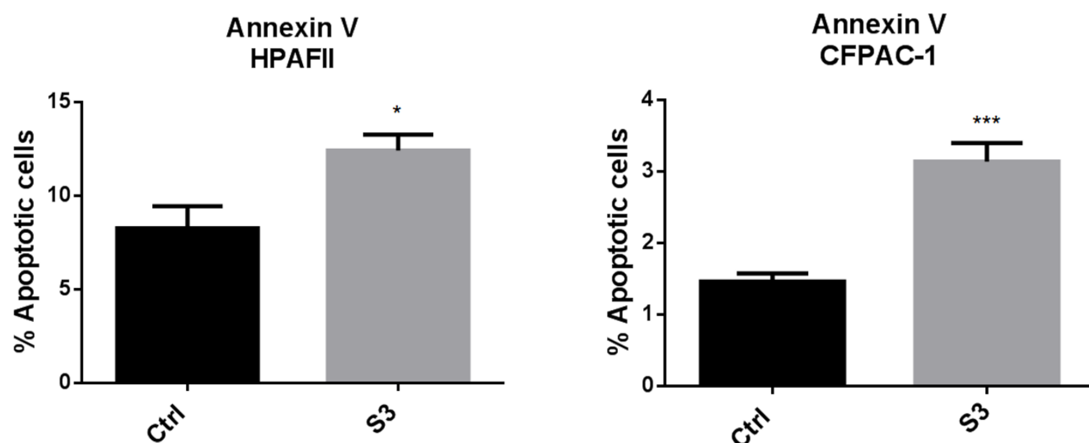


Figure 5.9 Inhibition of S3 induces apoptosis in PDAC cell lines in vitro. *Analysis of Annexin V staining in HPAFII and CFPAC-1 cells after treatment with S3. Each experiment was performed in triplicate. Results are presented as mean ± SEM, *p<0.05, ***p<0.001*

5.3.6.2 Knockdown of ABCC3 blocks cell cycle progression in PDAC cells

The involvement of ABCC3 in the cell cycle regulation was also verified as another potential factor contributing to the observed reduction of PDAC cell growth. Therefore, the effects of ABCC3 knockdown on the cell cycle was assessed. Cells were fixed with ice-cold 70% ethanol, stained with PI and analysed by flow cytometry as described in Materials and Methods (chapter 2.2.9). At the same time, the expression of cyclins, proteins, which expression changes depending on the cell cycle, like cyclin B1, cyclin E or cyclin D1, was verified by Western blot analysis.

Previous studies conducted by Dr Riccardo Ferro, demonstrated that transient knockdown of ABCC3 in the HPAFII cell line increased the percentage of cells in the G1 population, suggesting blockage of cell cycle in the G1 phase (Appendix, Figure 5A). Similarly, the preliminary data obtained by me with the use of CFPAC-1 cell line with stable knockdown of ABCC3 (CFPAC-1 shABCC3) also suggested the arrest of cell cycle in G1/S phase in these cells, compared to the cells transfected with control plasmid (CFPAC-1 4mut) (Figure 5.10-A). At the same time, a significant decrease in the expression of Cyclin B1, a protein involved in G2/M transition, was observed in these cells, suggesting lower percentage of the cells in G2/M phase, consistent with cell cycle analysis (Figure 5.10-B). Similarly, transient knockdown of ABCC3 in CFAPC-1 cells with two separate siRNAs resulted in the considerable decrease in Cyclin B1 expression, while no differences in the levels of Cyclin D1 were observed (Appendix, Figure 5B).

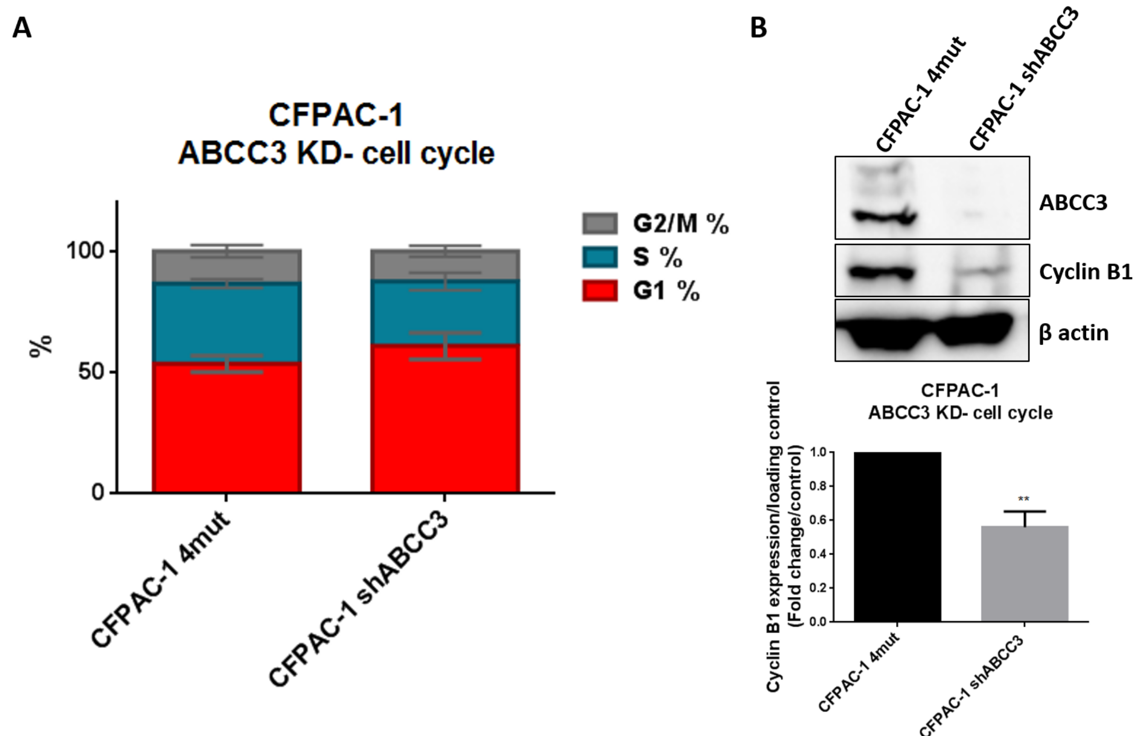


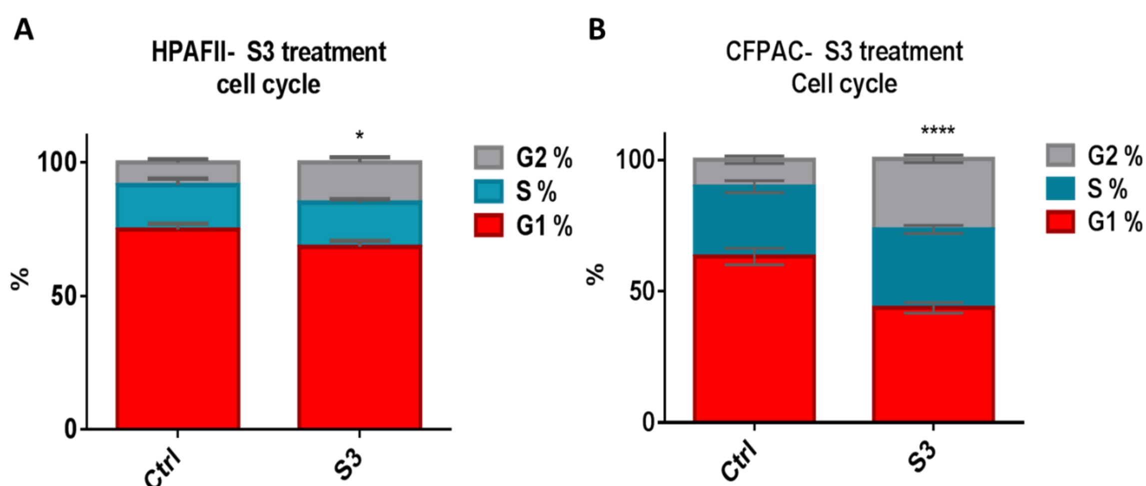
Figure 5.10 ABCC3 regulates cell cycle in PDAC cell lines*. (A) Cell cycle analysis of CFPAC-1 cell line with stable ABCC3 knockdown (CFPAC-1 shABCC3) compared to control CFPAC-1 cells (CFPAC-1 4mut). (B) Representative Western blot image and quantitative analysis of 3 independent experiments showing the expression of Cyclin B1 in CFPAC-1 shABCC3 and CFPAC-1 4mut cells. Results are presented as mean ± SEM, **p<0.01.*

Taken together, the observed effect of ABCC3 knockdown on the decrease of PDAC cell growth and proliferation is partly due to the induction of cell apoptosis and cell cycle arrest in G1/S phase.

5.3.6.3 Pharmacological inhibition of ABCC3 with S3 blocks cell cycle in PDAC cell lines

To verify the effects of the pharmacological inhibition of ABCC3 on PDAC cell cycle, CFPAC-1 and HPAFII cells were seeded in a 6-well plate and treated with 10µM S3 or DMSO for 48h. After that time cells were collected, fixed with ice-cold 70% ethanol, stained with PI and analysed by FACS.

A small but statistically significant increase in the percentage of cells in G2/M phase was detected after the treatment of HPAFII cells with S3 compared to the control cells (Figure 5.11-A). A more substantial and highly significant arrest of the cell cycle in the G2/M phase was detected in CFPAC-1 cells treated with S3 (Figure 5.11-B). At the same time, the Western blot analysis indicated a significant increase in the expression of Cyclin B1, protein expressed predominantly in G2/M phase of cell cycle, while the levels of the protein indispensable for cell progression through G1 phase, Cyclin E, remained unchanged (Figure 5.11-C).



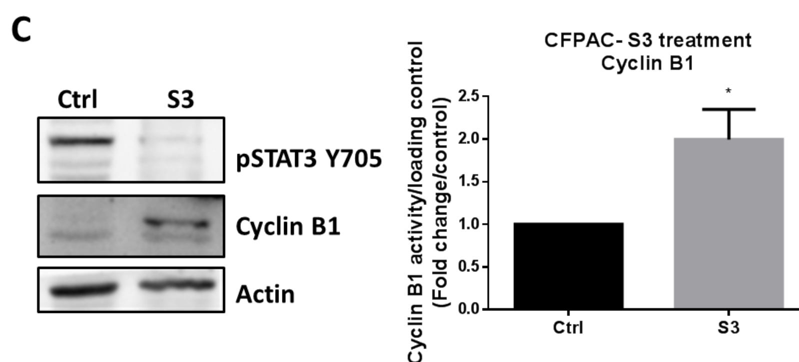


Figure 5.11 ABCC3 inhibition blocks cells in G2/M phase of cell cycle*. Cell cycle analysis of HPAFII (A) and CFPAC-1 (B) cell line treated with S3, compared to DMSO-treated cells; (C) Representative Western blot image and quantitative analysis of the expression of Cyclin B1 in CFPAC-1 cells treated with S3. Results are presented as mean ± SEM of 4 (cell cycle) and 3 (WB) independent experiments, *p<0.05, ****p<0.0001*

Taken together, these results demonstrate the mechanisms by which ABCC3 activity regulates PDAC cell growth. I showed that the cell cycle arrest, together with the induction of apoptosis, is mediated by the ABCC3 inhibition or genetic knockdown, leading to the reduction in the growth of all tested pancreatic cancer cells.

5.3.7 Pharmacological inhibition of ABCC3 in animal models of PDAC

Following the promising data obtained with the ABCC3 inhibition with S3 *in vitro,* the preclinical validation of S3 safety and efficiency in decreasing PDAC progression was performed in different murine models of PDAC. A cell-based xenograft mouse model, a patient-derived xenograft (PDX) model and transgenic model of PDAC were used for the analysis.

5.3.7.1 Pharmacological inhibition of ABCC3 with S3 slows tumour growth and PDAC progression in xenograft mouse models

For the initial validation of S3 efficacy, a xenograft mouse model was used. With this model, it is possible to test the effects of therapeutics on the growth of tumours grown from human cancer cell lines implanted subcutaneously in immunosuppressed mice. The HPAFII xenograft mouse model was created by subcutaneous injection of human HPAFII cells into the flank of female athymic CD-1 nu/nu mice (Charles River Laboratories, Calco, LC, Italy) as described in Materials and Methods. HPAFII cell line was chosen for the experiments due to proven and confirmed capability of fast growth in the *in vivo* xenograft models without the need to use any additional biologically active matrix, like matrigel.

Following the initial screening of several doses of S3, the safe dose of 25 mg/kg S3 showing at the same time good anti-tumour effects was chosen. HPAFII xenografts were randomized in two groups: S3-treated group (n=6) and control group (n=6), treated with vehicle used for S3 dilution (0.5% CMC/0.25% tween-80). Treatment started when the tumours reached the volume of 100 mm^3 and mice were treated with 25 mg/kg of S3 by oral gavage three times a week for 28 days. Tumour growth was measured every 3 days using a surgical calliper and volumes calculated according to the formula: tumour volume= $(\text{length} * \text{width}^2)/2$. Mice were sacrificed once the tumour volume reached the critical limit of 1500 mm^3.

We could demonstrate a clear and significant reduction in the tumour growth in the xenograft mice treated with S3 compared to vehicle-treated mice (Figure 5.12-A). The majority of control mice (vehicle-treated) developed tumours that advanced quickly and reached the threshold volume in a short period. Importantly, in the S3 cohort, apart from two mice, which progressed quite quickly and had to be sacrificed early on, most of the treated mice showed a significant decrease in tumour growth, which continued even after the termination of the treatment. Remarkably, two of the mice experienced complete remission of the tumour growth, following the treatment with S3, which is rarely observed in pancreatic cancer trials and research (Figure 5.12-B). As a result, a significant prolongation of the survival was also reported for the S3-treated group, in which the two mice with tumour remission continued to survive beyond the time of the experiment (median survival: 17 days for vehicle vs 84.5 days for S3; $p=0.0033$) (Figure

5.12-C). At the same time, no visible side effects could be noted and no weight loss was reported for any of the treatment arms (Figure 5.12-D).

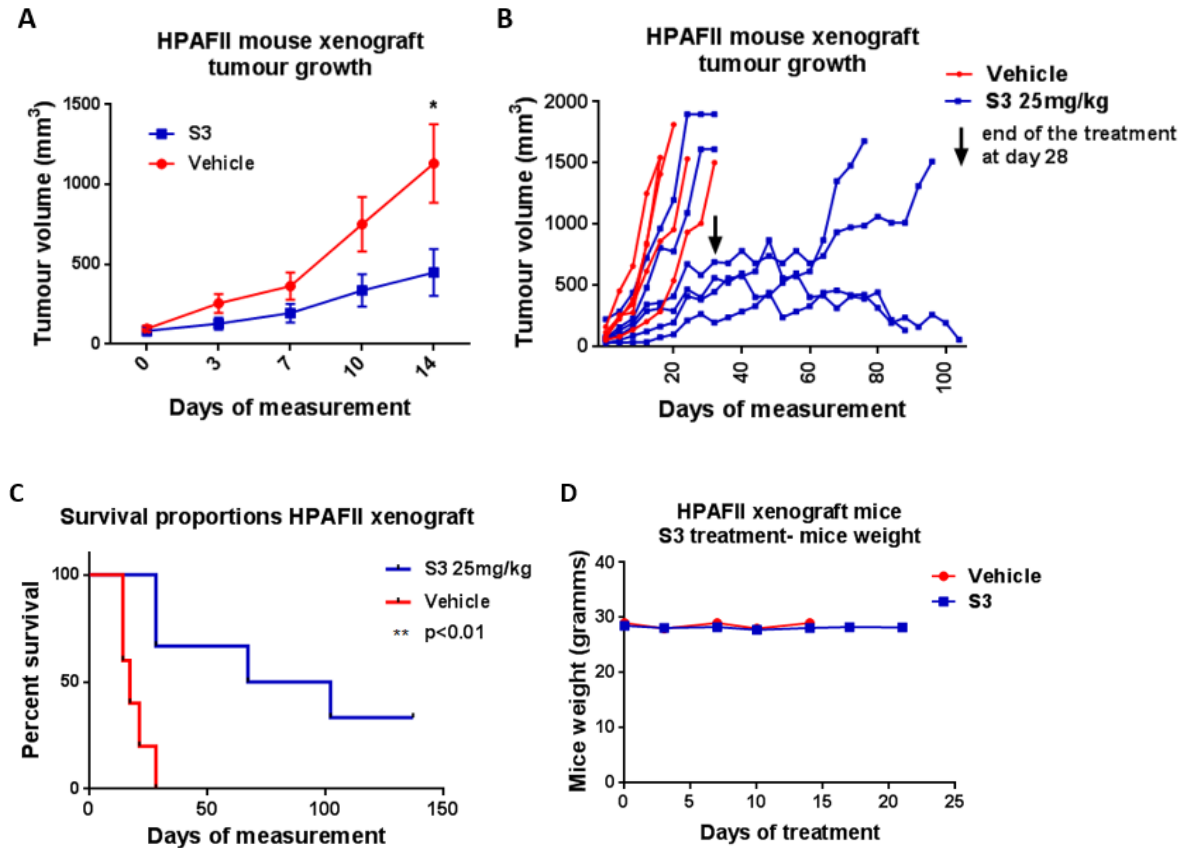


Figure 5.12 Pharmacological inhibition of ABCC3 shows high efficacy in xenograft mouse model of PDAC. *(A) Comparison of the tumour growth in the HPAFII xenograft mouse model treated with vehicle (n=4) and 25 mg/kg S3 (n=6); (B) Comparison of the tumour growth of the individual HPAFII xenograft mice treated with vehicle or S3. Arrow indicates the end of the treatment period; (C) KM survival curve of the HPAFII xenograft mouse model treated with S3 (blue) and vehicle (red), p=0.0033; (D) Comparison of the weights of the HPAFII xenograft mice treated with S3 or Vehicle over the period of the treatment. Xenograft experiments were performed by Dr Emily Capone and Verena Damiani under supervision of Dr Gianluca Sala at the University of Chieti.*

These results confirm the high potential of ABCC3 as a pharmacological target in PDAC therapy and show the notable effectiveness of ABCC3-targeting therapy with S3,

providing the base for further investigation and development of ABCC3- targeting molecules, such as S3.

5.3.7.2 Pharmacological inhibition of ABCC3 with S3 slows tumour growth in patient-derived xenograft (PDX) mouse model

Cell-based xenograft mouse models provide useful information on the tumour responsiveness towards investigated therapies. However, they do not fully recapitulate the genetic and phenotypic heterogeneity of the tumours, therefore restraining the proper validation of collected data. Patient-derived xenograft (PDX) mouse models, based on implantation of patients-derived tumour fragments into immunocompromised mice provide more complex and patient-specific information. In collaboration with Dr Pierluigi Di Sebastiano from Department of Surgery, SS. Annunziata Hospital, G. D'Annunzio University, Chieti, we obtained PDAC tumour fragments collected during the surgical resection of the tumour from the PDAC patient encoded as number 08. Tumours were implanted into the CD-1 mice and propagated according to the Material and Method section (chapter 2.3.3). Similar to HPAFII xenografts, mice were randomized into two treatment groups (25 mg/kg of S3 and vehicle) of six mice and treated by oral gavage three times a week for 3 weeks. Confirming the results obtained with the HPAFII xenograft, a clear and significant reduction in the tumour growth rate was demonstrated for the S3-treated mice, in which lower tumour growth rates were maintained after the end of the treatment (Figure 5.13). In addition, similarly to HPAFII xenografts, no evident side effects of the S3 activity could be detected, reinforcing the safety profile and effectiveness of S3 in the ABCC3 inhibition and its remarkable effect on the reduction of PDAC progression in the xenograft models of the disease.

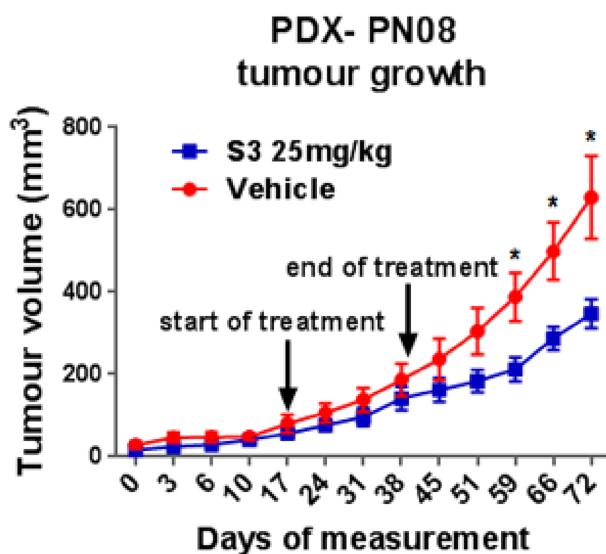


Figure 5.13 Pharmacological inhibition of ABCC3 shows high efficacy in PDX mouse model of PDAC. *Tumour growth in PDX mouse model on pancreatic cancer treated with vehicle (n=5) and 25 mg/kg S3 (n=5) shows significant difference in both parameters between the two treatment groups. Arrows indicate start and the end of the treatment period, *p<0.05. Xenograft experiments were performed by Dr Emily Capone and Verena Damiani under supervision of Dr Gianluca Sala at the University of Chieti.*

5.3.7.3 Pharmacological inhibition of ABCC3 with S3 remarkably increases survival in PDAC transgenic mouse model

The data obtained with the xenograft and PDX mouse models provided a solid preclinical validation of the potential of targeting of ABCC3 with the developed inhibitor in PDAC therapy. However, the lack of tumour heterogeneity and complex microenvironment including a fully competent immune system restrains the full and proper pre-clinical evaluation of S3 effectiveness and safety profile. KRAS$^{WT/G12D}$, P53$^{WT/R172H}$, PDX-1CRE$^{+/+}$ (KPC) mouse model, a genetically modified mouse model of pancreatic cancer was chosen for the preclinical evaluation of S3 as a potential anti-PDAC therapeutic. Due to the introduced mutations, KPC mice spontaneously develop pancreatic cancer with the genetic events and histopathology mimicking the development of human PDAC. In addition, a dense desmoplastic reaction is formed

around the tumour bulk, providing the environment resembling human disease. Importantly, development of metastasis and other disease-related consequences, including ascites or cachexia is also observed. Therefore, it is the best model for the most accurate evaluation of the developed therapies as it allows for the assessment of drug delivery and its pharmacokinetics, its safety profile and effectiveness in counteracting the progression of the disease or development of metastasis.

KPC mice were bred, maintained and genotyped by the Animal Research Centre (Murdoch, Western Australia) according to the original protocol (392). Mice were palpated daily for the evaluation of the tumour growth after they reached 80 days, the age around which PDAC development should be commencing. Two independent investigators palpated mice and treatments commenced if the tumour was palpable for three consecutive days. Mice were randomized into two different groups- S3 treated (6 mice) and vehicle treated (control group, 8 mice). The treatment arm, to which the mice were subjected, was selected by card drawing by an unbiased person. Considering the effectiveness and safety profile of 25 mg/kg dose of S3 in the xenograft models of PDAC, this dose was selected for the experiment. Mice were treated daily by oral gavage until they displayed visible signs of pain and distress due to tumour progression. Mice were checked daily and their weight was documented. Mice survival was compared between two treatment arms. In addition, pancreatic tumours and the organs, in which PDAC- related metastases are usually detected (liver, spleen, lungs) were resected form the mice.

Outstanding and significant prolongation of the survival of mice treated with S3 was demonstrated. Remarkably, a two-fold increase in the lifespan was observed from 72.5 days (median survival) in the control group to 146.5 days in the treatment group (Figure 5.14-A). Although the size of the tumours at the end of the experiment did not vary between the two groups, suggesting that S3 does not regress the development of the tumours, the remarkable increase in survival indicated the a significant slowdown of the disease progression caused by the pharmacological inhibition of ABCC3. Importantly, no side effects related to the S3 regimen could be observed, confirming the safety profile of the drug. No treatment-related weight loss

was observed for the S3- treated group (Figure 5.14-B; fold change of the weight to the weight at the start of the treatment).

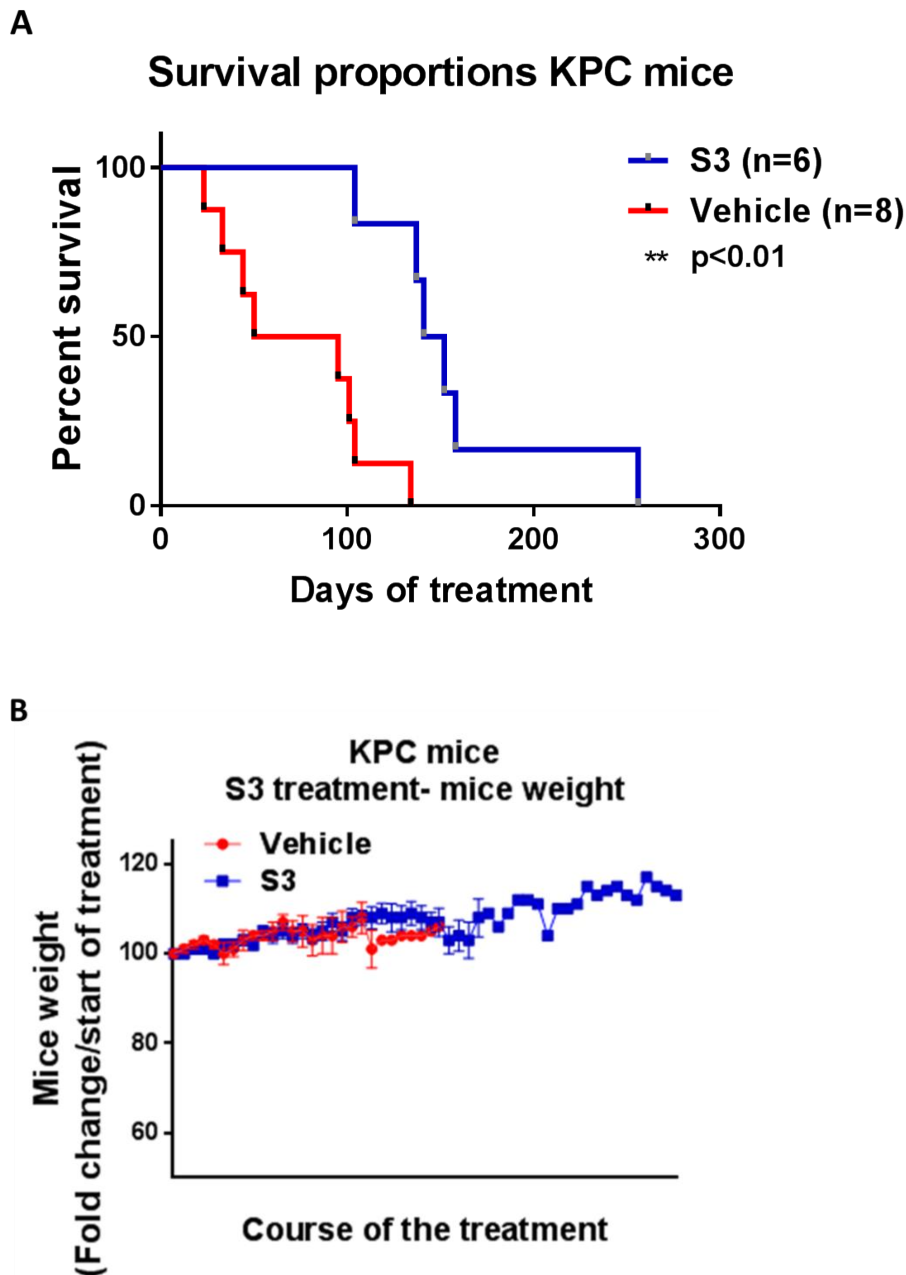


Figure 5.14 Pharmacological inhibition of ABCC3 shows safety and high efficacy in transgenic mouse model of PDAC. *(A) Kaplan Meier survival curve of KPC mice treated with vehicle (n=8) and S3 25mg/kg (n=6). Logrank (Mantel-Cox) test (p=0.0010) and Gehan-Breslow-Wilcoxon test (p=0.0026) were performed for statistical analysis; (B) Comparison of the weights of the KPC mice treated with Se and vehicle over the course of the treatment. The weights are presented as a fold change of the weigh at the start of the treatment.*

A remarkable increase in the survival of S3- treated KPC mice compared to the untreated (vehicle-treated) mice was observed. However, in order to be explored clinically, it is pivotal to demonstrate the superiority of the tested therapeutic over standard chemotherapy or to show its potential additive or complimentary effect. As discussed in the introductory chapter, the chemotherapy regimen currently applied as standard-of-care treatment includes gemcitabine, Abraxane and FOLFIRINOX. However, their efficacy is not optimal and their application is coupled with severe adverse events. Thus, combining the chemotherapy with additional, targeted therapeutic approaches is currently explored clinically.

Based on the *in vitro* analysis of the combination of S3 with several chemotherapeutics used in PDAC therapy (Figure 5.5), Abraxane (albumin-bound paclitaxel) + S3 was selected as the most optimal S3-based drug combination and applied as a third treatment arm (n=7) for the KPC mice. No data exists so far on the effects and conditions of long-term administration of Abraxane in murine models of PDAC. Chemotherapy was applied once a week by tail vein injection at the dose of 60 mg/kg. The chemotherapy administration followed the regimen used in PDAC patients' treatment, consisting of three rounds of chemotherapy (day 1, 8, and 15) every 28 days. In addition, a fourth experimental arm (n=6), combining daily gavage of the mice with 25 mg/kg of S3 and weekly chemotherapy (Abraxane) was also investigated.

No significant increase in mice survival could be observed for the chemotherapy group, with the survival comparable to the control, vehicle-treated mice (Figure 5.15-A). These results mirror the effects seen in human therapy, in which the use of standard chemotherapeutics only marginally improves patients' survival. Additionally, significant adverse effects of Abraxane reported in human therapy were also observed in KPC mice. Chemotherapy-associated weakness and apathy, development of lymphoma and weight loss were observed in the mice. We might speculate that these treatment-associated adverse events were partly the reason of the low survival of the KPC mice in the Abraxane-treatment arm. Accordingly, the deterioration of PDAC patients' quality of life is related to the side effects caused by

aggressive therapies and treatment-associated adverse events including weight loss, cachexia, ascites or thrombosis are frequently considered as direct cause of death. Surprisingly, the combination of S3 and Abraxane seemed to partly alleviate these effects, by e.g. decreasing the number of mice with developed lymphoma. This in consequence, slightly, but significantly increased the survival of S3+ Abraxane-treated mice comparted to the chemotherapy alone (Figure 5.15-A). However, S3 as a single agent remarkably outperformed other tested treatment arms, proving ABCC3 inhibition as a novel and very potent approach in targeting PDAC (Figure 5.15-B).

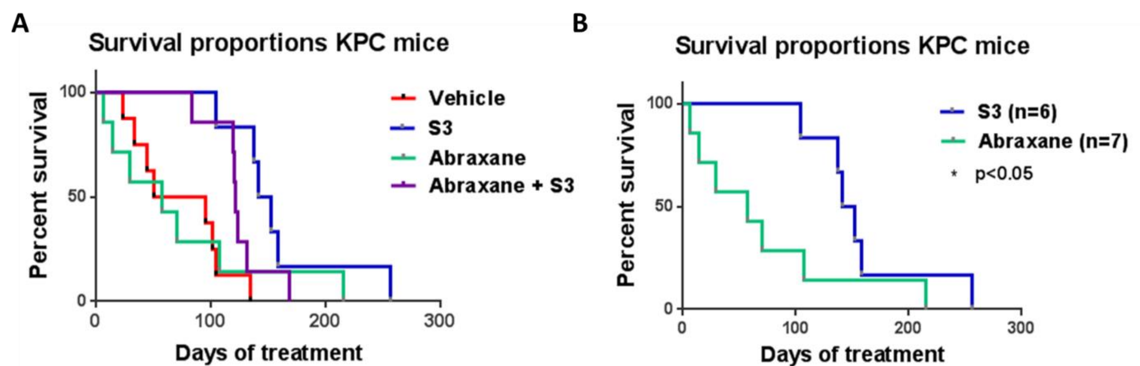


Figure 5.15 S3 shows higher efficacy than Abraxane in prolonging survival of KPC mouse model of PDAC. *(A) Kaplan Meier survival curve comparing the effects of S3 treatment and chemotherapy (Abraxane) on the survival of the transgenic KPC mouse model; (B) Kaplan Meier survival curve comparing the survival of the transgenic KPC mice treated with vehicle, S3, Abraxane and combination of S3 and Abraxane*

A different chemotherapy regimen, currently applied as one of the standard-of-care in PDAC treatment was also explored in a pilot study. Abraxane combined with gemcitabine (Gem/Abraxane) was injected weekly by lateral tail vein injection as a standard-of-care. In addition, a combination of Gem/Abraxane and S3 was investigated. The chemotherapy treatment showed visible side effects, causing nausea and severe weakness in some of the mice following the injection. The survival of the Gem/Abraxane-treated KPC mice remarkably surpassed vehicle or Abraxane alone treatment. However, even that combination could not outperform S3 applied as a single agent in terms of its effectiveness in increasing mice survival. The pharmacological inhibition of ABCC3 showed a slight improvement compared to the

Gem/Abraxane chemotherapy; however, the difference was not statistically significant.

Nonetheless, the remarkably higher tolerance of S3 compared to chemotherapy treatments, paired with the increased therapeutic effects make the application of S3 as a single agent a strong contender as a future treatment regimen in PDAC therapy.

5.3.7.4 Pharmacological inhibition of ABCC3 with S3 potentially slows down metastatic spread in transgenic mouse model of PDAC

Histopathological (H&E) analysis of the resected tissues was performed to confirm the presence of the tumours or premalignant lesions in the resected pancreatic tissues. In addition, histopathological analysis of liver tissues, the most frequent metastatic site observed in PDAC, was also performed.

Formalin-fixed paraffin-embedded (FFPE) tissues were processed as described in Materials and Methods section using the standard protocol (chapter 2.3.6). Briefly, tissues were cut into 4 µm sections and mounted on slides. Hematoxylin and Eosin (H&E) staining was then performed to visualize the cytoplasmic and nuclear components of the tissues. Haematoxylin is a basic dye, which binds DNA and RNA (of acidic character), staining these structures purple. On the other hand, negatively charged Eosin binds cytoplasmic proteins, resulting in the pink staining of the cytoplasmic structures.

H&E analysis of the pancreatic tissues confirmed that all of KPC mice presented with the PDAC tumours at the time of the resection. Clear histopathological features of fully invasive tumours were detected in the mice from both treatment arms (Appendix, Figure 6). These data confirm the presence of the pancreatic tumours in the treated mice. It also shows that, despite the increased survival of the S3-treated mice, the mice still develop invasive tumours, even though at a remarkably lower pace.

Interestingly, both macroscopic and microscopic observations of the livers resected from the KPC mice showed the differences in liver status between the vehicle-treated

and S3- treated mice. Enlarged and inflamed livers with potentially higher number of metastatic nodules were noted in the mice enrolled in the control arm.

Importantly, the histopathological analysis of the resected FFPE liver tissues demonstrated visible differences between both treatment groups (Figure 5.16). Clear morphological changes of liver tissue, suggesting the development of metastatic and pre-metastatic sites as well as liver fibrosis, were noted for all liver tissues from vehicle-treated mice. The changes were observed even in the livers from mice sacrificed early on from the start of the treatment. On the other hand, no clear signs of liver metastasis were visible in the liver tissues from S3-treated mice. Slight morphological changes were noted in the livers of the mice treated with S3 for an extended period of time, however, the effects were less evident than in vehicle-treated mice.

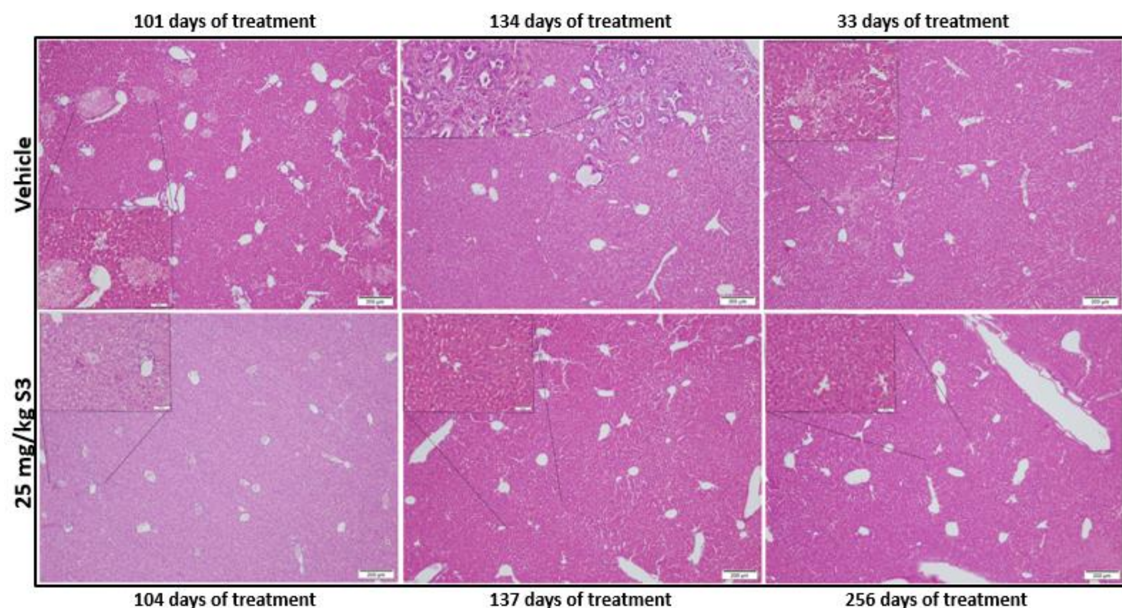
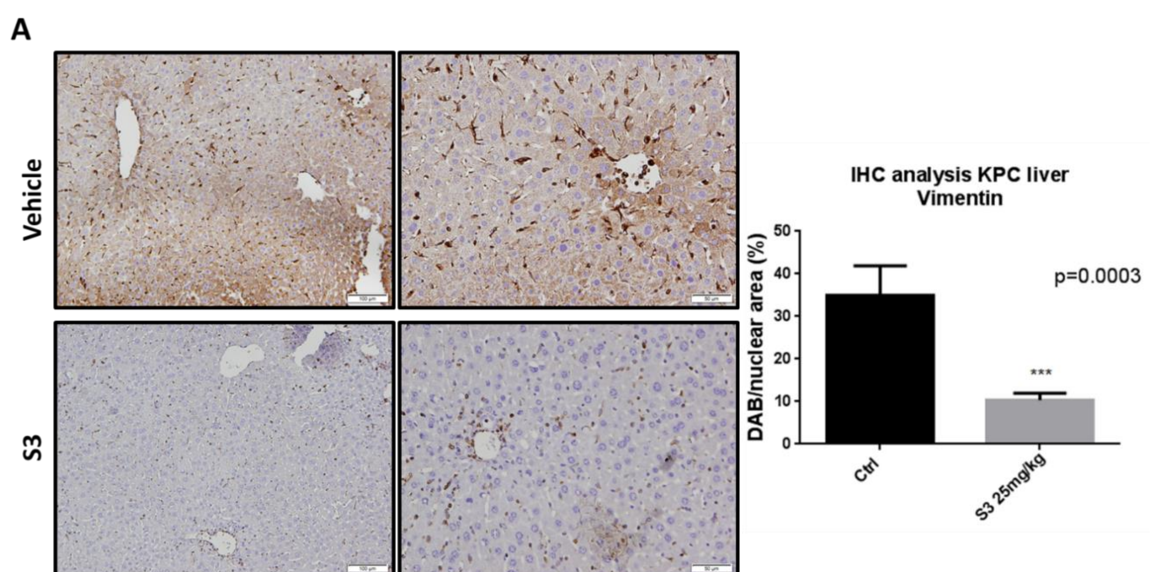


Figure 5.16 S3-treated and vehicle-treated KPC mice show different liver status. *Histopathological analysis of the liver tissues resected at the end point of the experiment (duration of the treatment shown) from the KPC mice treated with vehicle or S3*

These results suggest that the observed effects of the pharmacological inhibition of ABCC3 with S3 in the prolongation of KPC mice may be partly due to the slowdown in the metastatic spread of the PDAC tumours.

The potential mechanisms involved in the observed ABCC3-associated differences in liver status were then explored. Intrahepatic monocyte infiltration of the damaged liver tissue is one of the events of the innate immune response (472). The levels of CD11b, marker of liver inflammation, expressed on inflammatory monocytes, were therefore analysed in the liver tissues by Immunohistochemistry (IHC) staining. Lower levels of CD11b in the S3-treated group could suggest the impairment of the liver inflammation process, and consequently inflammation-induced hepatocarcinoma. However, no clear difference in CD11b expression between liver tissues from vehicle-treated and S3-terated groups could be demonstrated, suggesting that lack of involvement of ABCC3 in inflammation-related processes (Appendix, Figure 7). However, more analysis is necessary.

Nevertheless, IHC staining showed a significant reduction of the expression of vimentin in the livers from S3-treated mice (Figure 5.17-A). Moreover, Western blot analysis of snap-frozen liver tissues showed considerable, although not statistically significant decrease in the expression of vimentin and α-SMA in the livers from S3-treated mice (Figure 5.17-B), compared to vehicle-treated mice.



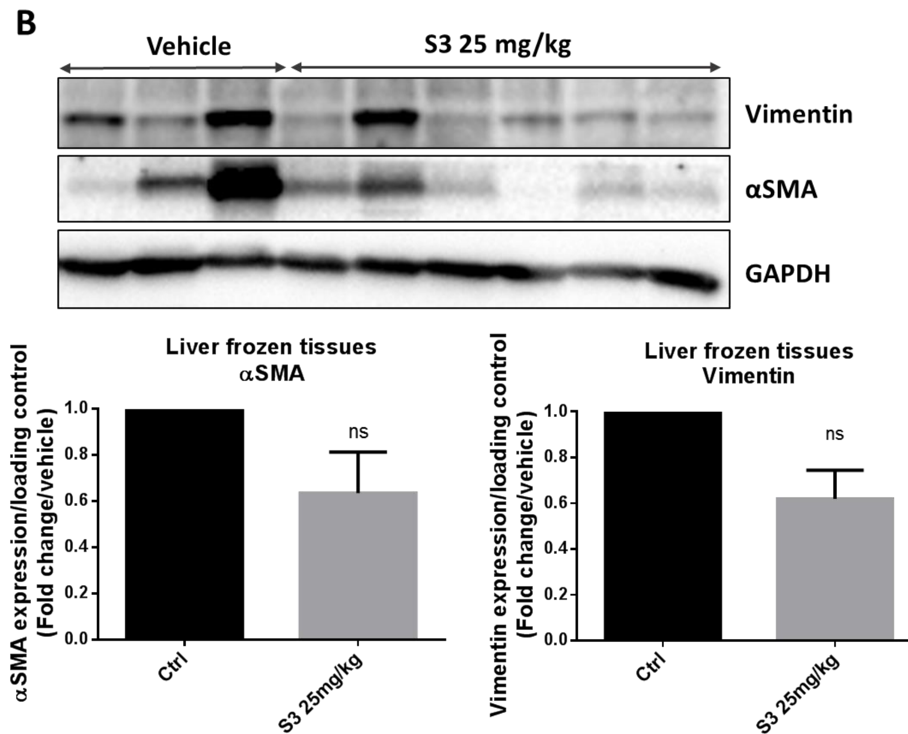


Figure 5.17 S3 treatment reduces vimentin and αSMA expression in murine livers. *(A) Representative Immunohistochemistry (IHC) staining of the livers resected form the KPC mice at the end point of the experiment comparing the expression of vimentin between the vehicle-treated and S3-treated animals, scale bar: 100μM, 50μM. The quantitative analysis of IHC staining was performed with the use of ImmunoRatio software and the results are presented as mean ± SEM of 9 (Vehicle group) and 15 (S3) different images from at least 3 different animals, p=0.0003; (B) Representative Western blot image and quantitative analysis of the expression of vimentin and αSMA in the snap frozen liver tissues resected from the KPC mice treated with vehicle (N=3) and S3 (N=6)*

Overexpression of vimentin has been associated with a more aggressive status in several cancer types. Importantly, it was previously demonstrated by the analysis of tissue microarray of 60 pairs of primary and matched metastatic hepatocellular carcinoma (HCC) samples, that the overexpression of vimentin plays important role in liver metastasis and its expression was detected in the metastatic or recurrent HCC (473). Similarly, high expression of αSMA in the activated fibroblasts was detected in various liver diseases, including hepatitis, liver cirrhosis and HCC (474). Moreover, high levels of αSMA corresponded with poor survival of HCC patients (475).

5.3.8 Pharmacological inhibition of ABCC3 with S3 slows down tumour growth by downregulation of STAT3 and HIF1α signalling and induction of apoptosis in transgenic mouse model of PDAC

Immunohistochemistry (IHC) analysis of resected pancreatic and liver tissues was additionally performed to verify if the effects of the S3 treatment on the activity of the oncogenic signalling pathways and the induction of apoptosis observed *in vitro* is reproduced *in vivo*. The effects of the long-term inhibition of ABCC3 transporter with S3 on the expression of HIF1α, pSTAT3 Y705, Bcl-xl and cleaved caspase 3 were then analysed. The expression pattern of these proteins was verified in pancreatic tissues resected from S3-treated mice and compared with vehicle-treated mice. IHC staining of the tissues was performed according to the standard protocol and antibody manufacturer's instructions. Briefly, FFPE tissues were cut into 4µm and mounted on slides. Tissues were deparaffinised using standard procedures and the heat-induced antigen retrieval was carried out in an appropriate buffer (EDTA or Citrate buffer). Tissues were blocked and stained overnight at the antibody concentration recommended by the manufacturer. The following day, tissues were incubated with secondary antibody and the signal was developed using DAB reagent. Tissues were then counterstained with Hematoxylin. For each verified protein, the tissues of S3-treated (n=6) and vehicle-treated (n=6) mice were processed at the same time and in the same conditions. For each of the antibodies used for the analysis, an optimization process was performed prior to the analysis of the whole treatment group. Two control tissues and two tissues form S3-treated mice were used for the optimization. The optimal antigen retrieval and antibody staining conditions were selected for each of the target proteins. When necessary, the signal was enhanced using a biotinylated secondary antibody. The quantitative analysis of all the IHC analysis was performed with the use of free online software, ImmunoRatio. At least three separate images for each sample, representing different areas of the same tissue were taken for analysis for each of the tissues. The results were presented as mean ± SEM of all the images of control (vehicle-treated) and S3-treated tissues.

Strong nuclear signal of phosphorylated STAT3 could be detected in the pancreatic tissues. Both ductal and stromal staining of the tumours from vehicle-treated mice was observed, suggesting a high expression of activated STAT3 in both fibroblast and epithelial tissues (Figure 5.18). This dual expression confirms the role that STAT3 signalling plays in PDAC tumour and stroma cross-talk shown in literature. Importantly, strong STAT3 staining could be observed for each of the tissues form the control group. As comparison, only one out of six tissues resected from the S3-treated mice showed strong STAT3 staining. The rest of the analysed tissues showed very sparse signal that could be mainly localized in the PDAC stoma, whereas the majority of the ducts did not show expression of STAT3.

These results might suggest that long-term administration of S3 and inhibition of ABCC3 in the KPC model of PDAC significantly reduces the activity of STAT3 proteins, especially targeting PDAC ducts.

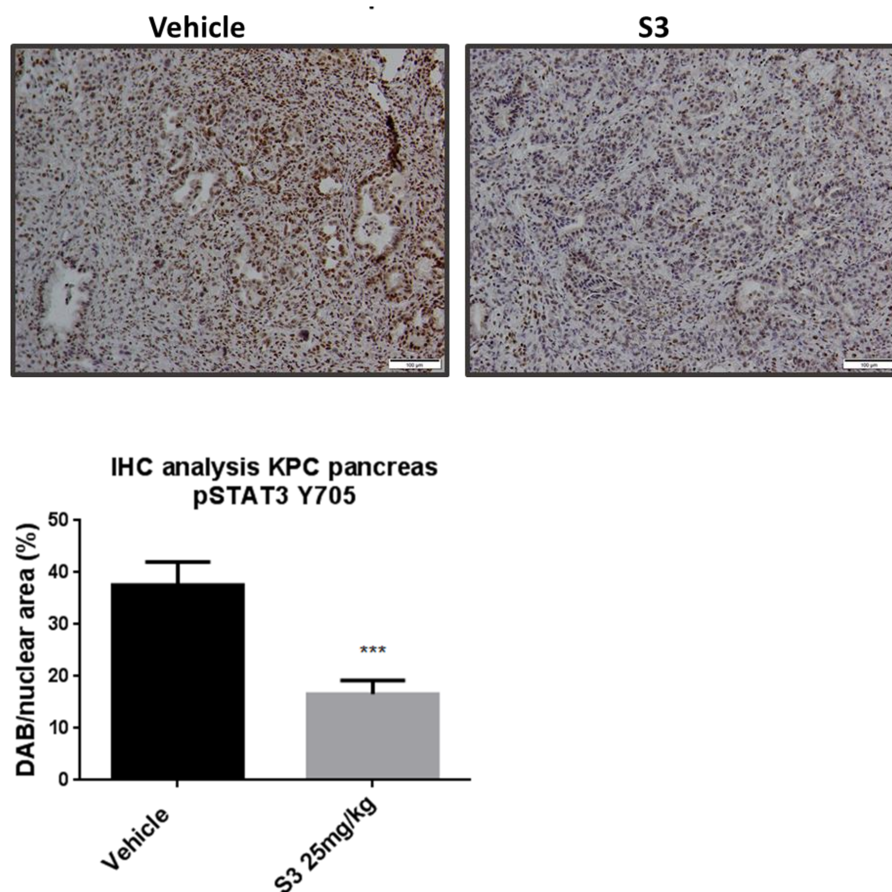
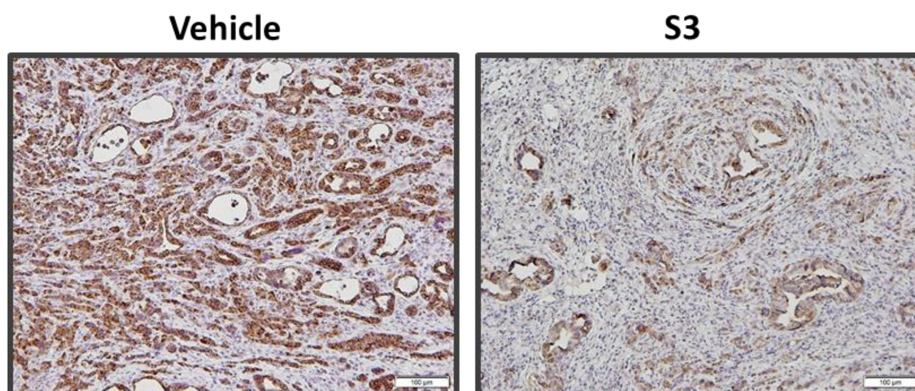


Figure 5.18 S3 reduces STAT3 activation in vivo. *Representative IHC staining of FFPE pancreatic tumor tissues resected from KPC mice showing differential expression of pSTAT3 Y705 between vehicle and S3- treated mice samples. Scale bar: 100μm. The quantitative*

*analysis of IHC staining was performed with the use of ImmunoRatio software and the results are presented as mean ± SEM of 17 (Vehicle group) and 14 (S3 group) different images from at least 4 different animals, ***p<0.001*

Downstream effectors of STAT3 were also verified. Bcl-xl is a protein belonging to Bcl2 family that participates in the regulation of the apoptotic machinery in PDAC. The correlation between STAT3 activity and Bcl-xl anti-apoptotic function has been previously demonstrated and presented as one of the mechanisms of STAT3-mediated regulation of carcinogenic processes. During malignancies, the upregulation of the expression of Bcl-xl, induced by increased STAT3 activity, inhibits apoptosis in cancer cells. Decreased levels of

The expression levels of Bcl-xl were compared between tissues from S3 treated and vehicle-treated mice groups (Figure 5.19). Very strong ductal expression of Bcl-xl could be shown for the majority of the tumours samples dissected from vehicle-treated KPC mice. No stromal expression could be observed in these samples. Still ductal, however less intense and sparse signal could be detected in the tumours form S3-treated mice.



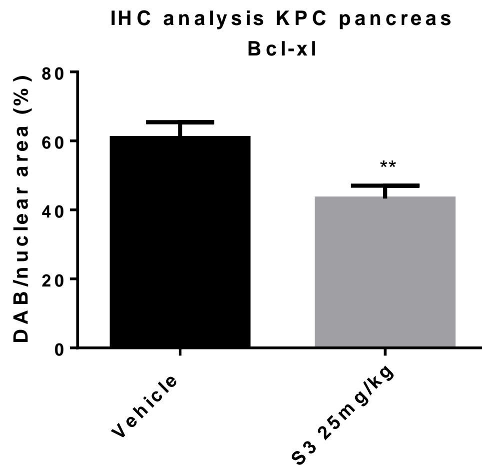


Figure 5.19 S3 treatment reduces Bcl-xl expression in vivo. *Representative IHC staining of FFPE pancreatic tumor tissues resected from KPC mice showing differential expression of Bcl-xl between vehicle and S3- treated mice samples. Scale bar: 100µm. The quantitative analysis of IHC staining was performed with the use of ImmunoRatio software and the results are presented as mean ± SEM of 17 (Vehicle group) and 14 (S3) different images from at least 4 different animals, **p<0.01*

The reported data suggest therefore that the decreased expression of Bcl-xl in the pancreatic ducts of the KPC mice is subjected to the long-term pharmacological inhibition of ABCC3. These results support the previously shown decrease in the activity of the phosphorylated STAT3 (STAT3 Y705). They also suggest the involvement of ABCC3 in the regulation of apoptosis, as the reported decrease of Bcl-xl expression following blockage of ABCC3 induces apoptosis activation.

Similar results were observed for the HIF1α staining of the pancreatic tissues. Strong ductal and slight stromal staining was detected in the tumour tissues from KPC mice. Pharmacological targeting of ABCC3 remarkably reduced the intensity of the detected signal in both epithelial cells and in fibroblasts (Figure 5.20). Interestingly, in the tumours resected from S3-treated mice, the areas of considerably lowered expression of HIF1α were present together with the rich hypoxic regions. This observation may suggest that the drug was not unanimously delivered to the whole tumour bulk.

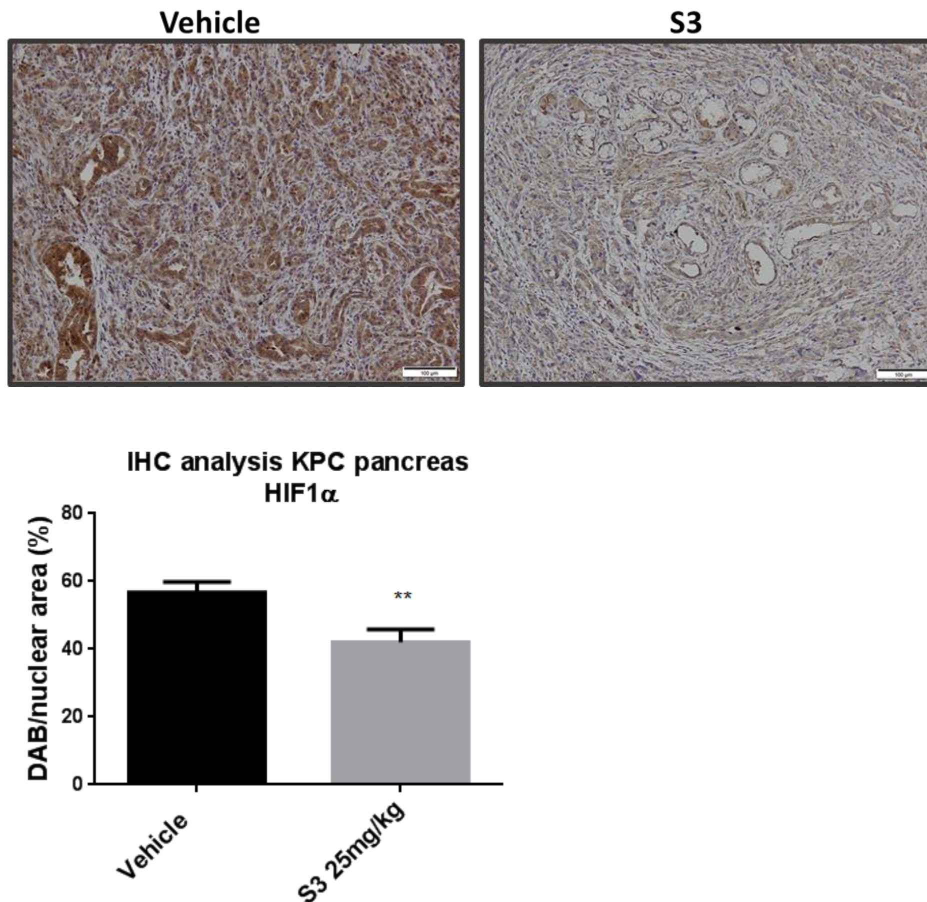


Figure 5.20 S3 treatment reduces HIF1α expression in vivo. *Representative IHC staining of FFPE pancreatic tumor tissues resected from KPC mice showing differential expression of HIF1α between vehicle and S3 treated mice samples. Scale bar: 100µm. The quantitative analysis of IHC staining was performed with the use of ImmunoRatio software and the results are presented as mean ± SEM of 13 (Vehicle group) and 17 (S3) different images from at least 4 different animals, **p<0.01*

5.3.8.1 ABCC3 targeting with S3 affects STAT3 and HIF1α signalling in metastatic sites in PDAC

As a complementary approach, liver tissues resected from the same animals were analysed for the activated STAT3 expression. Interestingly, exclusive nuclear staining in the livers of the KPC mice with sham treatment could be observed. In contrast, no expression of pSTAT3 Y705 could be observed in the mice subjected to the ABCC3-targeting therapy (Figure 5.21-A). In addition, snap frozen tissue fragments of about 25mg each were minced and lysed in 250µl of RIPA buffer. Analysis of whole lysate

liver tissues showed significant decrease in HIF1α expression in the livers of S3-treated KPC mice (Figure 5.21-B).

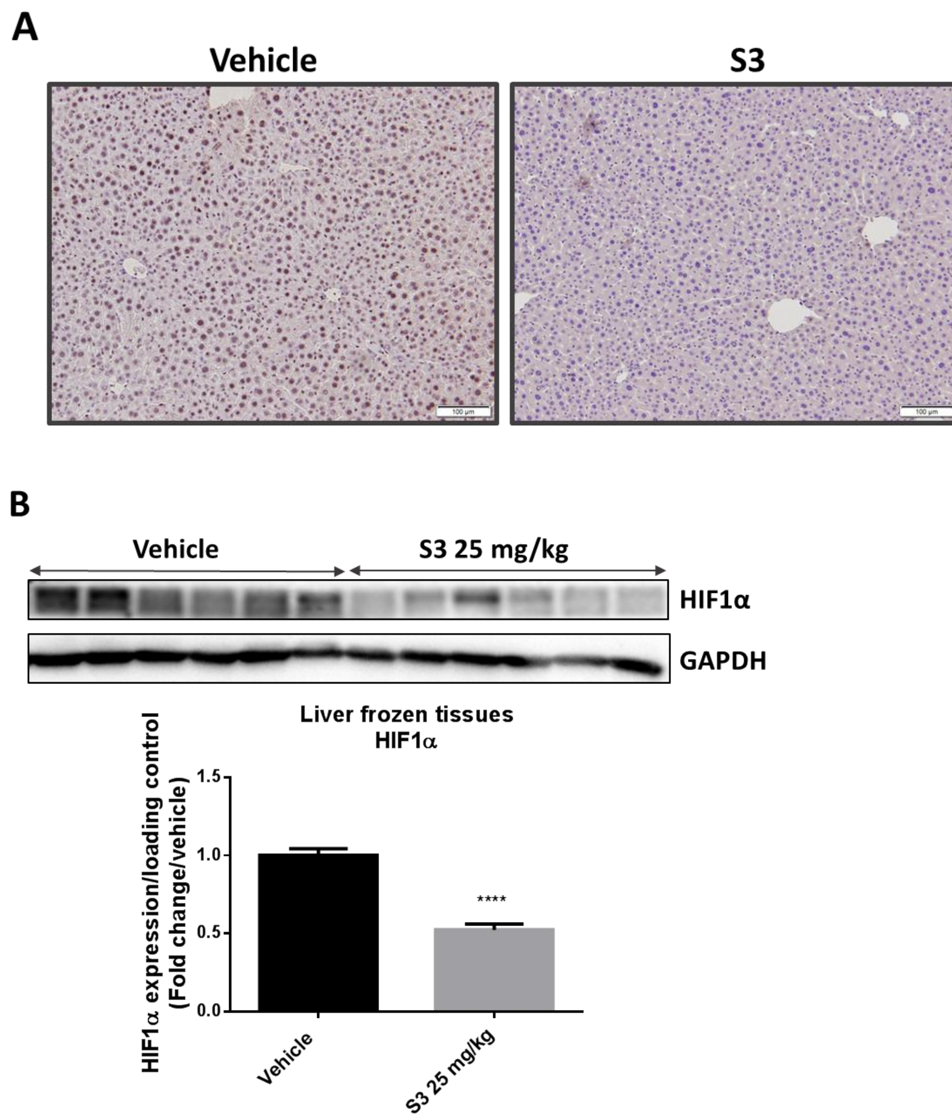


Figure 5.21 S3 treatment changes STAT3 and HIF1α signalling in livers of KPC transgenic mice*. (A) Representative IHC staining of FFPE liver tissues resected from KPC mice showing differential expression of pSTAT3 Y705 between vehicle and S3-treated mice samples. Scale bar: 100µm.; (B) Representative Western blot analysis of snap frozen liver tissues resected from KPC mice showing the expression of HIF1α in vehicle and S3- treated mice samples. Quantitative analysis is presented as mean ± SEM, ****p<0.0001*

It has been previously demonstrated that STAT3 promotes the migration and proliferation of hepatocellular carcinoma cells. Our data might suggest that in PDAC,

the development of metastasis in the liver is supported by the transcriptional activity of STAT3 regulated by ABCC3. Blocking of ABCC3 might disrupt the functional activity of STAT3 pathway, preventing its transcriptional functions not only in the primary tumours but also in the metastatic sites. This stays in agreement with the demonstrated role of STAT3 signalling in the liver inflammation and tumorigenesis. A therapeutic approach targeting STAT3 has also been proposed for the treatment of hepatocellular carcinoma.

High expression of pSTAT3 Y705, Bcl-xl and HIF1α could be also demonstrated in the regional lymph nodes, another metastatic site observed for the KPC mice. In contrast, remarkably lowered levels of the proteins were detected in the lymph nodes of S3-treated mice, with some of the tissues showing almost complete reduction of their expression (Figure 5.22).

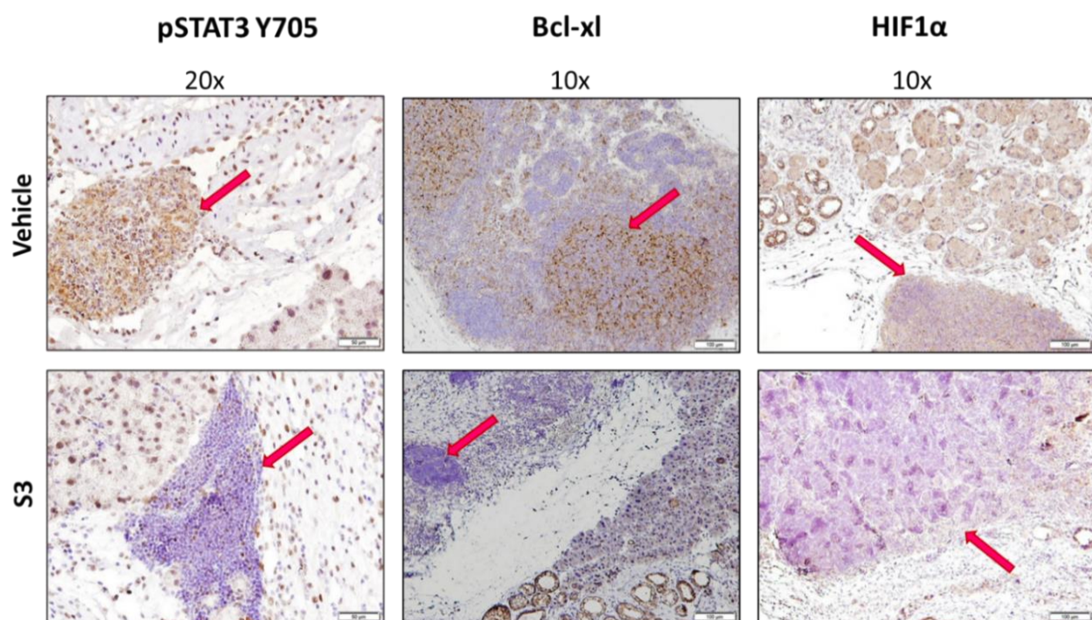


Figure 5.22 S3 treatment reduces STAT3 and HIF1α signalling in regional lymph nodes of KPC transgenic mice. *Representative IHC staining of FFPE regional lymph node tissues resected from KPC mice showing differential expression of pSTAT3 Y705, Bcl-xl and HIF1α between vehicle and S3 treated mice samples. Scale bar: 100μm, 50μM; arrows indicate the lymph nodes.*

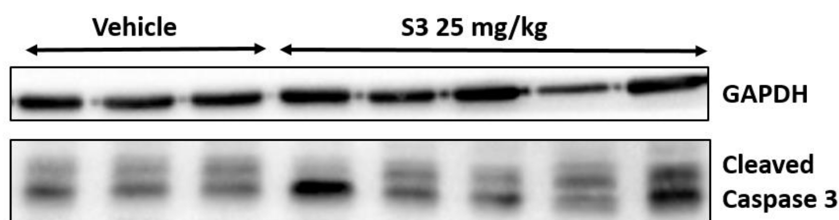
These observations suggest the role of ABCC3 in the control of key regulatory pathways in PDAC and PDAC-related metastasis. They also show that the increase in survival of S3-treated KPC mice might be due to the downregulation of the activity of

key oncogenic pathways not only in the primary tumour but also in potential metastatic sites, decreasing the development of metastatic tumours.

5.3.8.2 ABCC3 targeting with S3 causes induction of apoptosis in vivo

Induction of apoptosis following ABCC3 knockdown and its pharmacological inhibition with S3 was demonstrated *in vitro* in commercially available PDAC cell lines.

To confirm that ABCC3 downregulation induces apoptosis *in vivo,* FFPE and snap frozen tumour tissues resected from the KPC mice treated with S3 or vehicle were also analysed. IHC staining showed a small increase in the expression of cleaved caspase 3 in the pancreatic tissues from S3- treated KPC mice, although the difference was not statistically significant (Appendix, Figure 8). Similarly, Western blot analysis of whole snap frozen tumour lysates showed a considerable, although not statistically significant increase in cleaved caspase 3 levels in the mice treated with S3 (Figure 5.23).



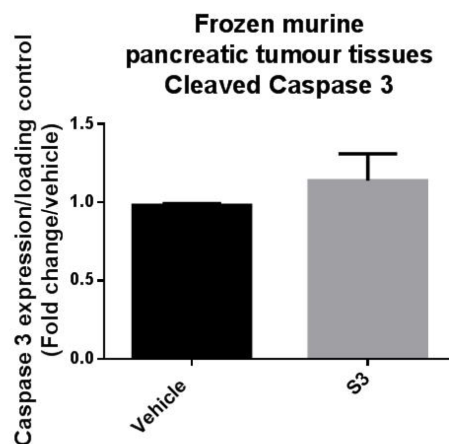
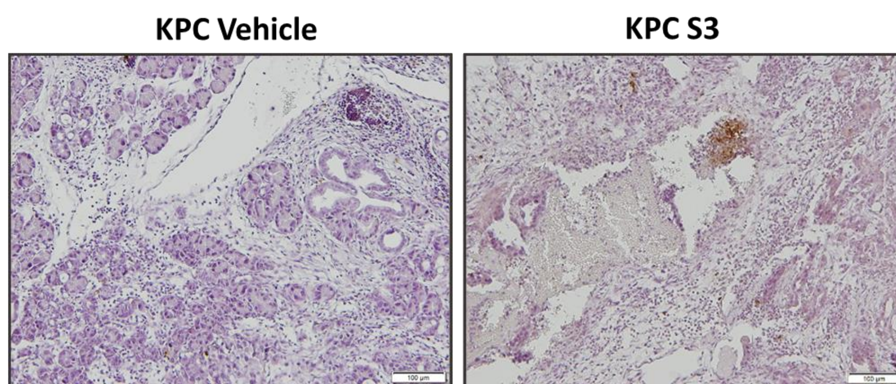


Figure 5.23 S3 treatment induces apoptosis in vivo. *Western blot analysis of the levels of cleaved caspase 3 in the snap frozen pancreatic tumours resected from vehicle-treated (N=3) and S3-treated (N=5) mice*

As a final validation of the involvement of ABCC3 inhibition in the induction of apoptosis, the pancreatic tissues resected from KPC mice treated with vehicle and S3 were analysed via Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. TUNEL assay is a staining method that detects fragmented DNA by the labelling of 3'- hydroxyl termini of dsDNA fragmentation occurring during apoptosis. Therefore, increased signal detected by TUNEL staining correlates with higher levels of apoptotic cell death.

FFPE pancreatic tissues from both treatment groups were subjected to the TUNEL labelling as described in the Material and Method section (chapter 2.3.8). Comparison of the number of apoptotic cells, expressed as a number of detected signal points was compared between the two groups. The number of apoptotic cells detected in the pancreatic tumour tissues from the S3-treated mice was significantly higher than in the mice treated with vehicle, as assessed by the quantitative analysis with the use of ImmunoRatio software (Figure 5.24).



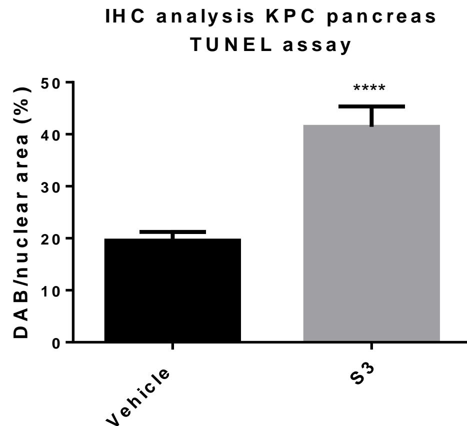


Figure 5.24 S3 treatment increases apoptosis in vivo. *Representative image of the TUNEL assay performed on FFPE pancreatic tissues resected from KPC mice treated with vehicle and S3 (Scale bar: 100µm). The quantitative analysis of IHC staining was performed with the use of ImmunoRatio software as mean ± SEM of 17 (Vehicle group) and 21 (S3 group) images; **** p<0.0001*

Interestingly, increased TUNEL signal was detected in the lymph nodes of S3-treated mice, suggesting the induction of apoptosis also in the metastatic sites in the KPC mice. These results confirm the involvement of ABCC3 in the regulation of apoptosis in PDAC and demonstrates that its pharmacological inhibition induces apoptosis not only *in vitro* but also in mouse animal model of PDAC. It also suggests the potential anti-metastatic effect that ABCC3 blockage with S3 may cause; however, further validation of this hypothesis needs to be performed.

5.3.9 Validation of the ABCC3 downregulation in primary KPC cell line

The potential mechanisms by which ABCC3 activity might influence PDAC progression have been so far demonstrated *in vitro* in the commercially available pancreatic cancer cell lines. To corroborate *in vivo*, a primary cell line was established from the pancreatic tumours from the KPC mice. Combining several protocols for the establishment of the primary cell lines from the tumours, we isolated the tumour cells form resected pancreatic tumours and cultured them *in vitro*. Cells were grown

in standard cell culture flasks in complete DMEM cell growth media. Interestingly, established primary cell line mimicked the phenotype observed for pancreatic tumours. Tumour epithelial cells formed cellular clusters resembling tumour bulk, surrounded by elongated fibroblast cells, main component of PDAC stroma. Therefore, the responsiveness and processes observed in the KPC primary cells could reflect to the greater extend the biology of tumours *in vivo*.

In vitro experiments were performed on the primary KPC cell line to confirm the results obtained with the commercially available pancreatic cancer cell lines. The obtained results could complement the *in vivo* results obtained in the KPC mouse model and could reinforce the efficiency of S3 in PDAC treatment. Primarily, the expression of ABCC3 was verified in the KPC cell line and compared with its expression in the commercial PDAC cell lines. Strong expression of ABCC3 was detected in the KPC cell line, which was comparable with its expression in other PDAC cell lines (Figure 5.2). These data confirm the overexpression of ABCC3 in the KPC transgenic mouse model.

To verify the role of ABCC3 in the KPC cell line, knockdown of ABCC3 was performed by transient siRNA transfection. The effect of ABCC3 downregulation on cell viability and on the activity of the demonstrated ABCC3-regulated signalling pathways was verified. Two separate KPC cell lines, established from tumours from two KPC mice were tested in order to verify the obtained results. I could see that both the knockdown of ABCC3 and its pharmacological inhibition with S3 significantly reduced the number of viable KPC cells, as shown by manual counting of the live cells 72h post-treatment. Importantly, almost identical results were obtained for both primary cell lines, showing the accuracy of obtained results (Figure 5.25-A). Moreover, the response of the KPC cells to S3 treatment was comparable to the effects seen in the commercial cell lines.

As a complimentary approach, the responsiveness of the KPC primary cell line to pharmacological inhibition of ABCC3 was also tested in the 3D soft agar assay. Similarly to other PDAC cell lines, the formation and growth of colonies was significantly decreased after cell treatment with increasing doses of S3 (Figure 5.25-B, C). In addition, the size of the colonies was lowered with higher doses of S3. These

results confirmed the efficiency of S3 in decreasing the proliferation of PDAC cells and slowing the progression of the disease observed *in vivo*. They also confirm the effectiveness of S3 in the KPC mouse model.

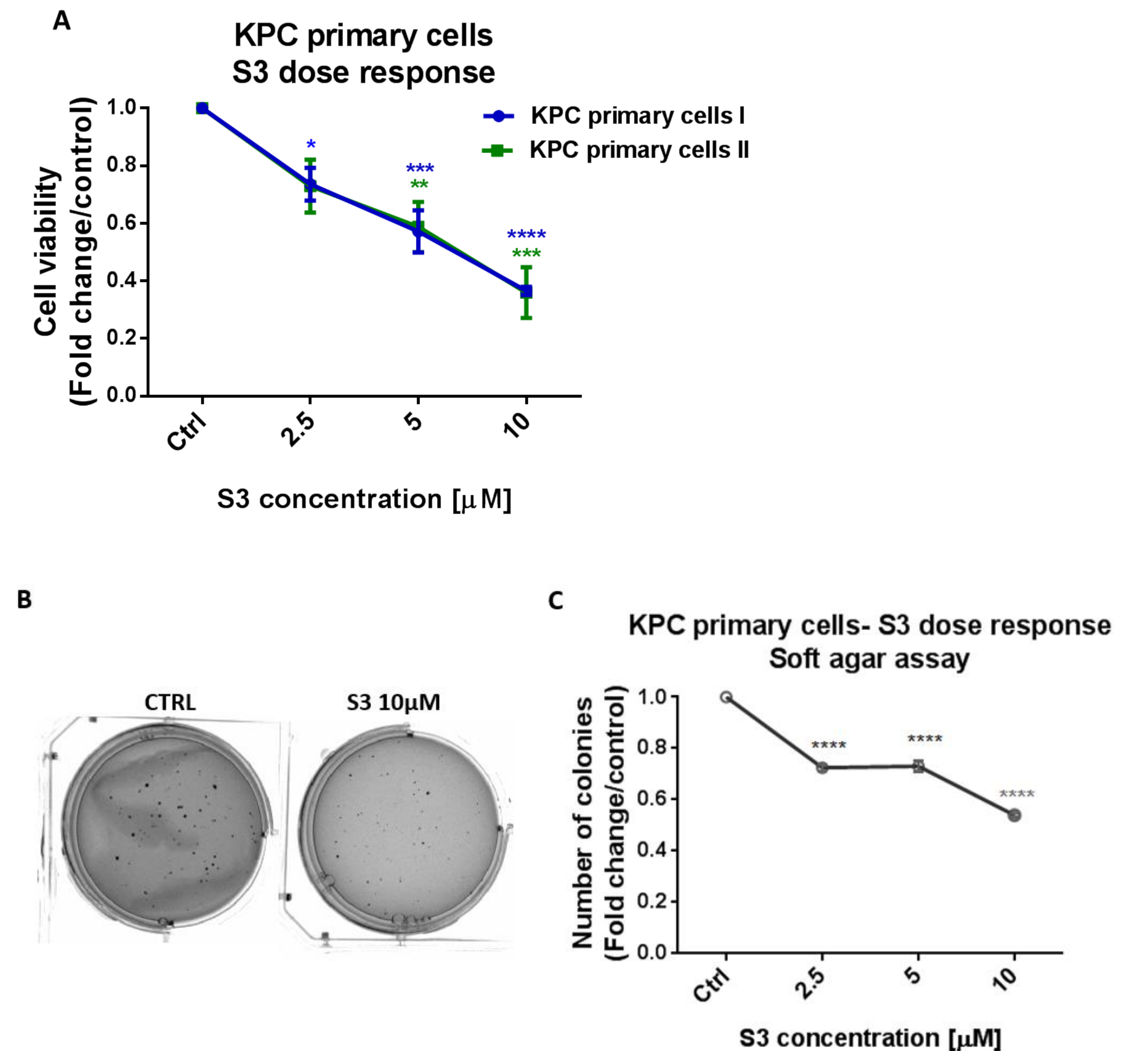


Figure 5.25 Pharmacological inhibition of ABCC3 with S3 reduces growth and clonal expansion of primary KPC cell line. *The effects of S3 dose response treatment in established KPC primary cell lines on anchorage dependent (A) and independent (B,C) cell growth. Blue: I^st^ established cell line, Green: 2nd established cell line. The results are presented as mean ± SEM of 5 (blue) and 3 (green) independent experiments, *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001.*

In addition, the signalling pathways regulated by ABCC3 were analysed in the primary cell line to verify the findings obtained with the use of the commercial PDAC cell lines.

After the knockdown of ABCC3 and its pharmacological inhibition with S3, cells were collected and protein analysis was performed by Western blotting. Cells were collected 48h post treatments. Similarly to the previously tested commercial cell lines (AsPC1, HPAFII, CFPAC-1), the genetic knockdown of ABCC3 in the KPC primary cell line remarkably decreased the phosphorylation of STAT3 at tyrosine 705 (pSTAT3 Y705) and reduced the expression of HIF1α, confirming the involvement of ABCC3 in the regulation of these pathways not only *in vitro* but also *in vivo* (Figure 5.25-A). Similarly, the inhibition of ABCC3 with S3 induced similar effects in the KPC cell line, significantly reducing the activity of STAT3 and HIF1α pathways (Figure 5.25-B).

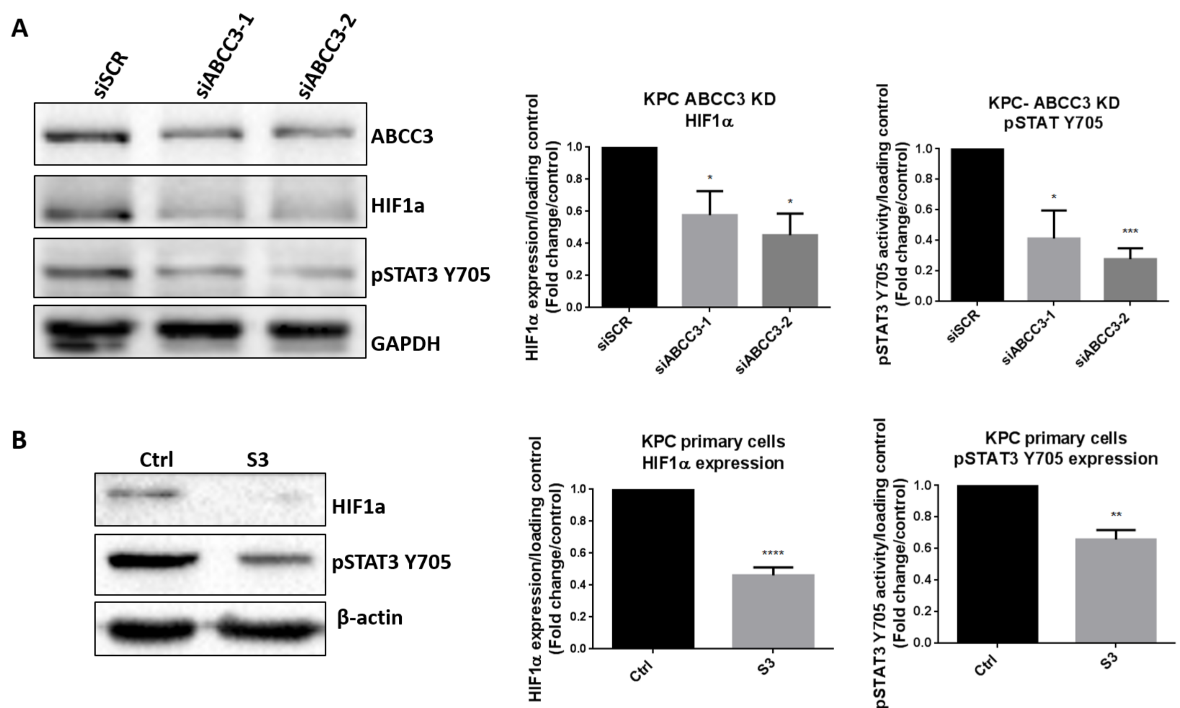


Figure 5.25 Downregulation of ABCC3 reduces the activity of STAT3 and HIF1α signalling in KPC primary cell line. *Representative Western blot images and quantitative analysis of the effects of ABCC3 knockdown (A) and pharmacological inhibition with S3 (B) on the expression of pSTAT3 Y705 and HIF1α in the KPC primary cell line; Results are presented as mean ± SEM of at least 3 independent experiments, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

Interestingly, the decrease in the expression of Bcl-xl, a protein exhibiting anti-apoptotic functions in cancer cells, was also detected after downregulation of ABCC3 in the KPC cell line by both, ABCC3 silencing and pharmacological inhibition,

confirming the link between ABCC3 and STAT3 signalling and the induction of apoptosis (Figure 5.26-A,B). Similarly, induction of apoptosis was demonstrated by the analysis of cleaved caspase 3 expression in the KPC cells with downregulated ABCC3. Both knockdown of ABCC3 and its pharmacological inhibition with S3 considerably enhanced the activation of caspase 3 (Figure 5.27-A, B).

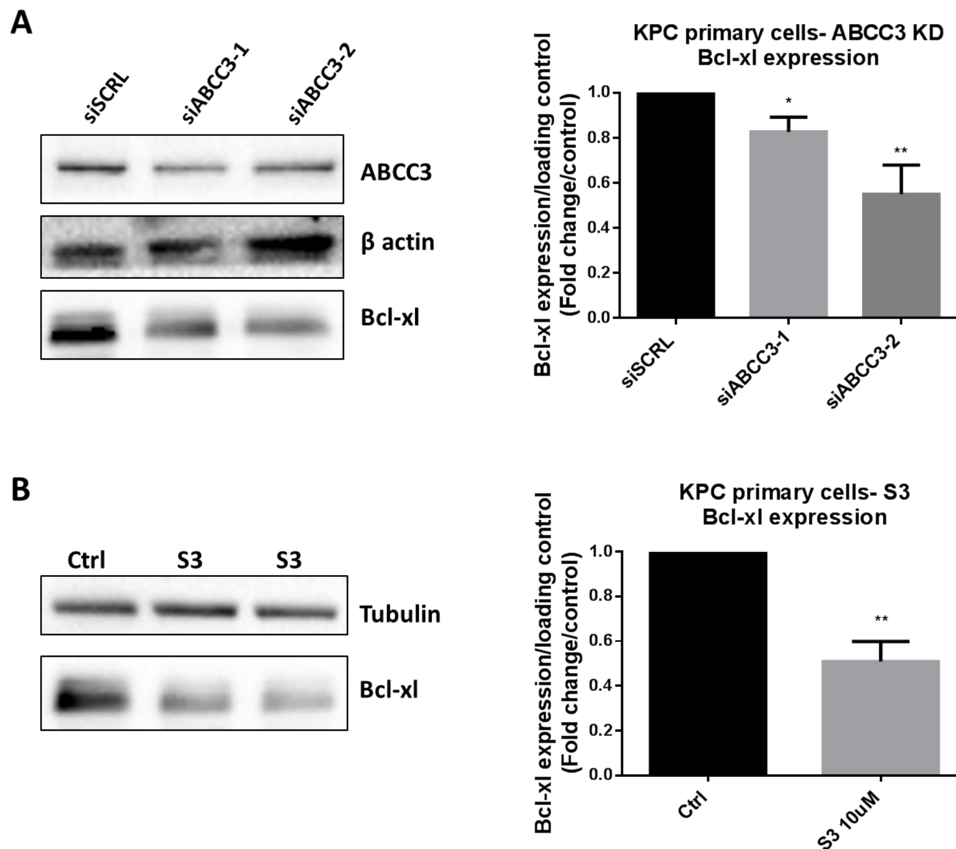


Figure 5.26 Downregulation of ABCC3 reduces the expression of Bcl-xl protein in KPC primary cell line. *Representative Western blot images and quantitative analysis of the effects of ABCC3 knockdown (A) and pharmacological inhibition with S3 (B) on the expression of Bcl-xl in the KPC primary cell line; Results are presented as mean ± SEM of at least 3 independent experiments, *p<0.05, **p<0.01*

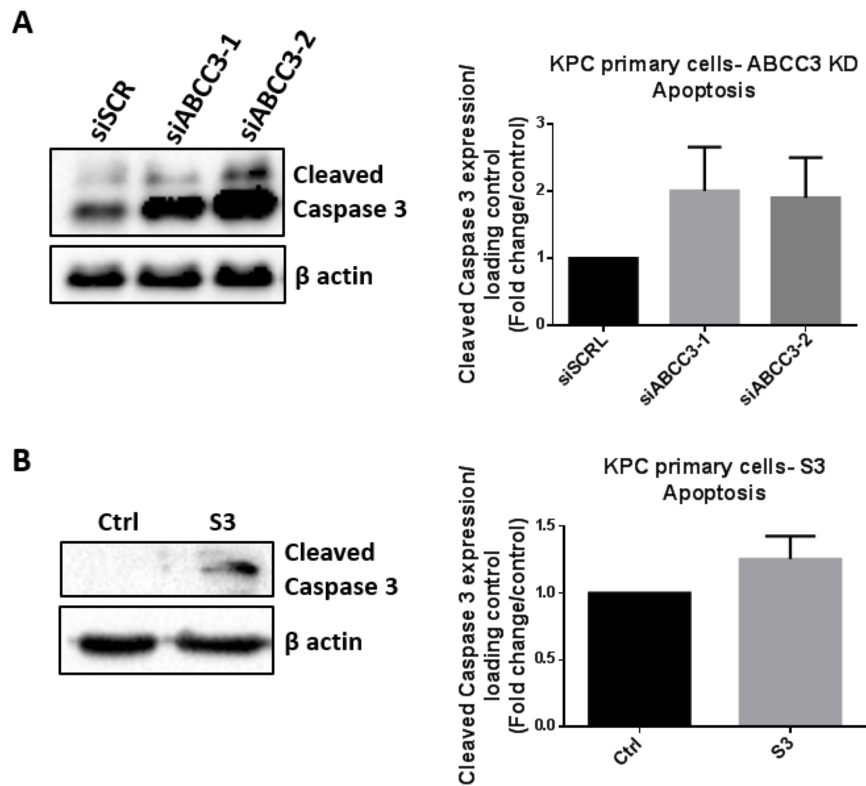


Figure 5.27 Downregulation of ABCC3 induces apoptosis in primary KPC cell line. *Representative Western blot images and quantitative analysis of the effects of ABCC3 knockdown (A) and pharmacological inhibition with S3 (B) on the levels of cleaved caspase 3 in the KPC primary cell line. Results are presented as mean ± SEM of at least 3 independent experiments*

These data provide additional *ex vivo* confirmation of the involvement of ABCC3 in PDAC progression through the regulation of STAT3 and HIF1α signalling and apoptosis. They also give additional evidence of the effectiveness of ABCC3 inhibition with S3 in the slowdown of PDAC progression in the KPC model, the most clinically relevant model of PDAC.

5.3.10 ABCC3 as a player in tumour- stroma interactions?

The importance of stroma in PDAC has been widely documented. The interplay between tumour cells and surrounding environment fuels cancer progression and protects it from the therapies. Therefore, targeting PDAC environment has emerged as a novel approach in anti-PDAC therapies.

5.3.10.1 ABCC3 is overexpressed in PDAC stroma

The Immunohistochemical (IHC) staining analysis of the pancreatic tissues resected from the KPC mice and control mice demonstrated significant overexpression of ABCC3 in the KPC mice. Similarly to human pancreatic tissues, pancreas of the control mice showed low expression of ABCC3, which was localized mainly in the Islets. Surprisingly, pancreatic tissues from the KPC mice, apart from the ductal expression of ABCC3 showed stromal staining, suggesting stromal expression of ABCC3 in PDAC samples. In particular, strong stromal expression of ABCC3 was detected with the use of Santa Cruz antibody (Figure 5.28-A). PDAC stroma is a complex microenvironment composed of fibroblasts, stellate cells, immune cells and a variety of ECM proteins. Therefore, to verify the potential overexpression of ABCC3 in PDAC stroma, its presence in cancer-associated fibroblasts was analysed. Two human fibroblast cell lines- human fibroblasts (BJ) cell line and human pancreatic cancer-associated fibroblasts (CAFs) were investigated. Western blot analysis revealed a strong expression of ABCC3 in CAFs cell line, whereas insignificant levels of ABCC3 were observed in the BJ cells. At the same time, lower levels of STAT3 phosphorylated at Tyr705 and HIF1α were detected in BJ cell line (Figure 5.28-B). Interestingly, CAFs cell line showed higher expression of ABCC3, compared to the tested PDAC tumour cell lines (Figure 5.28-C). These results are consistent with the high stromal expression of ABCC3 in the pancreatic tissues form KPC mice, as well as with the analysis of ABCC3 expression in the primary KPC cell line.

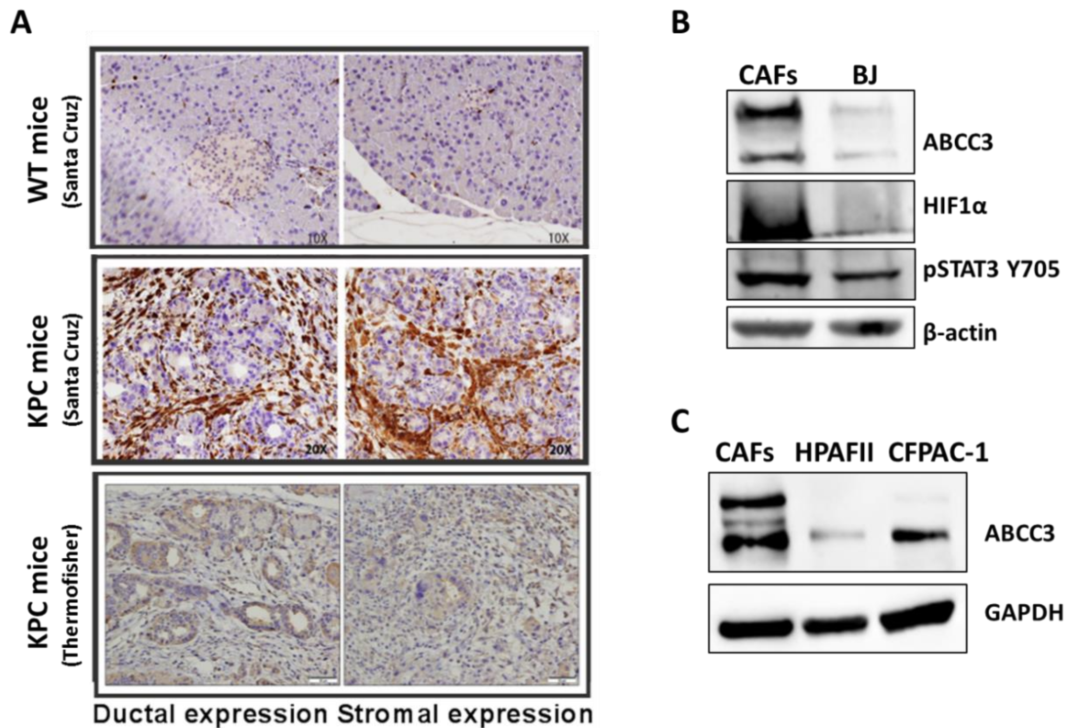


Figure 5.28 ABCC3 is overexpressed in PDAC stroma. *(A) Representative IHC staining showing ABCC3 expression in the KPC wild type (WT) mice compared to the expression in the KPC mice, as shown with the use of two different anti-ABCC3 antibodies (Santa Cruz, Thermofisher). Scale bar: 100µM, 50µM; (B) Western blot analysis of the expression of ABCC3 and its downstream effectors in the human immortalized cancer-associated fibroblasts (CAFs) compared to normal fibroblasts (BJ); (C) Western blot analysis of ABCC3 expression in CAFs and commercially available PDAC cell lines.*

5.3.10.2 Targeting of ABCC3 with S3 slows down PDAC progression through loosening of PDAC stroma

5.3.10.2.1 S3 shows high efficiency in the CAF cell line

The effects of ABCC3 inhibition on the viability of the CAF cells were then verified in order to assess the potential of ABCC3 targeting on the stroma reprogramming. Consistent with the increased ABCC3 expression in the stromal cells, higher responsiveness to S3 treatment was observed for the CAF cell line. Comparison of the response of the CAF and BJ cells to the treatment with increasing doses of ABCC3

inhibitor showed a significant and efficient decrease in the number of the CAF cells, whereas BJ cells remained unresponsive even when exposed to high drug concentrations (Figure 5.29-A). Importantly, the response of CAF cells to S3 treatment was higher than its effectiveness in the commercially available PDAC cell lines (AsPC1, CFPAC-1, HPAFII), consistent with elevated expression of ABCC3 in this cell line (Figure 5.29-B). These results confirm the increased activity of ABCC3 in PDAC stroma and demonstrate the specificity of S3 towards ABCC3.

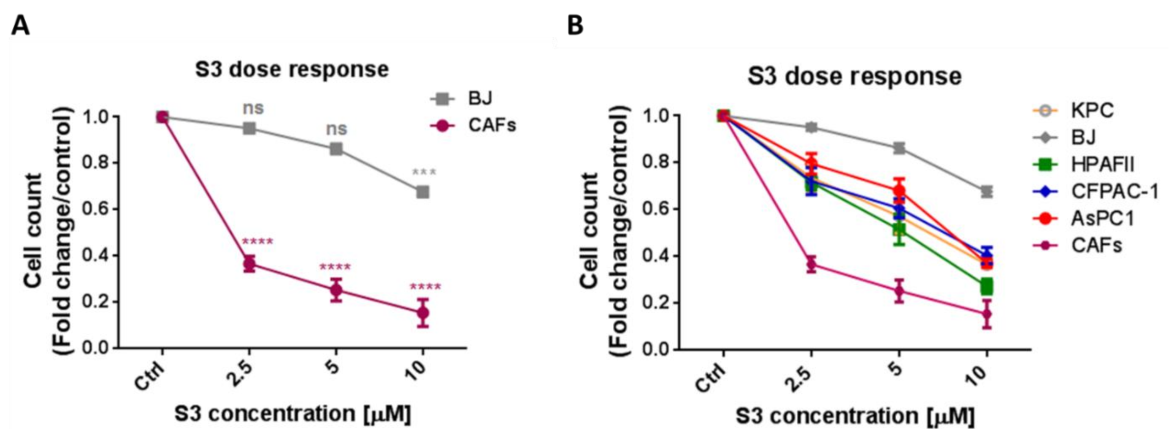


Figure 5.29 ABCC3 inhibition shows higher efficacy in cancer-associated fibroblasts. *Comparison of the effects of S3 dose response treatment on the viability of CAFs and BJ cell line (A) and in comparison with all tested PDAC cell lines (B). Each experiment was performed in triplicate, results are presented as mean ± SEM, ***p<0.001, ****p<0.0001*

5.3.10.2.2 Inhibition of ABCC3 with S3 decreases levels of stromal markers in vivo

Having demonstrated the overexpression of ABCC3 in PDAC stroma, it was tempting to speculate whether the observed effects of ABCC3 inhibition on PDAC progression *in vivo* are due to modification of stromal signalling and stroma-tumour interplay. To verify the potential involvement of ABCC3 in the modification of PDAC stroma, the expression of vimentin, a fibroblast marker, was analysed. I have previously demonstrated that vimentin expression was remarkably reduced in the livers of S3-

treated KPC mice, suggesting the blockage of PDAC cells in the epithelial phenotype. IHC analysis of pancreatic tumour tissues resected from KPC mice treated with S3 or vehicle was performed and the quantitative analysis of the vimentin-positive tissues was carried out with the use of ImmunoRatio software. Significant decrease in the vimentin content could be observed in the pancreatic tumours from the mice treated with S3 compared to the mice receiving vehicle, suggesting the involvement of ABCC3 and its inhibition in PDAC stroma reprogramming. Higher magnification images showed that not only the expression of vimentin was decreased in these tumours, but also the number of vimentin-expressing fibroblasts was remarkably lowered (Figure 5.30).

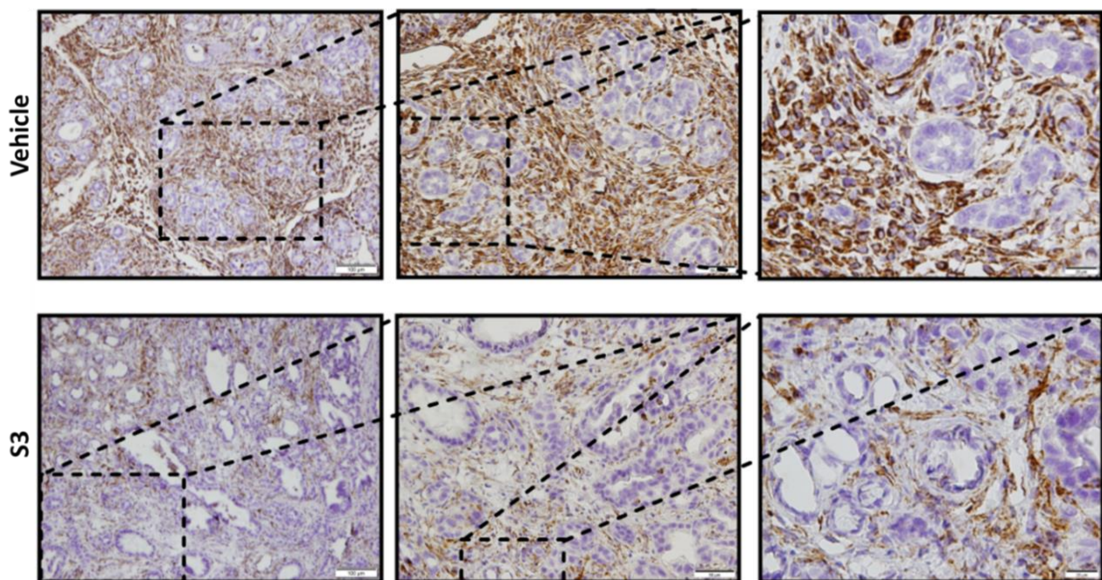


Figure 5.30 S3 treatment reduces vimentin expression and loosens PDAC stroma. *Representative IHC staining comparing vimentin expression in FFPE pancreatic tissues from KPC mice treated with vehicle or 25 mg/kg of S3 (Scale bar: 100µm, 50µm, 20µm). Quantitative analysis is presented as mean ±SEM of 8 (Vehicle) and 12 (S3) separate images ***p<0.001*

Moreover, Western blot analysis of the snap frozen pancreatic tissues from the same mice confirmed a significant downregulation of vimentin in the mice subjected to S3 treatment compared to control (vehicle-treated) mice. In addition, the same analysis demonstrated the downregulation of the expression of αSMA- another marker of

activated pancreatic stellate cells, confirming the effects of ABCC3 targeting on the reprogramming of PDAC stroma (Figure 5.31).

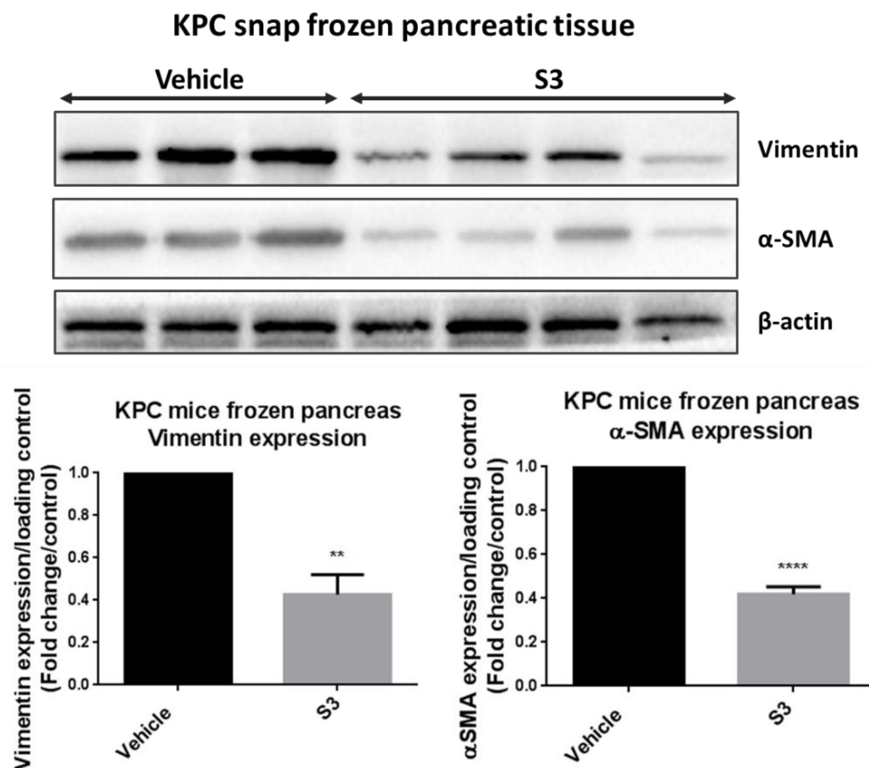


Figure 5.31 S3 treatment reduces levels of stromal markers*. Western blotting analysis of vimentin and αSMA expression in snap frozen pancreatic tissues resected from KPC mice treated with vehicle (n=3) or 25 mg/kg S3 (n=4), **p<0.01, ****p<0.0001*

5.3.10.2.3 Inhibition of ABCC3 with S3 targets stroma-associated fibroblasts in the KPC primary cell line

To gain additional confirmation of the ABCC3-mediated targeting of PDAC stroma, the KPC primary cell line was analysed. Phenotypically, the primary cells adopted a tumour-like morphology with the formation of epithelial clusters surrounded by a matrix of fibroblast-like cells, demonstrating the presence of both cell types in the established cell line. Thus, the effects of ABCC3 downregulation on both epithelial tumour cells and associated fibroblast may be assessed with the use of this cell line. Downregulation of ABCC3 in the primary KPC cells reduced the viability of both cell

types (Figure 5.32). Interestingly, fibroblast-like cells were remarkably more affected by ABCC3 downregulation than the epithelial cells that they surround. The number of the fibroblasts significantly decreased after ABCC3 knockdown and inhibition with S3, confirming the results obtained with the cancer associated fibroblast cell line (CAFs). The increased expression of ABCC3 observed in CAF cell line and its enhanced responsiveness to S3 lays in agreement with the high effectiveness of S3 in the KPC cell line.

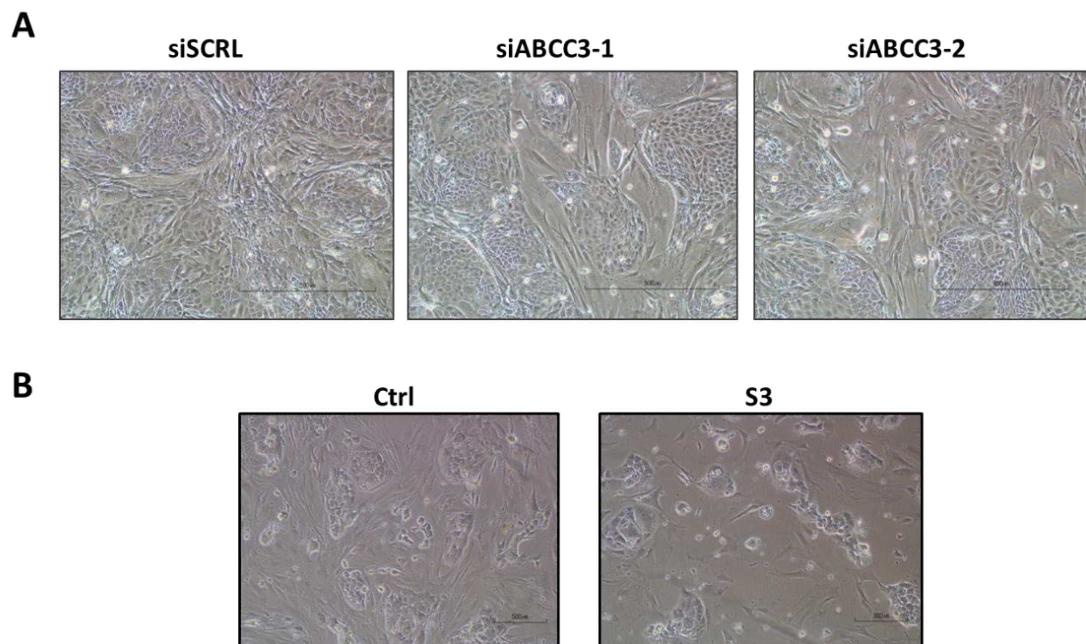


Figure 5.32 ABCC3 downregulation targets both epithelial and fibroblast cells in KPC primary cell line. *The effects of ABCC3 genetic knockdown (A) and pharmacological inhibition with S3 (B) on the viability and morphology of KPC primary cells. The reduction in epithelial and fibroblast-like cells was observed following both procedures. Photos were taken with the inverted microscope, scale bar: 500µm*

These data reinforce the hypothesis of the expression and role of ABCC3 in both PDAC ducts and stroma. Moreover, Western blot analysis of the KPC primary cell line following both ABCC3 genetic silencing and its inhibition with 10 µM S3 also showed a significant decrease in the expression of both stromal markers (αSMA, vimentin) (Figure 5.33).

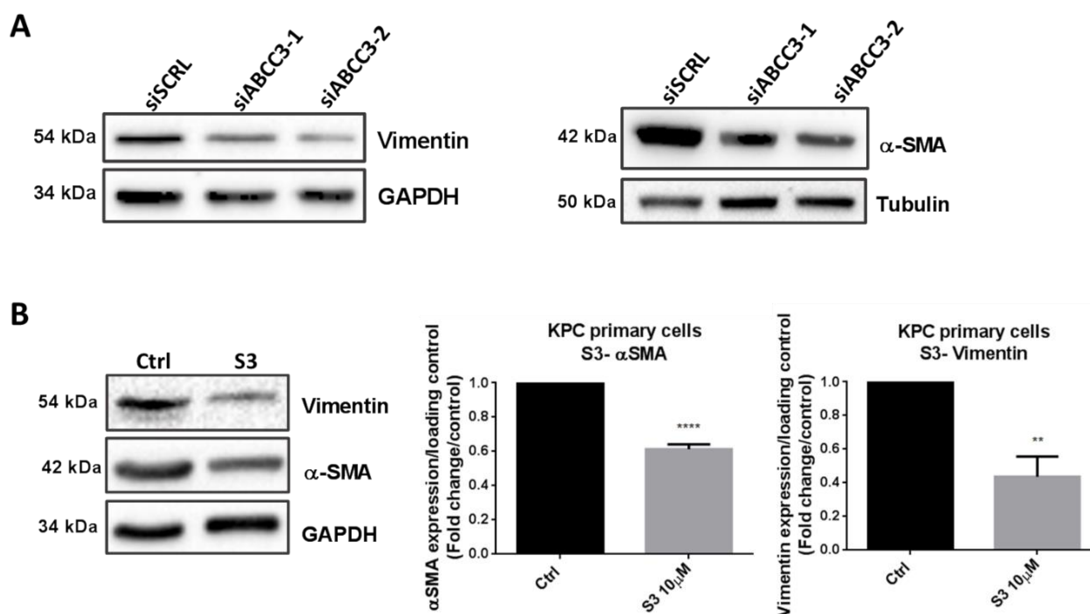


Figure 5.33 ABCC3 downregulation reduces levels of stromal markers in the KPC primary cell line. *Representative Western blot images showing the effects of ABCC3 transient knockdown with 2 specific siRNAs (siABCC3-1, siABCC3-2) and 10μM S3 treatment of KPC primary cell line on the expression of stromal markers: vimentin and α-SMA. Quantitative results are presented as a mean ± SEM of 3 independent experiments, **p<0.01, ***p<0.001.*

Reduction in vimentin expression in the S3-treated PDAC cells might additionally suggest blocking of the epithelial to mesenchymal transition (EMT). Vimentin is a fibroblast-expressed marker that is overexpressed in the cells of the mesenchymal phenotype. Therefore, less vimentin detected in the cells with ABCC3 downregulation may suggest blocking of the cells in the epithelial, less invasive phenotype. Consistent with these data, treatment of the KPC cell line with S3 resulted in the upregulation of E-cadherin and reduction in Slug levels (Appendix, Figure 9). Loss of E-cadherin has been associated with enhanced EMT process and more invasive phenotype. Similarly, Slug is a transcription factor expressed by cells of mesenchymal phenotype. Therefore, observed upregulation of E-cadherin and downregulation of Slug levels might suggest less aggressive, epithelial phenotype of the cells with non-active ABCC3.

5.4 Discussion

Pancreatic cancer is a very aggressive and malignant disease lacking effective therapeutic approaches. Heterogeneity and plasticity of the tumours and development of dense stroma around the tumours contribute to the high chemotesistance of PDAC, making available chemotherapies mostly ineffective. In addition, genetic heterogeneity leading to dysregulation of multiple pathways highly restrained the effectiveness of targeted therapies developed so far, leaving space for identification of novel therapeutic targets. In the previous chapter, I presented ABCC3 as a novel key player in PDAC progression. The involvement of ABCC3 in PDAC progression through regulation of STAT3 signalling and apoptosis was demonstrated *in vitro*. Moreover, *in vivo* data, as well as database analysis of clinical samples indicated ABCC3 as an important player in PDAC progression, mainly in patients bearing *TP53* mutations, suggesting it as a potential therapeutic target.

Therefore, in this chapter I explored the potential of ABCC3 as a novel druggable target in PDAC. I showed that sulindac derivative developed in our group, referred to as S3, is a specific inhibitor of ABCC3, showing high anti-tumorigenic potential and, unlike sulindac, lacking inhibitory activity on COX pathway. I also demonstrated that S3 activity mirrors the effects of ABC genetic silencing, significantly reducing PDAC cell growth and clonal expansion and slowing down PDAC progression *in vivo*, and at the same time not showing visible side effects. The specificity of S3 towards cancer cells has been also demonstrated. Low expression of ABCC3 in non-malignant pancreatic cells, as well as in the cells bearing wild type p53 correlated with lack of efficacy of S3 in decreasing cell number. However, more studies investigating the specificity of S3 towards ABCC3 compared to other ABCC transporters need to be performed. Using different animal models of pancreatic cancer, I showed that S3 treatment of xenograft mice significantly reduced tumour growth and increased mice survival. Remarkably, complete tumour remission was observed in 30% of the S3-treated xenograft mice, showing the high potency of this ABCC3-targeting approach. Importantly, high effectiveness of S3 was also demonstrated in the transgenic model of pancreatic cancer, which reproduces the biology and progression of the human disease, therefore providing an important pre-

clinical validation for targeting ABCC3 with small-molecule inhibitors like S3. Importantly, higher effectiveness of S3 over the standard-of-care chemotherapy, Abraxane, was observed. I speculate that the minimal effectiveness of chemotherapy was partly due to treatment-related deterioration of mice welfare. The results mirror the effects observed in human therapy, in which marginal effectiveness of chemotherapy is coupled with increased rates of adverse events and deterioration of the quality of life. In contrast, no side effects were reported for the S3-treated group.

I demonstrated that the mechanisms regulating the observed decrease in cell proliferation and clonal expansion involve induction of apoptosis, control of cell cycle and regulation of STAT3 and HIF1α signalling. Using different approaches I showed that both ABCC3 silencing and its pharmacological inhibition with S3 increase apoptosis in PDAC cells. These data confirm the role of ABCC3 in regulation of PDAC progression. I could also show that modulation of ABCC3 activity influences cell cycle progression in PDAC cells. However, differences in the cell cycle arrest were observed depending on the way of ABCC3 dysregulation. Silencing of ABCC3, both stable and transient, blocked cell cycle in the G1 phase as demonstrated with FACS analysis and Western blot analysis of cyclins expression. On the other hand, S3 treatment resulted in the arrest of the cells in G2/M phase. These differences suggest that different mechanisms might be induced by ABCC3 silencing and pharmacological inhibition that lead to cell cycle arrest at different phases. More studies, exploring more in detail the intermediate events triggered by ABCC3 modulation, need to be considered to verify the reason for observed differences. More importantly, downregulation of activated STAT3 and HIF1α was observed both *in vitro* and *in vivo*, following S3 treatment. Demonstrated results 'mimick' the data obtained after ABCC3 knockdown, confirming the specificity of S3 towards ABCC3. These data also provide a potential novel approach for indirect targeting of STAT3 in pancreatic cancer, one of main therapeutic targets in PDAC.

In this project, I also showed that overexpression of ABCC3 was demonstrated in the stroma of pancreatic cancer and importantly, targeting of ABCC3 expressed in the stroma highly reduced the number of ABCC3-expresing fibroblast cells, leading to stroma disruption. Several attempts in targeting PDAC stroma have been previously made through inhibition of Hedgehog signalling combined with chemotherapy. However, majority of the trials failed due to increased metastatic spread caused by disruption of

the stroma. In this study, we showed that targeting of ABCC3 in the KPC transgenic mouse model highly prolonged the survival, not showing evident side effects. In fact, my initial observations suggest that the liver status of the KPC mice treated with S3 is different from vehicle-treated animals, which show lower number of lesions and significantly decreased vimentin expression. These data might partly explain the observed the increase in the survival of KPC mice treated with S3, suggesting reduced metastatic spread in these mice. As a support, differential expression of pSTAT3 Y705 in the livers of the KPC mice suggests the loss of the transcriptional activity of STAT3 and, as a consequence, slowdown in the progression of tumour development in the liver. Moreover, a reduced expression of pSTAT3, Bcl-xl and HIF1α in the regional lymph nodes additionally supports the hypothesis of the reduction of metastatic spread after S3 treatment. Decreased vimentin expression presented in both, pancreas and liver of the S3-treated KPC mice additionally supports these observations. Vimentin is a fibroblast marker expressed by the cells of mesenchymal phenotype. The remarkably reduced levels of vimentin observed in the tissues resected from S3-treated mice suggest the blockage of EMT transition. Similarly, increase in E-cadherin levels was observed in the KPC cells with inhibited ABCC3. Consequently, I may hypothesise that PDAC cells with downregulated ABCC3 are characterized by less invasive, epithelial phenotype, less likely to disseminate and metastasise. However, more in depth analysis of the effects of ABCC3 downregulation on migration and invasion of PDAC cells needs to be performed. Both *in vitro* migration studies and *in vivo* studies including experimental metastasis mouse model could substantially add to presented data.

6. ABCC3- mediated LPI release regulates PDAC progression

6.1 Introduction

Lysophospholipids constitute a group of lipids playing essential role in many physiological and pathological processes. As components of plasma membrane, lysophospholipids modulate the curvature of the membrane by altering the functions of membrane proteins, such as ion channels. The role of lysophospholipids as secondary messengers has been also distinguished and it involves the regulation of cell signalling pathways controlling a variety of physiological processes, such as angiogenesis, inflammation, nervous system regulation or tumorigenesis. The secretion of lysophospholipids by cancer cells, but also by tumour environment components has been previously described, suggesting a role as extracellular signalling molecules in the regulation of cell growth and modulation of cancer immune system. Furthermore, mitogenic function of lysophospholipids has been also demonstrated.

Until recently, the majority of studies focused on lysophosphatidic acid (LPA) or sphingosine-1-phosphate (S1P). However, increasing evidence has pointed to the active biological role of other lysphospholipids in the stimulation of cellular responses. Lysophosphatidylinositol (LPI) is a bioactive lipid belonging to the lysophospholipids family. It is synthesised from phosphatidylinositol (PI) by the phospholipase A (PLA) enzyme family. Depending on the PLA subtype (PLA_1 or PLA_2), it cleaves PI releasing fatty acids and generating 1-Acyl-LPI (PLA_2) or 2-Acyl-LPI (PLA_1) (203). Although identified in early 1960s, the first notion of the potential role of LPI was not discovered until 20 years later when the stimulation of the insulin release from β cells was linked with LPI activity (476). The involvement of LPI in a variety of physiological processes has been demonstrated, including Ca^{2+} mobilisation and insulin release from pancreatic islets (476). Additionally, LPI has been shown to activate different signalling pathways, e.g. through $ERK_{1/2}$ or Akt phosphorylation, Rho activation or modulation of TRP and K^+ channels (477). The identification of G

protein-coupled receptor 55 (GPR55) as the specific receptor for LPI fuelled more interest in LPI, leading to an increased knowledge on its biological functions. Indeed, the involvement of LPI in many physiological processes such as cell growth and motility has been identified in several cell types, mainly in the nervous system, endothelial cells and cancer. LPI has been shown to activate cell migration in neutrophils as well as in ovarian and breast cancer cell lines. Other than the mitogenic function in tumorigenesis, several GPR55-regulated biological functions were established for LPI, including impact on bone physiology, nervous system and pain perception (203). Involvement of LPI in lipid metabolism has been also demonstrated and its increased plasma levels have been detected in some metabolic diseases, including obesity (478).

Falasca et al. first described the role of LPI as a mitogenic factor in cancer cells in 1994, who noted highly elevated LPI levels, because of increased PLA_2 activity, in the Ras mutated thyroid cells (479, 480). More importantly, the ability of Ras-transformed fibroblasts to secrete LPI was demonstrated, showing remarkably higher LPI levels released in the media of transformed cells, compared to normal fibroblasts where LPI was undetectable. Following this observation, several clinical studies confirmed the increased levels and direct role of LPI in stimulation of cell proliferation and in the progression of e.g. ovarian, breast or prostate cancer (481). Elevated levels of LPI, but no increase in total phospholipids, were observed in ovarian cancer or peritoneal cancer patients and they were additionally enhanced in more advanced or recurrent patients (482). LPI- induced migration of breast cancer cells additionally suggests the potential role of LPI in metastatic spread. LPI- mediated activation of signalling pathways essential for cancer cell survival was also documented (78, 330).

Importantly, a novel mechanism of LPI-stimulated proliferation of prostate and ovarian cancer cells was discovered in our group (334). It was shown that LPI, together with GPR55 form an autocrine loop in these cells, which triggers the activation of tumorigenic signalling pathways. This loop involves the $cPLA_2$-mediated synthesis of LPI in malignant cells and its release to extracellular space by the ABCC1 transporter (334). Increased levels of LPI in the extracellular medium, caused by the enhanced synthesis and export in the neoplastic tissues, lead to mobilisation of

intracellular Ca^{2+} and activation of GPR55-mediated signalling, e.g. $ERK_{1/2}$ phosphorylation or Akt activation. Furthermore, $cPLA_2$ silencing in prostate and ovarian cancer cell lines significantly decreased their proliferation. A similar reduction in cell proliferation was observed with GPR55 silencing, an effect that could not be restored by exogenously added LPI. These data demonstrated the existence of a mechanism involving ABCC1-LPI-GPR55 loop in ovarian and prostate cancers that propelled cell proliferation and cancer progression. This evidence was further corroborated by the elevated levels of LPI detected in the plasma of ovarian and prostate cancer patients (481). Therefore, LPI was additionally proposed as a potential biomarker for early detection of malignant cell transformation (483).

6.2 Aims of the project

As previously described, several ABC transporters exert their functions through release of bioactive lipids, which, by triggering the activation of various signalling pathways induce cell proliferation, contributing to carcinogenesis. Thus, molecules involved in the synthesis or release of these lipids represent a potential target for anti-carcinogenic therapies. Having demonstrated that the activation of the LPI receptor GPR55 triggers the proliferation of PDAC cells, the next aim was to investigate the role of LPI in PDAC and to identify the proteins involved in the release of LPI from cells. As previously described, in prostate and ovarian cancers, the existence of ABCC1-LPI-GPR55 was demonstrated. Considering the demonstrated key role of ABCC3 in PDAC progression and the ability of ABC transporters to shuffle bioactive lipids across plasma membrane, in this part of the project we aimed to:

- Investigate the potential role of ABCC3 in LPI release in pancreatic cancer
- Investigate the role and potential mechanisms of LPI-mediated PDAC progression
- Analyse the existence and potential role of an ABCC3-LPI-GPR55 axis in PDAC
- Investigate the vertical targeting of ABCC3 and GPR55 in PDAC therapy

6.3 Results

6.3.1 ABCC3-released LPI regulates PDAC proliferation

The mitogenic activity of LPI has been suggested in a variety of human cancers and elevated LPI levels were correlated with tumours aggressiveness. Therefore blocking of the molecules responsible for their release presents the opportunity for the decrease of cancer progression. In the previous chapters, I demonstrated the involvement of ABCC3 in LPI release from PDAC (Figure 4.3-A, B, C). In order to confirm the importance of LPI in PDAC progression the effect of long-term LPI stimulation on PDAC cells proliferation was verified. I demonstrated that LPI stimulation of AsPC1, CFPAC-1 and HPAFII cell lines resulted in considerable and statistically significant increase in cell proliferation, compared to the control cells, which could solely rely on endogenously synthesized LPI (Figure 4.3-D).

These results confirmed the involvement of LPI in regulating the proliferation and growth of PDAC cells. It is thus tempting to hypothesize that the inhibition of LPI synthesis or release from the cells could substantially reduce PDAC cell proliferation and decrease disease progression. In fact, in chapter 4 I showed that downregulation of ABCC3 transporter, protein responsible for LPI efflux in pancreatic cancer cells, significantly reduced PDAC cell number.

To further verify the role of LPI as mitogenic factor in PDAC, I used a CFPAC-1 cell line stably silenced for ABCC3 (CFPAC-1 shABCC3) and the respective control CFPAC-1 cells (CFPAC-1 4Mut). To maintain the selection of the infected cells, both populations were cultivated in 1µg/ml puromycin-containing medium. Cells were serum-starved and stimulated with LPI as previously described and number of viable cells was tested by manual counting with trypan blue exclusion. Long-term stimulation with exogenous LPI increased the proliferation of the control CFPAC-1 4mut cells, confirming previous results. On the other hand, consistent with our previous data, genetic silencing of ABCC3 reduced the number of viable cells in serum-free conditions. However, stimulation of CFPAC-1 shABCC3 cells with exogenous LPI

reversed the effects of ABCC3 silencing, increasing the number of viable cells almost to the initial levels (Figure 6.1).

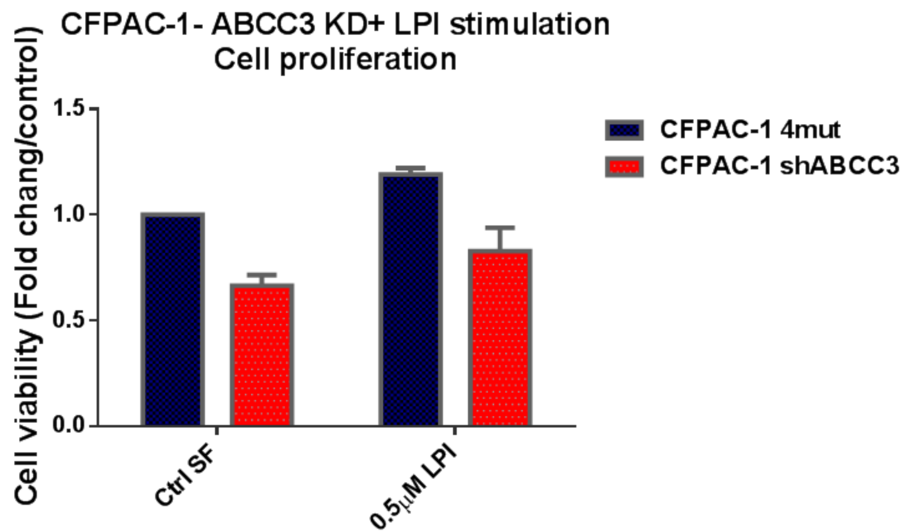


Figure 6.1 ***Mitogenic activity of LPI depends on ABCC3 activity.*** *Effects of long-term LPI stimulation on proliferation of CFPAC-1 control cells (4-mut, blue) and CFPAC-1 cells with stable knockdown of ABCC3 (shABCC3, red). The results are presented as mean ± SEM of 2 independent experiments. All samples were maintained in serum free conditions with addition of 1µg/ml of puromycin*

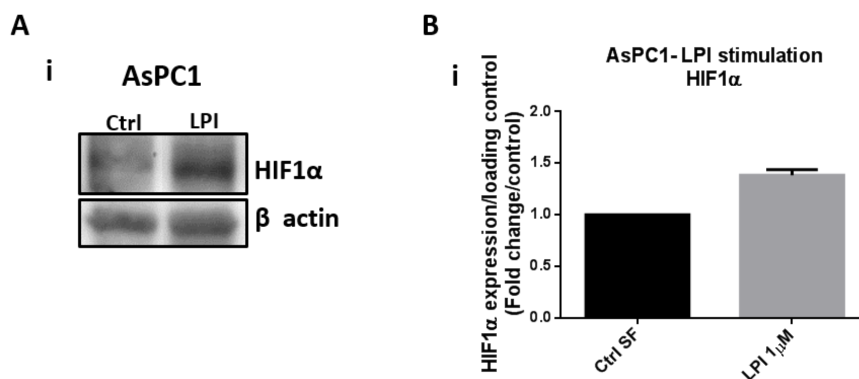
These results, although preliminary, once again confirm the involvement of ABCC3 in LPI transport. Using several approaches I showed that blocking the cellular release of LPI by ABCC3 knockdown slows down PDAC cell growth. On the other hand, observed effects are restored by LPI stimulation.

6.3.2 ABCC3-mediated LPI release regulates PDAC proliferation through STAT3 and hypoxia

Having confirmed the importance of LPI in PDAC progression *in vitro* and the link between ABCC3 expression and LPI release, I wanted to verify whether activation of signalling pathways shown to be affected by ABCC3 activity, such as STAT3 and HIF1α, is mediated by LPI. Acute stimulation of serum-starved cancer cells with LPI was

carried out and changes in the activation of STAT3 protein (phosphorylation of STAT3 at tyrosine 705) was analysed by Western blotting. A set of experiments were performed to optimize the conditions for LPI acute stimulation of PDAC cells. An incubation with 1µM LPI for 8 min, following overnight starvation of the cells (incubation in the serum-free media) was set as the optimal reaction conditions. Following the acute LPI stimulation, cells were harvested and lysed using the standard protocol and the protein content of the cells was analysed by western blot analysis. As shown in previous chapter, significantly increased levels of phosphorylated STAT3 (pSTAT3 Y705) could be detected in the AsPC1 and CFPAC-1 cells after LPI stimulation, compared to the control serum- starved cells (Figure 4.4).

Another signalling pathway regulated by ABCC3 activity is HIF1α signalling. Therefore, the influence of LPI stimulation on HIF1α expression was also verified. Time optimization of the LPI stimulation was performed on PDAC cell lines and a clear stimulation of HIF1α expression could be detected 24h following stimulation with 1µM LPI. Thus, this time point and concentration were selected as optimal conditions for the investigation on the LPI- induced expression of HIF1α. Remarkably, increased levels of HIF1α were detected in all three tested cell lines (AsPC1, HPAFII, CFPAC-1) after LPI stimulation (Figure 6.2).



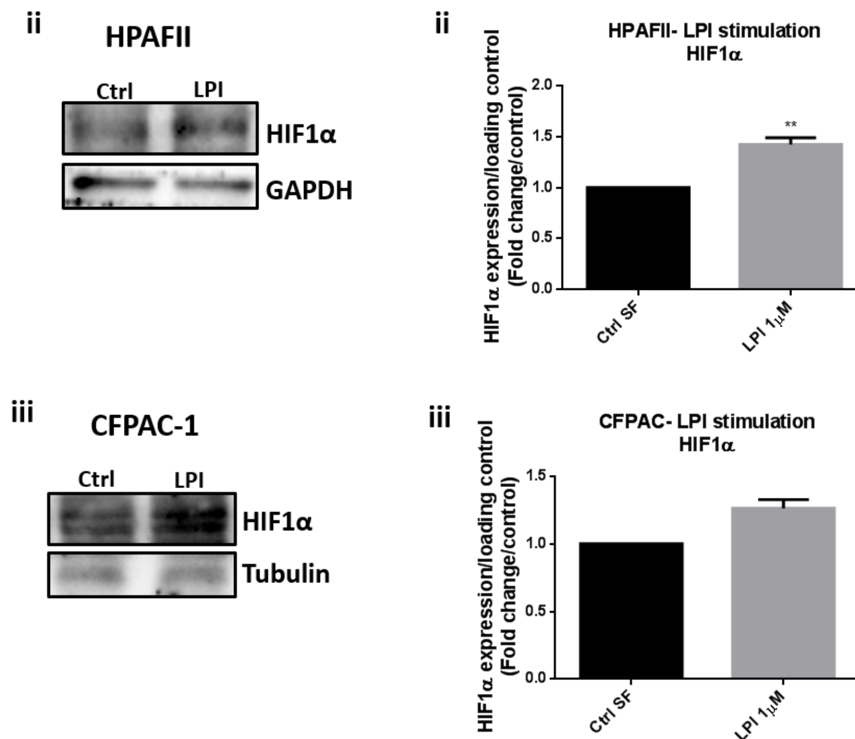


Figure 6.2 ***LPI stimulates HIF1α expression in PDAC cell lines.*** *(A) Representative western images showing the effects of 24 stimulation of (i) AsPC1, (ii) HPAFII and (iii) CFPAC-1 cells with 1μM LPI on the expression of HIF1α; (B) Quantitative analysis of N=2 (AsPC1, CFPAC) and N=3 (HPAFII) separate experiments. Results are presented as mean ± SEM; **p<0.01*

Taken together, these data confirm the involvement of ABCC3-released LPI in the control of PDAC cell growth through regulation of hypoxia and STAT3 signalling in PDAC.

To gain additional evidence for the involvement of ABCC3-LPI axis in the regulation of the STAT3 signalling, transient knockdown and pharmacological inhibition of ABCC3 with small molecule inhibitor S3, followed by LPI stimulation was performed. ABCC3 was transiently knockdown in AsPC1, CFPAC-1 and HPAFII cell lines. Transfected samples were serum-starved and acute stimulation with 1μM LPI was performed. The levels of pSTAT3 Y705 expression in the knockdown samples with and without LPI stimulation were verified by Western blot analysis (Figure 6.3).

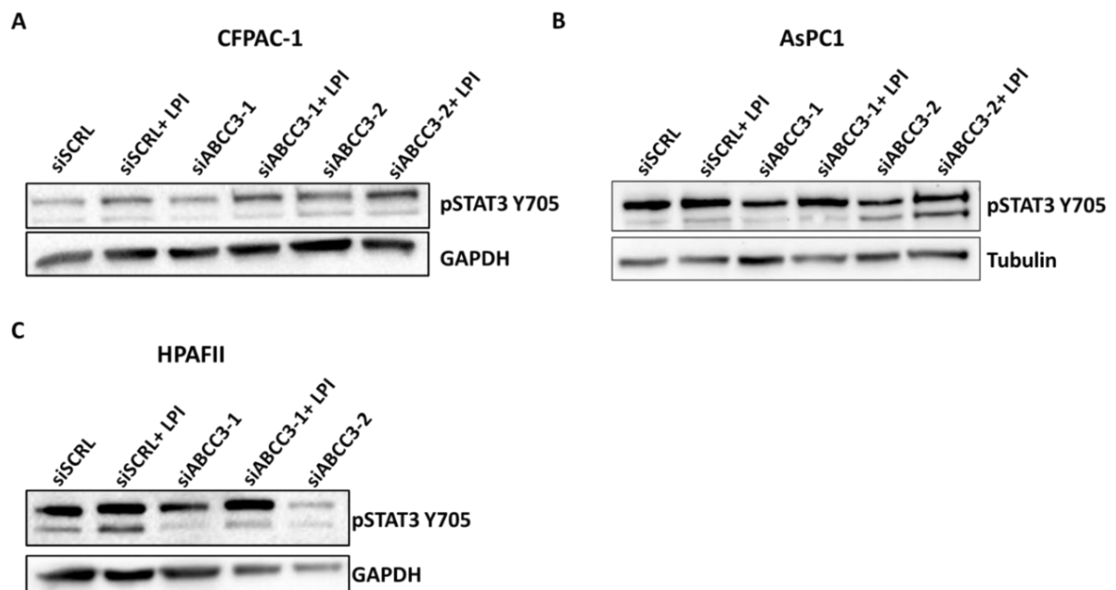


Figure 6.3 ***LPI stimulation of STAT3 activation is dependent on ABCC3 expression.*** *Representative Western blot images showing the effects of knockdown of ABCC3 followed by acute LPI stimulation on the levels of phosphorylated STAT3 (STAT3 Y705) in CFPAC-1 (A), AsPC1 (B) and HPAFII (C) PDAC cell lines .*

Although only preliminary, these results confirm the role of ABCC3 in LPI release. Knockdown of ABCC3 with two siRNAs decreased the levels of activated STAT3 (STAT3 Y705), consistent with previously presented data, potentially through the blocking of LPI release. Acute stimulation with exogenous LPI increased phosphorylation of STAT3 in control cells and restored its levels in the knockdown samples. Silencing of ABCC3 in HPAFII cell line with sequence siABCC3-2 reduced cell number so significantly that the serum starvation and LPI stimulation could not be performed.

Additionally, CFPAC-1 cells were treated with 10 µM S3 in serum-deprived environment for 24h before the acute stimulation with LPI in the conditions described above. As previously shown, significant decrease in pSTAT3 Y705 levels were detected in cells, in which ABCC3 activity was blocked by S3 treatment. These preliminary results show that lowered pSTAT3 Y705 levels observed after S3 treatment of CFPAC-1 cells were restored following the acute stimulation of the cells with 1µM LPI (Figure 6.4).

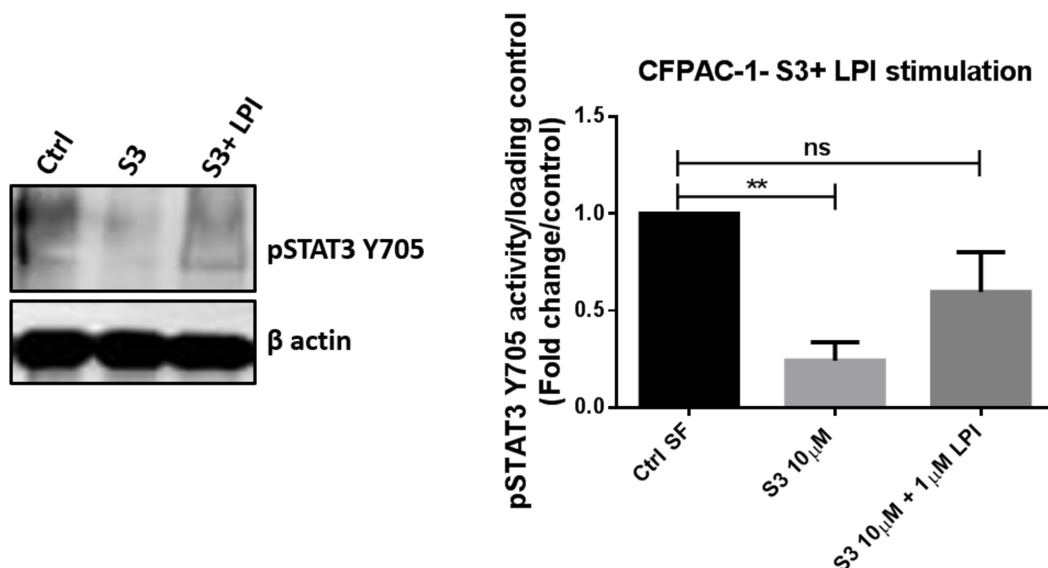


Figure 6.4 ***LPI stimulation of STAT3 activation is dependent on ABCC3 activity.*** *Representative Western blot image and quantitative analysis of the levels of phosphorylated STAT3 (pSTAT3 Y705) following the pharmacological inhibition of ABCC3 with S3 and acute LPI stimulation in CFPAC-1 PDAC cell line. The results are presented as mean ± SEM of 3 independent experiments, **p<0.01*

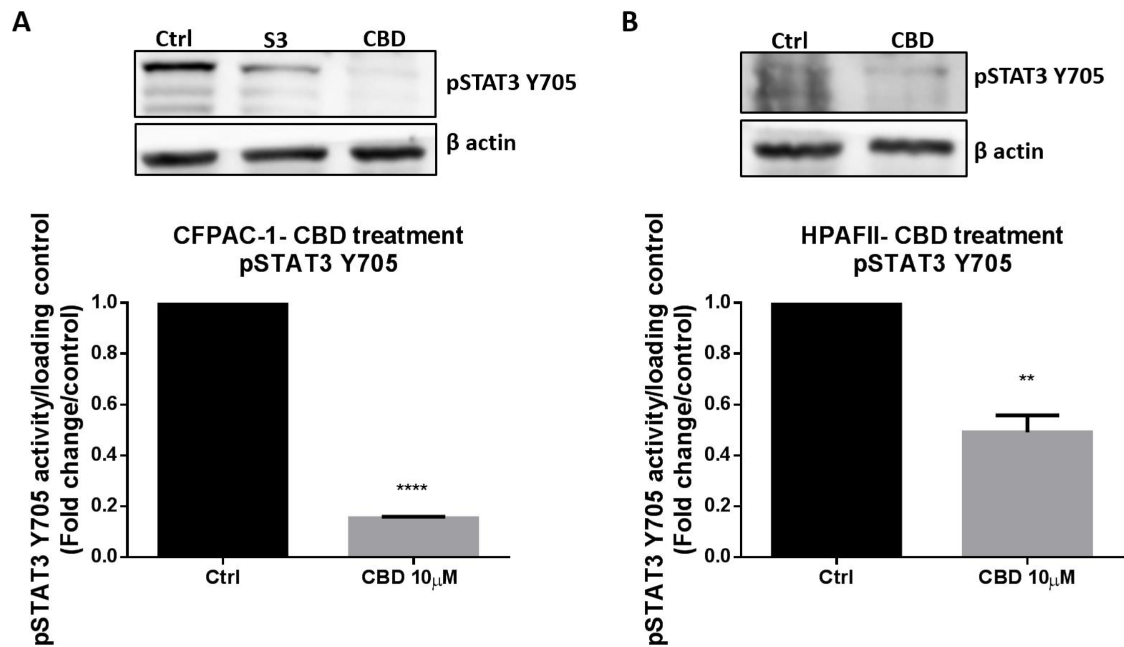
These results confirm the involvement of LPI in the activation and regulation of STAT3 signalling. Combined with the demonstrated involvement of ABCC3 in STAT3 regulation, the data also support the hypothesis of ABCC3-mediate release of LPI and confirm the importance of ABCC3-LPI axis in the stimulation of PDAC progression through activation of STAT3 signalling.

6.3.3 ABCC3-LPI-GPR55 loop regulates the activity of STAT3 signalling pathway in PDAC

We have previously suggested that ABCC3 and GPR55 form a loop in pancreatic cancer, in which ABCC3 is responsible for LPI release to the extracellular medium, where it can bind and stimulate GPR55, triggering the activation of several signalling pathways and consequently influencing PDAC cell proliferation and disease progression. Previous studies conducted in our group confirmed the involvement of

LPI-GPR55 axis in the induction of MAPK signalling in pancreatic cancer. Increased phosphorylation of ERK1/2 and S6 proteins induced by LPI stimulation was shown by Western blot analysis. Consistently, genetic downregulation of GPR55 and its pharmacological blockade reduced the levels of phosphorylation of both ERK1/2 and S6, confirming the involvement of LPI-stimulated GPR55 in activation of MAPK signalling (Ferro R, Adamska A et al (398) Figure 4e, Supplementary Figure 3f)

In this study, I demonstrated the involvement of ABCC3 in LPI cellular release. I showed that downregulation of ABCC3 by transient siRNA knockdown and pharmacological inhibition reduces STAT3 phosphorylation at tyrosine 705. On the other hand, stimulation of STAT3 Y705 activation was linked with ABCC3 released LPI. However, no link between GPR55 and STAT3 pathway in pancreatic cancer has been shown so far. To verify if ABCC3-LPI induced regulation of STAT3 levels in PDAC is also mediated by GPR55, I used the GPR55 antagonist CBD. PDAC cell lines (HPAFII, CFPAC-1) were treated with 10µM CBD for 48h and the levels of activated STAT3 (pSTAT3 Y705) were verified by Western blotting. Consistent with the LPI-stimulated STAT3 phosphorylation, I could demonstrate that following pharmacological blocking of GPR55, a significant downregulation of pSTAT3 Y705 levels was detected in the two cell lines (Figure 6.5-A, B). In particular, an almost complete blocking of STAT3 phosphorylation was noted in CFPAC-1 cell line, which is characterized by higher levels of STAT3 activity. These results suggest that apart from the GPR55-mediated activation of MAPK signalling, GPR55 is involved in regulation of STAT3 signalling in PDAC.



6.5 ***Downregulation of GPR55 reduces the expression of activated STAT3.*** *Representative Western blot images and quantification of the effects of treatment of (A) CFPAC-1 and (B) HPAFII cells with 10µM CBD on the levels of phosphorylated STAT3 (STAT3 Y705). The results are presented as mean ± SEM of 3 independent experiments, **p<0.01, ****p<0.0001*

The remarkable decrease in pSTAT3 Y705 activation caused by GPR55 pharmacological inhibition supports the hypothesis of the existence of a loop involving LPI, ABCC3 and GPR55 in PDAC.

We previously showed that LPI stimulation significantly increases levels of phosphorylated ERK1/2 in pancreatic cancer. Thus, to gain additional evidence to support that hypothesis, the potential inhibition of ERK/12 phosphorylation by downregulation of ABCC3 activity was investigated. Genetic knockdown and pharmacological inhibition of ABCC3 with S3 was performed in AsPC1, HPAFII and CFPAC-1 cell lines and the expression of pERK1/2 was verified. A slight reduction in phosphorylation of ERK1/2 could be detected following ABCC3 silencing in the analysed cell lines (Appendix, Figure 10). Similarly, downregulation of the levels of phosphorylated ERK1/2 were detected in these cells after ABCC3 inhibition with S3 (Figure 6.6). Although only preliminary, these results confirm the involvement of ABCC3-released LPI in ERK stimulation.

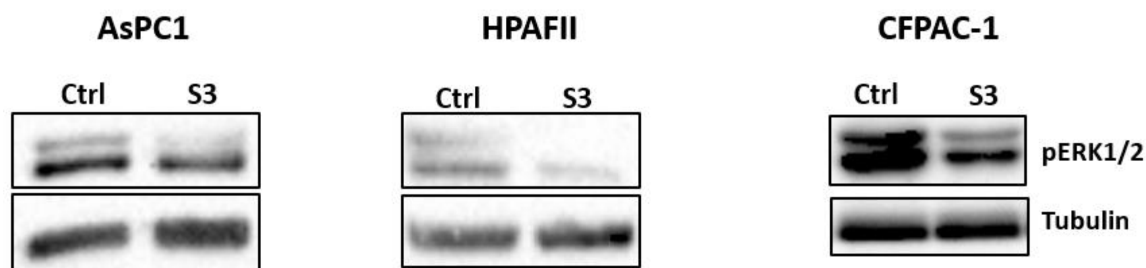


Figure 6.6 ***Downregulation of ABCC3 reduces expression of activated ERK.*** *Representative Western blot images of the effects of treatment of AsPC1, HPAFII and CFPAC-1 PDAC cells with 10µM S3 on the levels of activated ERK1/2 (pERK1/2).*

These preliminary data support the hypothesis of the existence of a loop, in which ABCC3-mediated release of LPI stimulates the proliferation of PDAC cells through activation of GPR55-regulated pathways.

6.3.4 Vertical inhibition of ABCC3 and GPR55 synergistically reduces proliferation of PDAC cells

As shown in chapters 3 and 4, high effectiveness of pharmacological inhibition of ABCC3 and GPR55 with S3 and synthetic CBD used as a single agents was demonstrated both *in vitro* and in animal models of PDAC. Based on the hypothesis of the existence of an ABCC3/LPI/GPR55 axis in PDAC, which activity perpetuates PDAC progression, the potential of vertical inhibition of both proteins to slow down PDAC progression was evaluated. The effects of a combination treatment coupling simultaneous inhibition of ABCC3 and GPR55 on proliferation of PDAC cells was initially tested *in vitro*. Suboptimal concentration of CBD was selected. Three PDAC cell lines (AsPC1, HPAFII, CFPAC-1) were treated with S3 (5µM, 10µM), 2.5µM CBD and their combination. The effect of the drug combination was verified by manual counting of the viable cells 72h post treatment. In all tested cell lines treatment of the cells with S3 significantly decreased the number of viable cells, confirming previously obtained results. Similarly, treatment with 2.5µM CBD caused slight decrease in cell viability in AsPC1 cell line. In two other cell lines (HPAFII, CFPAC-1)

higher effectiveness of CBD was evident. Notably, addition of CBD remarkably enhanced the effectiveness of S3, resulting in a consistent decrease in cell viability (6.7-i). The most significant results were detected following the treatment with CBD and higher concentration of S3. Similar effects could be observed in all three tested cell lines, in which combination of 10μM S3 and 2.5μM CBD reduced cell viability to 20-30%.

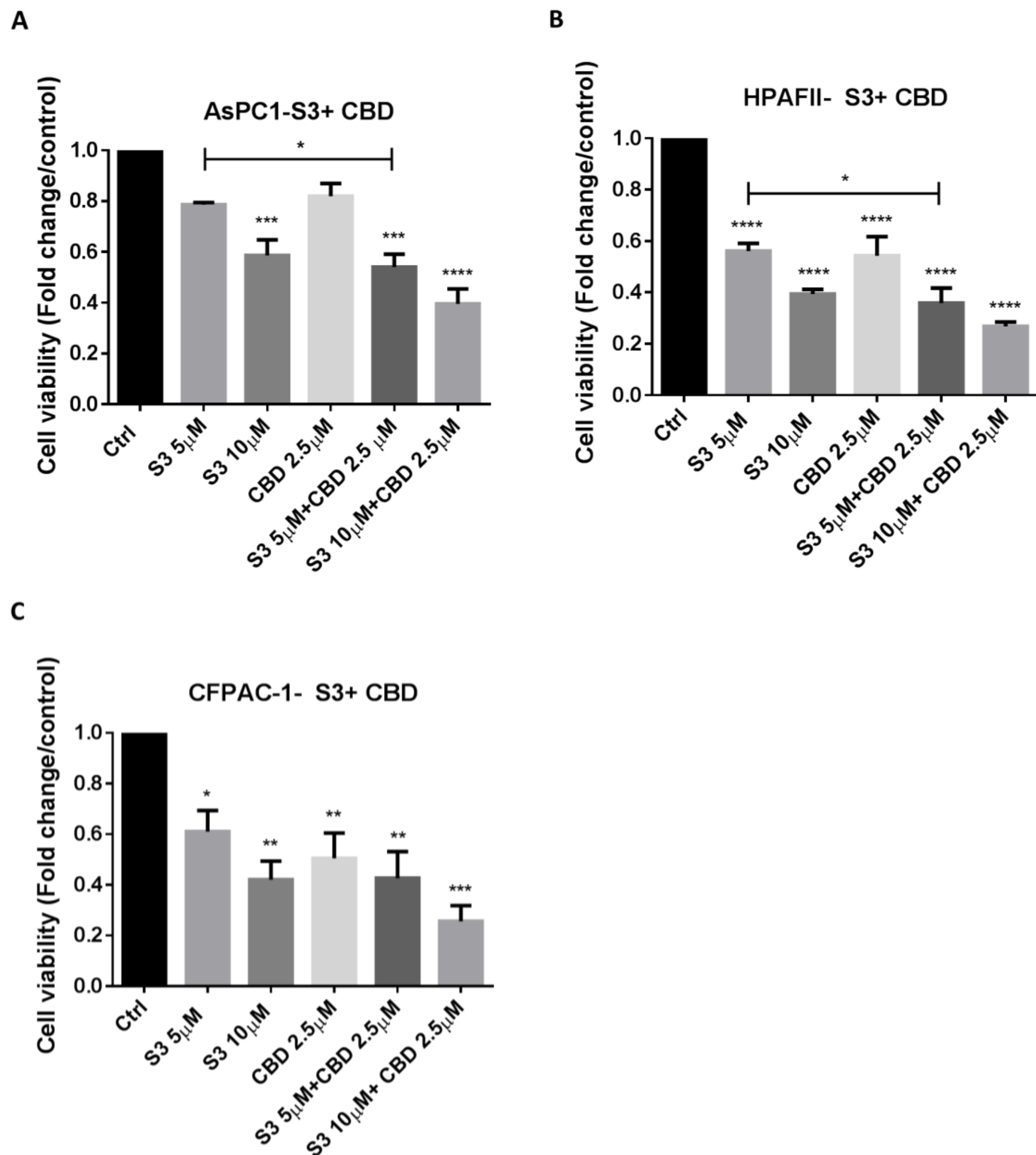


Figure 6.7 ***Horizontal inhibition of ABCC3 and GPR55 potentiates the effects of single treatment.*** *Effects of treatment with combination of 5μM and 10μM S3 and 2.5μM pure CBD on (A) AsPC1, (B) HPAF-II and (C) CFPAC-1 cell viability. Results are presented as mean ± SEM of 3 independent experiments, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001*

The potential additive or synergistic efficacy of tested combinations was also assessed using the CompuSyn software (Table 6.1). With the aid of the software, combination index (CI) values are calculated based on the effects of single drugs and their combination. CI values lower than one indicate a synergism between tested drugs, whereas values above 1 indicate an antagonism. Values around 1 (0.9 to 1.1) are indicative of additive effect. In most of tested cell lines, combination of S3 with CBD showed slight synergistic or additive effects in decreasing the number of viable PDAC cells. Importantly, the synergism between anti-tumorigenic compounds at their higher doses is considered more relevant in the CompuSyn analysis.

	AsPC1	**HPAFII**	**CFPAC-1**
S3 dose [μM]	**CBD 2.5 μM**	**CBD 2.5 μM**	**CBD 2.5 μM**
		CI value	
5	0.8806	0.9853	1.2149
10	0.9080	1.0345	0.8218

Table 6.1. ***Horizontal inhibition of ABCC3 and GPR55 shows high synergism.*** *CompuSyn analysis of the effects of combination of S3 and CBD on viability of AsPC1, HPAFII and CFPAC-1 cell lines. CI values <1 indicate synergism, values close to 1 show additive effects.*

These preliminary *in vitro* results provide the opportunity for the vertical inhibition of two molecules belonging to the same signalling loop to potentially increase the efficacy of individual targeted PDAC therapy. Considering the safety and efficacy of GPR55 and ABCC3 inhibitors demonstrated *in vivo,* these results provide the basis for the evaluation of the efficiency of these drug combinations on tumour growth and survival *in vivo*.

6.3.5 The synergistic effects of ABCC3 and GPR55 vertical inhibition involve downregulation of STAT3 signalling

Considering the remarkable decrease in the viability of cells treated with both S3 and CBD, the potential mechanisms regulating these effects were sought. The reduction of STAT3 phosphorylation at tyrosine 705 was shown to be affected by downregulation of both: ABCC3 and GPR55. Thus, I hypothesized that the increased effectiveness of the simultaneous inhibition of ABCC3 and GPR55 may be due to the synergistic inhibition of STAT3 signalling.

Considering that in CFPAC-1, 10 µM CBD caused almost complete downregulation of pSTAT3 Y705 levels, cells were treated with 5µM S3, 5µM CBD and the combination of both drugs for 24h and 48h and the levels of phosphorylation of STAT3 at tyrosine 705 were verified by Western blotting. Interestingly, similar effects in terms of pSTAT3 Y705 downregulation were obtained in CFPAC-1 cells after treating the cells with 5µM S3 compared to 10µM dose. Similarly, a remarkable decrease in pSTAT3 Y705 was detected following treatment of the cells with 5µM CBD. Strikingly, almost doubled reduction in pSTAT3 Y705 levels was observed in the cells treated with the combination of both drugs for 24h, compared to the effects of each drug alone (Figure 6.8-A). Treatment of the cells for 48h with S3 or CBD resulted in upregulation of pSTAT3 Y705 levels, confirming previous results of time-sensitive regulation of STAT3 phosphorylation. However, combination treatment with CBD reduced pSTAT3 Y05 to the initial levels.

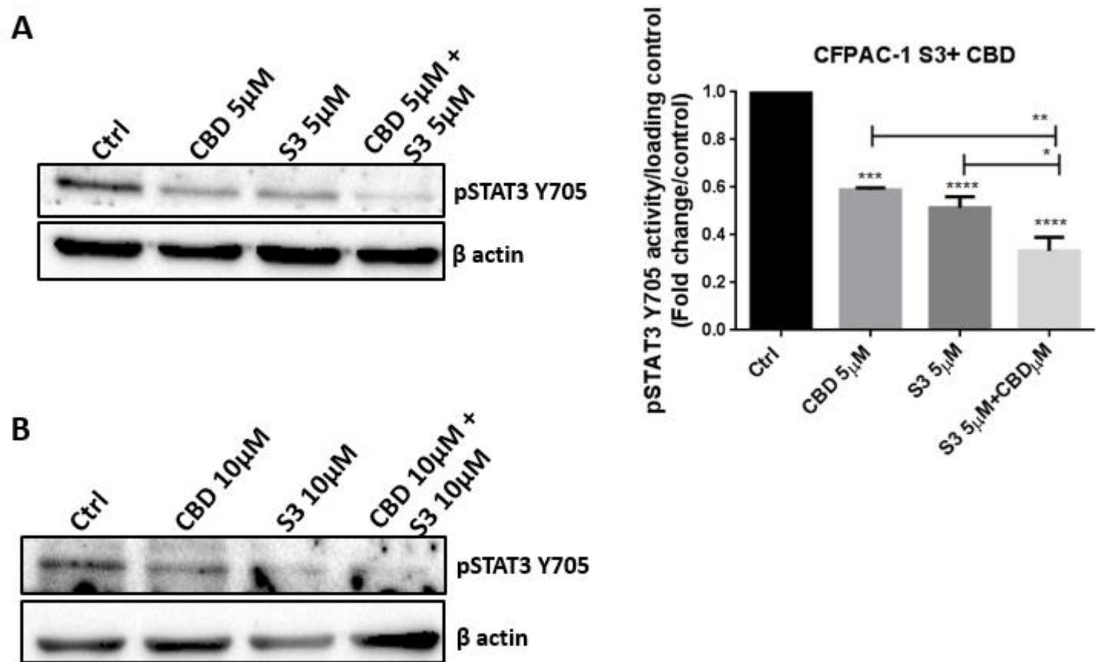


Figure 6.8 ***Horizontal inhibition of ABCC3 and GPR55 potentiates the blocking of STAT3 signalling.*** *Representative Western blot analysis and quantification of the effectiveness of vertical treatment of (A) CFPAC-1 and (B) AsPC1 cell line with combination of S3 and CBD on the levels of phosphorylation of STAT3 (pSTAT3 Y705) compared to the effect of single drugs. Results are presented as mean ± SEM of 3 (CFPAC-1) independent experiments, *p<0.5, **p<0.01, ***p<0.001, ****p<0.0001*

Consequently, similar, although not as striking, results were obtained in the other tested cell line, AsPC1, in which cells treated with the combination of 10µM S3 and 10µM CBD exhibited remarkably higher effects in inhibiting phosphorylation of STAT3 than each of the drugs alone (Figure 6.8-B).

In addition, slightly lowered expression of phosphorylated ERK1/2 was detected in the samples with vertical inhibition of ABCC3 and GPR55 in CFPAC-1 cell line, although no significant difference between the combination and single treatments could be observed (Figure 6.9).

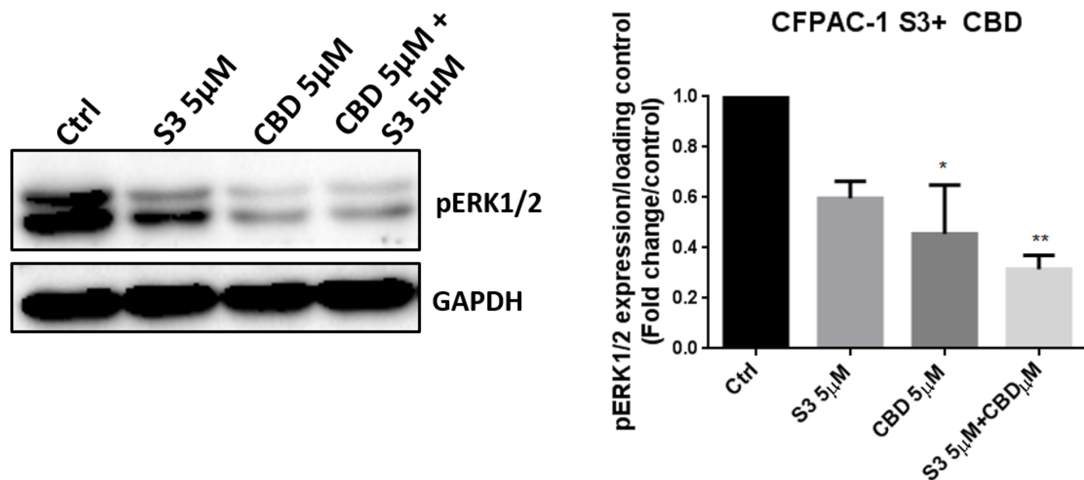


Figure 6.9 ***Horizontal inhibition of ABCC3 and GPR55 potentiates the blocking of STAT3 signalling.*** *Representative Western blot analysis and quantification of the effectiveness of vertical treatment of CFPAC-1 cell line with combination of S3 and CBD on the levels of phosphorylation of STAT3 (pSTAT3 Y705) compared to the effect of single drugs. Results are presented as mean ± SEM of 3 independent experiments, *p<0.5, **p<0.01*

Collectively, these data suggest the dual inhibition of STAT3 and MAPK signalling as the potential mechanism responsible for the observed synergistic effects of vertical GPR55 and ABCC3 inhibition in PDAC.

6.4 Discussion and future plans

In previous chapters, I demonstrated the key role of LPI-GPR55 axis in pancreatic cancer progression and the pharmacological potential of its inhibition *in vitro* and *in vivo* with synthetic Cannabidiol (CBD) was shown. I also demonstrated the remarkable role of ABCC3 in PDAC progression both *in vitro* and *in vivo*. Pharmacological inhibition of ABCC3 with a small molecule inhibitor, developed in this project, showed high potency in reducing PDAC cell growth and tumour growth *in vivo*. More importantly, a significant increase in the survival of a transgenic mouse model of PDAC was demonstrated following the treatment of the mice with the ABCC3 inhibitor. Original research from my group in prostate and ovarian cancer demonstrated the existence of an ABCC1-LPI-GPR55 autocrine loop, which activity stimulates cancer cell growth and proliferation. In light of these results, I investigated the role of LPI in PDAC progression and its function as a potential link between GPR55 and ABCC3 activity in PDAC. I also verified the pharmacological potential of targeting ABCC3-LPI-GPR55 axis in PDAC treatment.

I demonstrated that ABCC3 is responsible for LPI release in PDAC. I showed that ABCC3 expression is indispensable for transport of endogenous LPI to the extracellular space. Moreover, using cell viability assays I showed that ABCC3-mediated LPI release regulates PDAC cell proliferation, through activation of GPR55-induced signalling pathways. Taking into consideration the well-known role of LPI in stimulating proliferation and migration of cancer cells, the identification of a protein responsible for its release and activity is of high importance. I can therefore speculate that the striking effects of ABCC3 genetic silencing and inhibition demonstrated in Chapters 4 and 5 may be due to the impairment of LPI release, and as a consequence, LPI-induced stimulation of cell proliferation. Thus, ABCC3 inhibition may represent a novel mechanism for reduction of PDAC progression through depletion of extracellular LPI levels.

Moreover, I demonstrated the involvement of both ABCC3 and LPI in the regulation of the STAT3 pathway. Acute stimulation of cells with LPI enhanced the levels of STAT3 phosphorylated at tyrosine 705. On the other hand, ABCC3 downregulation

resulted in significant decrease in pSTAT3 Y705 levels that could be partly restored by addition of exogenous LPI. Considering that LPI exerts it functions through activation of GPR55 and its downstream signalling, the connection between ABCC3 and GPR55 was investigated. I could show that ABCC3, LPI and GPR55 are involved in an autocrine loop, which activation regulates the activity of key signalling pathways in PDAC biology and influences PDAC cell proliferation. I could demonstrate that similarly to ABCC3, the inhibition of GPR55 remarkably reduced pSTAT3 Y705 levels in two PDAC cell lines. Similarly, inhibition of ABCC3 resulted in downregulation of ERK1/2, although the effects were not as pronounced as after GPR55 inhibition. I may speculate that despite the inhibition of LPI release from the cells, the existence of exogenous LPI or other growth factors in the cell media may induce GPR55-activated pathways. In addition, remarkably higher downregulation of STAT3 by ABCC3 observed in serum-free media, accompanied by significantly more reduced cell proliferation may support this hypothesis. In complete media, existence of additional growth factors may influence cell proliferation through GPR55 activation, even after inhibition of ABCC3-mediated LPI release. However, downregulation of ABCC3 in serum-free conditions deprived the cells from additional stimuli, leading to almost complete reduction in pSTAT3 Y705 levels and cell viability. Nevertheless, additional experiments, such as the downregulation of GPR55 followed by LPI stimulation and analysis of the effects on pSTAT3 Y05 levels and cell growth, should be performed. Based on obtained data, the potential of vertical targeting of both proteins was also investigated. Previous *in vivo* data evaluating pharmacological potential of GPR55 and ABCC3 showed high efficacy of the inhibition of each individual protein in decreasing tumour growth and prolonging survival of KPC transgenic mouse model. I hypothesize that dual targeting of ABCC3 and GPR55, inhibiting both LPI release and its GPR-55 mediated activity, could potentiate observed effects. Initial *in vitro* validation showed that dual inhibition using S3 and CBD synergistically enhanced the activity of each drug applied as a single agent. A reduced cell number was observed in all analysed cell lines treated with this combination. Moreover, I proposed a potential mechanism involving the enhanced inhibition of STAT3 and MAPK pathways. However, more in-depth analysis of this and other potential mechanisms contributing to the enhanced activity of the combination of both inhibitors needs to

be performed in the future studies. In addition, the effects of the dual inhibition on apoptosis and cell cycle should be evaluated. Moreover, the combination of CBD and S3 with other drugs should be considered. For instance, considering the role of EGF in the induction of intracellular LPI synthesis, combination of S3 and CBD with inhibitors of the EGFR signalling could potentially enhance their activity. For this purpose, the use of FDA-approved drug such as the EGFR inhibitor Erlotinib (198), could facilitate the implementation of the treatment regimen into the clinic.

More importantly, having validated *o* the synergistic efficacy of S3 plus CBD *in vitro*, we plan to investigate the potential of this combination *in vivo*. Our previous data showed that pharmacological inhibition of both ABCC3 and GPR55 resulted in significant prolongation of survival of KPC transgenic mouse model, the most clinically relevant mouse model of PDAC. If the efficacy of the vertical inhibition of ABCC3 and GPR55 obtained *in vitro* could be translated into *in vivo* model, it would represent a breakthrough in PDAC therapy with improved survival combined with, possibly, marginal chemotherapy-related side effects.

7. PDAC stem-like cells are responsible for high chemoresistance of pancreatic cancer

7.1 Introduction

PDAC is characterized by a very high degree of heterogeneity, which makes this malignancy one of the most aggressive and challenging to treat. The main reasons for the lack of effectiveness of PDAC treatments have been attributed to the early manifestation of metastasis and chemoresistant nature of the tumours. Indeed, resistance of the cells to the broad repertoire of structurally diverse drugs, called multidrug resistance (MDR), has been indicated in PDAC and has been evidenced as one of the main factors contributing to the dismal prognosis of PDAC patients. It has been shown that development of MDR in PDAC cells is a multifactor consequence of several mechanisms developed by cancer cells during disease progression (intrinsic) or induced by the treatments (acquired chemoresistance). *Inter alia,* decreased drug uptake or its increased metabolism, blocking of apoptotic signals or the presence of highly resistant stem-like cells have been indicated as some of the mechanisms that on their own, or combined, influence the effectiveness of applied therapies (360, 484-488). Importantly, overexpression of the transmembrane proteins belonging to ATP-binding cassette (ABC) transporter family has been claimed to increase drug efflux from the tumour cells, contributing to their enhanced resistance to broad spectrum of therapeutics leading to the poor response of PDAC patients to treatments (433, 489-491). Human equilibrative nucleoside transporters (ENTs) is another family of transmembrane proteins that mediate transport of nucleosides through plasma membrane and the correlation between their expression and resistance of cancer cells to gemcitabine treatment has been investigated; however results are contradictory (492-494). Tumour environment also contributes to the chemoresistance in PDAC. Dense desmoplastic reaction formed around the tumour by activated stellate cells, ECM proteins, cytokines and growth factors forms a protective barrier, impeding the effective delivery of applied therapeutics. In

addition, dense stroma leads to poor vascularisation that further restricts the drug supply to the tumour.

The high heterogeneity of pancreatic cancer is an additional factor that plays a role in this peculiar chemoresistance. Different populations of cells exist within tumour bulk, characterized by different phenotype, surface markers expression and metabolic capacities (495). Importantly, recent evidence suggests the existence of slow cycling and highly invasive cells that possess self-renewal capacities. These cells, named cancer stem cells (CSCs), possess characteristics associated with normal stem cells. They have been first identified in the Acute Myeloid Leukaemia in 1994 and subsequently the presence of CSCs has been identified in a variety of solid tumours. This cell subpopulation has been hypothesized to possess increased tumorigenic potential and be responsible for tumour onset, relapse and metastatic spread (496). CSCs also possess the ability to evade pharmacological treatments, thus they have been associated with the development of chemoresistance by cancer cells. In fact, targeting of this tumour subpopulation of the tumour has been shown to increase tumour responsiveness to applied therapies (497). As an example, metformin has been recently demonstrated to increase sensitivity of pancreatic cancer cells to drugs (451). Interestingly, the same compound was previously shown to enhance the effectiveness of chemotherapy and slowing down of tumour growth by selectively targeting cancer stem cells subpopulation (498).

Several stemness markers have been proposed that distinguish this subpopulation from the whole tumour bulk, including increased expression of transcription factor Octamer-binding transcription factor 4 (OCT4) or G-protein coupled receptor CXCR4 (C-X-C chemokine receptor type 4). In PDAC, the presence of c-Met, the elevated expression of STAT3 phosphorylated at Tyrosine 705 and the expression of Aldehyde dehydrogenase 1 subfamily A member 1 (ALDH1A1) have been also proposed as CSCs markers, especially in subpopulations with increased tumorigenic potential (442). Interestingly, ALDH1A1 activity has been reported to mediate cell chemoresistance (499). Moreover, overexpression of ABC transporters in CSCs is another mechanism conferring high resistance of that subpopulation (453). Enhanced expression and activity of member of the ABC transporters family such as ABCG2 or ABCC1 has been

reported. Both transporters participate actively in the extrusion of a plethora of chemotherapeutics and have well-established role in the resistance of a variety of solid tumours. In addition, altered expression of the proteins regulating EMT, a process responsible for invasive phenotype of cancer cells, was also attributed to the subpopulation of CSCs (500). This phenomenon supports the hypothesis of role of CSCs in the metastatic spread.

The potential clinical impact of the hypothesized existence of CSCs has attracted particular attention due to their resistance to conventional treatments that leads to poor response to therapies and to an increase in metastatic spread and cancer relapse. Considering the failure of the currently available PDAC therapeutic options, the role and mechanisms of CSCs in the poor response of PDAC patients to conventional chemotherapy needs to be elucidated. Consequently, selective targeting of this cell subpopulation may provide a valuable tool to test new pharmacological interventions.

7.2 Aims of the project

Lack of effective therapeutic approaches in PDAC is partly due to its high chemoresistance. Available therapies, such as Gemcitabine or nab-paclitaxel only marginally improve patients' survival. It is known that increased survival abilities and high resistance to therapies characterize a subpopulation of cancer cells, CSCs. However, there are still discrepancies regarding the characterization and the role of CSCs in PDAC. Since pancreatic CSCs are particularly refractory to anticancer treatments, it is essential to gain a better understanding of the characteristics of these cells in order to develop more effective therapies. In this project, we aimed to:

- Establish a protocol for the isolation and identification of pancreatic stem-like cells
- Analyse the responsiveness of selected population to commonly applied chemotherapeutics in PDAC therapy
- Investigate the potential of CSCs subpopulation as a tool in testing of new therapeutic interventions in PDAC

7.3 Results

Parts of this chapter have been adapted from the following research article:

Domenichini A, Edmands JS, **Adamska A**, Begicevic RR, Paternoster S, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. Adv Biol Regul. 2019 Feb; 72: 63-77; doi: 10.1016/j.jbior.2019.02.001

The experiments that were performed by me that were included in the publication (isolation of tumorspheres and characterization of chemoresistance of PDAC thumorspheres) are presented in this chapter. Figures published in this article that were based on my work are presented and the reference to the appropriate figure in the publication is made under the figure legends. Whole publication is attached at the end of the chapter. All the authors have acknowledged my contribution to the publication and the statement is presented at the end of the thesis.

7.3.1 Isolation and characterization of stem-like cells population from PDAC cells

To verify the role of CSCs in PDAC therapy, the differences in the response of PDAC stem-like cells and their parental counterparts to several commonly used chemotherapeutics were investigated. Therefore, a reliable protocol for the isolation of the cancer stem-like population form the PDAC cells needed to be optimized.

CSCs were selected from the parental cell culture according to the Materials and Methods section (Chapter 2.1.3). When cultured in serum-free media, cancer cells undergo a series of changes that lead to the acquirement of more aggressive phenotype. Therefore, selected PDAC cell lines (AsPC1, HPAFII, CFPAC-1 and KPC) were serum-starved for 7 days. During that time, cells start to detach and form cell aggregates. After 7 days, adherent cells together with cells in suspension were

collected and grown in non-adherent conditions in stem cell media supplemented with growth factors (EGF, FGF_2) (Figure 7.1).

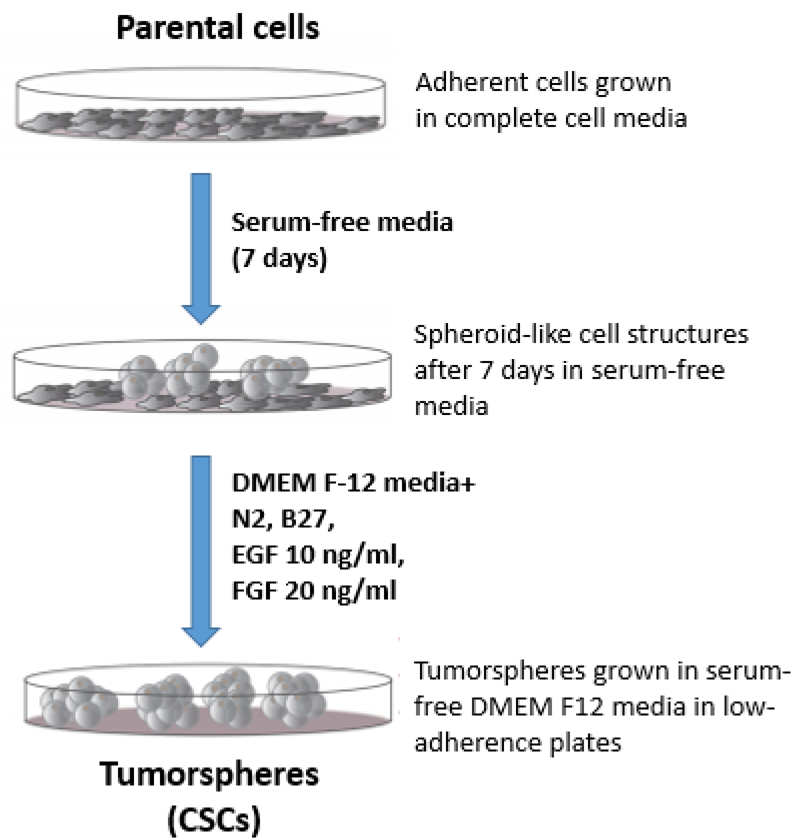


Figure 6.1 ***Protocol for CSCs isolation****. Schematic presentation of the established process of selection of stem cell-like subpopulation from the parental counterparts of the PDAC cell lines.* Adapted from figure 1 in: Domenichini A, Edmands JS, **Adamska A**, Begicevic RR, Paternoster S, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. Adv Biol Regul; 2019; doi: 10.1016/j.jbior.2019.02.001

Analysed pancreatic cancer cells (AsPC1, HPAFII, CFPAC-1, BxPC3) were grown in suspension in the form of tumorsphere aggregates, similarly to what has been previously reported for cancer stem-like cells (Figure 7.2). In contrast, non-malignant pancreatic cells (hTERT-HPNE) failed to form spheroids and showed remarkable decrease in viability when cultured in serum-free conditions. This supports the

correctness of established protocol and confirms the selection of invasive stem-like cell subpopulation from the parental counterparts.

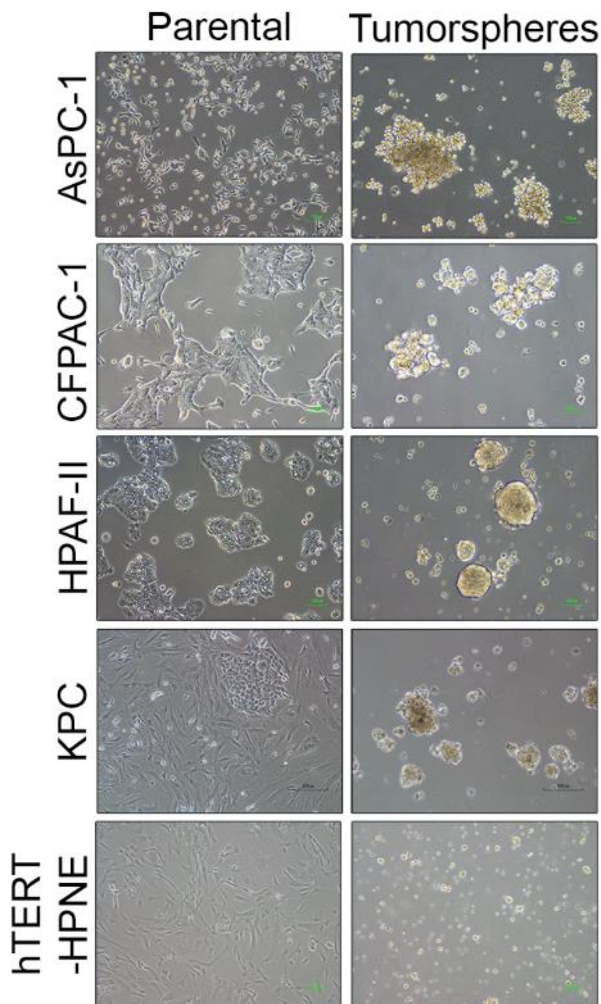


Figure 7.2 ***PDAC CSCs form tumorspheres in the low attachment conditions.*** *Comparison of the phenotype of the parental cells and isolated stem cell-like subpopulation (tumorspheres) of AsPC1, CFPAC-1, HPAFII pancreatic cancer cells, KPC primary cell line and non-malignant immortalized pancreatic cell line hTERT-HPNE.*

Figure 1 in the following publication: :Domenichini A, Edmands JS, **Adamska A**, Begicevic RR, Paternoster S, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. Adv Biol Regul; 2019; doi: 10.1016/j.jbior.2019.02.001)

Following the isolation of tumorsphere cultures form the parental cell population, the expression of markers of stem cells (e.g. c-Met, OCT4, CXCR4, ALDH1A1) was analysed in order to confirm the existence of the cells with stem-cell like phenotype in isolated tumorspheres. Western blot analysis of collected tumorspheres and parental cells, from which they were selected was performed. In addition, the expression of GPR55, ABCC3 and proteins belonging to signalling pathways essential for PDAC progression (e.g. STAT3) was compared between parental cells and induced stem cells. The three studied cell lines showed a heterogeneous expression of stemness markers, suggesting different tumorigenic potential of different cell lines (Figure 7.3). While AsPC1 and HPAFII cell lines shared similar characteristics, CFPAC-1 cell line showed differential expression of majority of the markers. Increased expression of ALDH1A1 was detected in the CSCs subpopulations of all the cell lines, whereas CXCR4 was expressed at higher levels in AsPC1 and HPAFII cell lines. Similarly, CSCs subpopulation of AsPC1 and HPAFII cell lines showed elevated expression of c-Met, whereas no difference in its expression between parental cells an tumorspheres was detected in CFPAC-1. The expression of ABCG2 was shown to be remarkably higher in the AsPC1 tumorspheres compared to the parental cells. Increase of its expression was also noticeable on the HPAFII; however, no expression in any of the populations could be detected in CFPAC-1. Importantly, activated STAT3 protein (STAT3 phosphorylated at tyrosine 705), one of oncogenic factors in pancreatic cancer, was highly expressed in the stem cell population compared to parental cells in two cell lines: AsPC1 and HPAFII, whereas its levels were equally high in parental and CSCs subpopulations in CFPAC-1 cell line. Similar expression pattern could be observed for total STAT3.

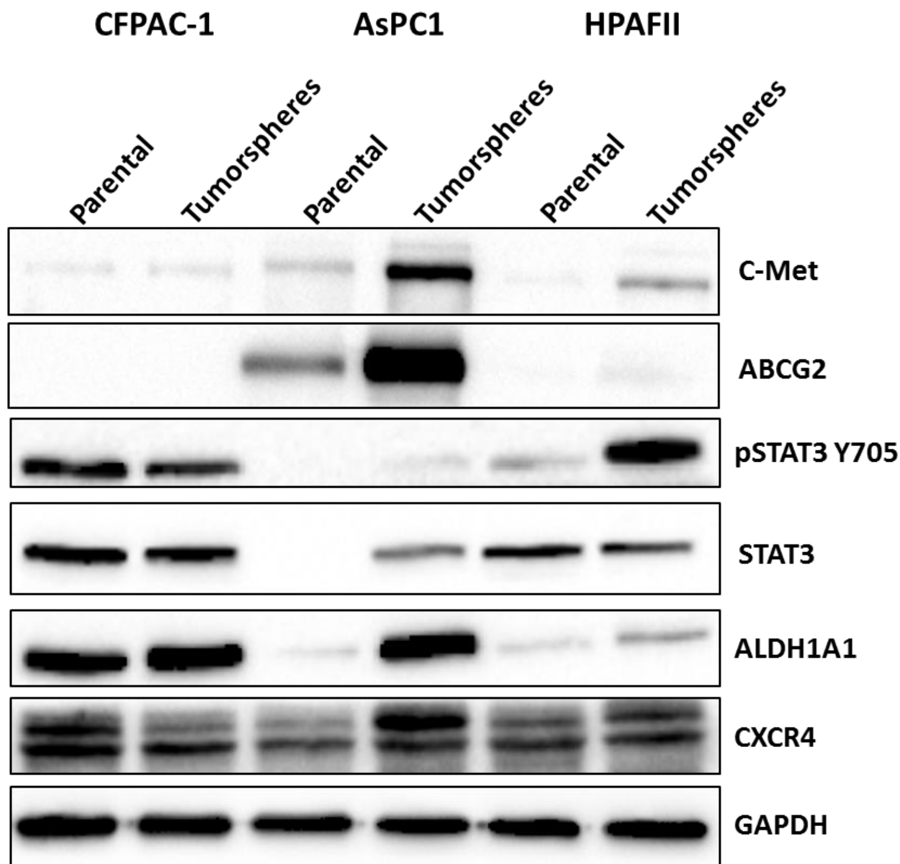


Figure 7.3 ***PDAC tumorspheres overexpress CSCs markers.*** *Comparison of the parental cells and selected tumorspheres in terms of the expression of stemness markers (ALDHA1, CXCR4, ABCG2, c-Met) and several proteins with proven function in PDAC.*

I demonstrated the expression of stemness markers in the selected tumorspheres, confirming the stem-like characteristics of selected sub-population. Additionally, observed enhanced expression of proteins of oncogenic character (like STAT3) in selected populations suggests the increased tumorigenic potential of CSCs.

7.3.2 PDAC stem-like cells show increased chemoresistance

Pancreatic cancer belongs to the most resistant types of cancer. Especially, plenty of mechanisms have been developed by PDAC cells to decrease the effectiveness of "gold standard" in PDAC treatment- gemcitabine. To verify the response of selected

population to chemotherapeutics commonly applied in PDAC therapy (Gemcitabine, Carboplatin, Paclitaxel, 5-FU), the effectiveness of mentioned drugs was tested and the effects were compared between parental cells and CSCs population. Equal number of parental cells and cancer stem-like cells were seeded in 24-well plates (adherent and low adherent respectively). Both cell populations were treated with the same concentrations of tested drugs and the results were assessed after 5 days of growth by cell counting with trypan blue extrusion. Tumorspheres population was disaggregated with Accutase prior to counting. In addition, the expression of GPR55 and ABCC3 in PDAC tumorspheres and the potency of their inhibitors, CBD and S3, was also verified.

We could see that the viability of AsPC1 tumorspheres was not remarkably affected by the treatment with increasing doses of Gemcitabine, even at the highest doses, compared to the parental counterparts, which viability was significantly decreased with respective treatment (Figure 7.4-A). Similarly, HPAFII tumorspheres were slightly less responsive to Gemcitabine compared to parental cells up to 100 nM dose (Figure 7.4-B). Interestingly, decreased difference in cell response observed between both populations was a result of increased resistance of HPAFII parental cells to Gemcitabine, compared to other tested cell lines. Consistent with the differential expression of stemness markers in the CFPAC-1 CSCs compared to AsPC1 and HPAFII subpopulations, CFPAC-1 tumorspheres possessed different drug sensitivity, and were found sensitive to Gemcitabine therapy. No significant difference could be detected between the response to Gemcitabine between CSCs subpopulation and parental counterparts (Figure 7.4-C), in agreement with decreased tumorigenic potential and no difference in expression of stemness markers in these cells. Importantly, KPC primary cell line, developed from the pancreatic tumours of the KPC mice was highly sensitive to Gemcitabine treatment, which almost completely reduced cell viability, whereas the tumorspheres derived from this cell line were unresponsive to the same treatments (Figure 7.4-D).

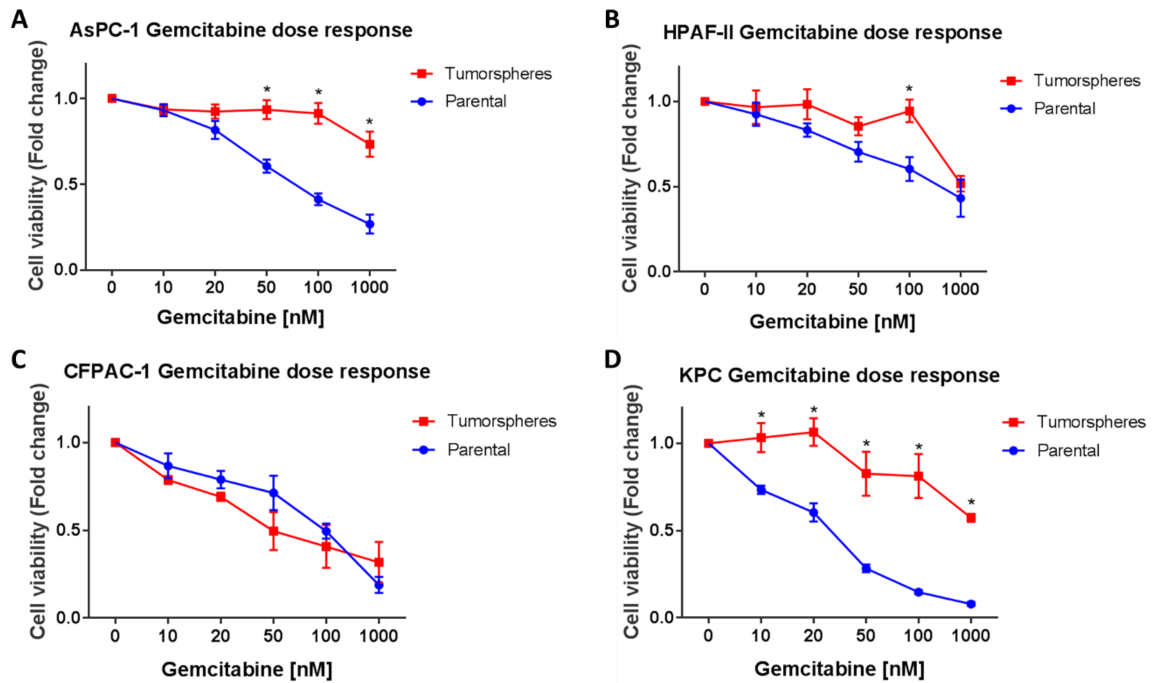


Figure 7.4 ***PDAC tumorspheres are more resistant to chemotherapy treatment.*** *Comparison of the responsiveness to Gemcitabine treatment between the parental cells and stem-like cells subpopulations of (A) AsPC1, (B) HPAFII, (C) CFPAC-1 and (D) KPC cell lines. Each experiment was performed in triplicate. The results are presented as mean ± SEM of 3 independent experiments*

Figure 7 in the following publication: Domenichini A, Edmands JS, **Adamska A**, Begicevic RR, Paternoster S, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. Adv Biol Regul; 2019; doi: 10.1016/j.jbior.2019.02.001

Similarly, treatment of AsPC1 cell line with two other therapeutics used in PDAC therapy: Carboplatin or Paclitaxel proved to be remarkably more effective in the parental sub-population compared to the CSCs population, which was resistant to a wide range of drug doses (Figure 7.5-A,B).

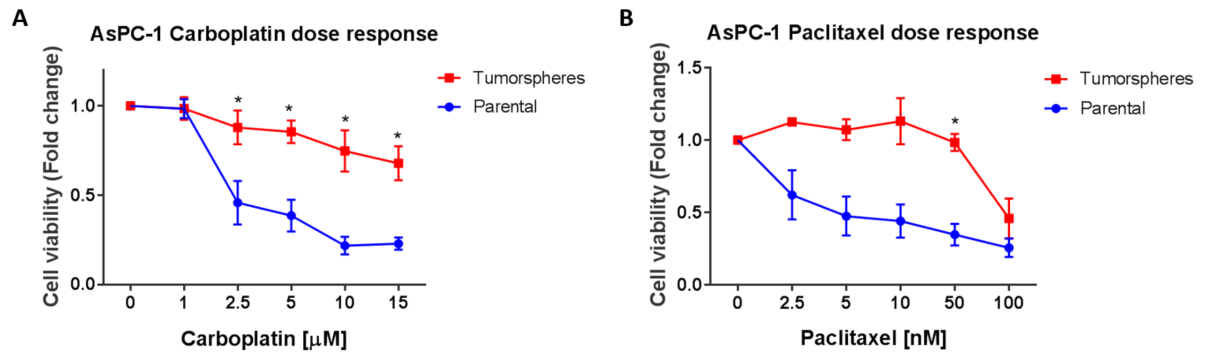


Figure 7.5 ***PDAC tumorspheres are more resistant to chemotherapy treatment.*** *Comparison of the responsiveness of AsPC1 parental cells and stem-like cells subpopulation to the treatment with (A) Carboplatin and (B) Paclitaxel. Each experiment was performed in triplicate. The results are presented as mean ± SEM of 3 (Carboplatin) and 2 (Paclitaxel) independent experiments.*

Figure 7 e, f in the following publication: Domenichini A, Edmands JS, **Adamska A**, Begicevic RR, Paternoster S, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. Adv Biol Regul; 2019; doi: 10.1016/j.jbior.2019.02.001

These data demonstrated the high resistance of CSCs subpopulation of PDAC cells and suggested that this subset is responsible for chemoresistance of PDAC. These data confirm the increased resistance of the stem-like subpopulation of cancer cells, even in the primary KPC cell line, established from the murine pancreatic tumour.

7.3.3 Potential of targeting of ABCC3 and GPR55 in PDAC tumorspheres

Targeted therapies and chemotherapy in PDAC have not provided expected effectiveness so far. My data demonstrates that, when developing a new therapies, not taking into account the CSCs subpopulation of PDAC cells highly overlooks a population that is highly tumorigenic and resistant to the majority of the treatments. Therefore, the treatment regimen that would effectively decrease the viability of CSCs population could significantly add to the prognosis for the PDAC patients.

Western blotting analysis of PDAC cell lines (AsPC1, HPAFII, CFPAC-1) and the primary KPC cell line demonstrated significantly increased expression of GPR55 in the stem cell populations, compared to parental counterparts (Figure 7.6-A), which reinforces the importance of GPR55 in PDAC and suggests the role of GPR55 in the PDAC cell resistance to drugs. Consistent with previous results, no difference in GPR55 expression was detected in CFPAC-1 cell line.

Therefore, I verified the effects of the inhibition of GPR55 on PDAC stem- cell survival compared to the parental subpopulation. The same quantity of stem- cells and parental cells were seeded in 24-well plates. AsPC1 and HPAFII tumorspheres and their parental counterparts were treated with 5μM and 10μM doses of CBD and 50nM Gemcitabine (Figure 7.6-B, C). Surprisingly, treatment of the cells with CBD strikingly and significantly impaired the growth of the cancer stem-like cells compared to the parental counterparts. At the same time, tumorspheres were mostly unresponsive to 50 nM Gemcitabine.

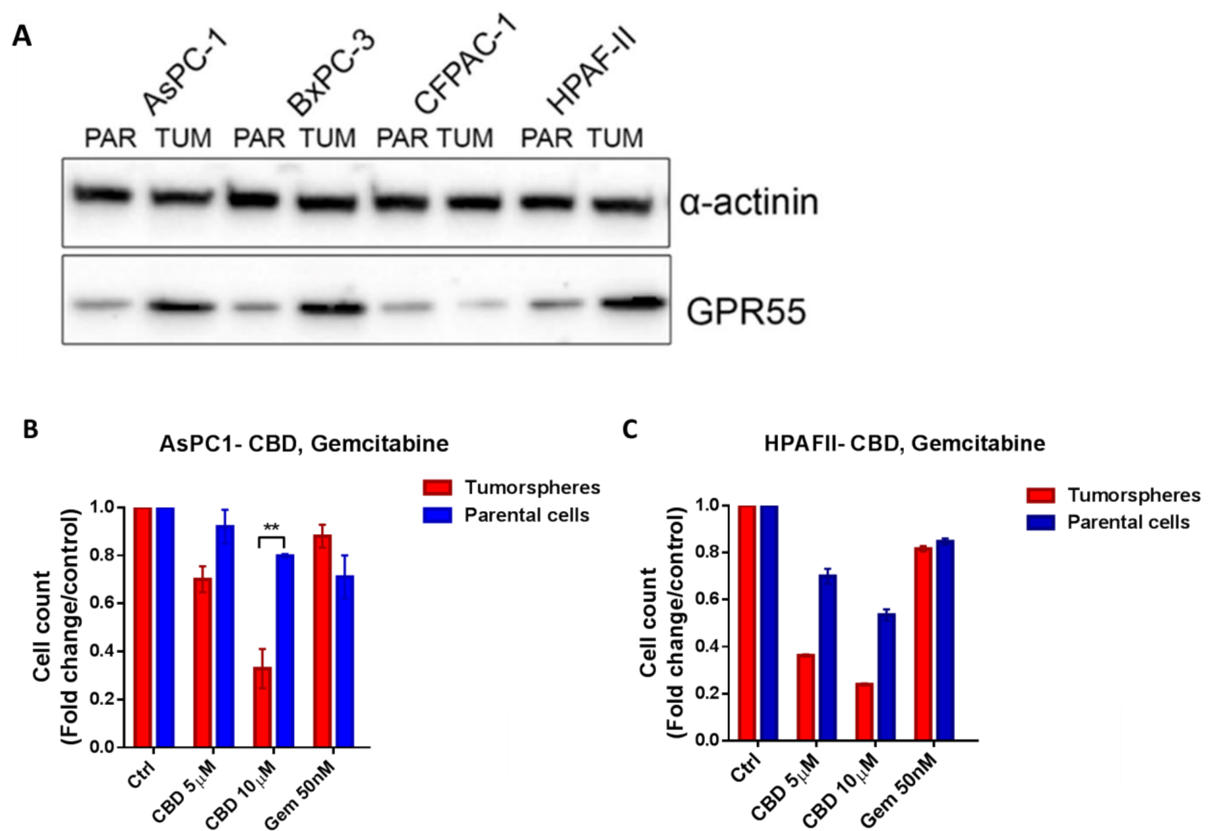


Figure 7.6 ***PDAC tumorspheres are highly sensitive to GPR55 inhibition.*** *(A) Representative Western blot comparing the expression of GPR55 in tumorspheres (Tum) and parental*

*counterparts (Par) of AsPC1, BxPC3, CFPAC-1 and HPAFII cell lines; Comparison of the effectiveness of CBD and gemcitabine treatment in reduction of the viability of tumorspheres and parental counterparts in AsPC1 (B) and HPAFII (C) cell lines. The results are presented as a mean ± SEM of 3 (AsPC1) and 2 (HPAFII) experiments, *p<0.05, **p<0.01. Western blot was performed by Dr Alice Domenichini.*

These results suggest that one of the mechanisms, by which CBD exerts its functions in PDAC may involve blockade of small population of tumour bulk composed of stem-like cells.

Similar approach has been subjected in the treatment of tumorspheres with ABCC3 inhibitor- S3. However, consistent with the lack of clear upregulation of ABCC3 in selected CSCs subpopulations (Figure 7.7-A), no improvement in cell response to increasing doses of S3 could be detected between tumorspheres and parental counterparts. In fact, parental cells showed higher responsiveness to S3 treatment (Figure 7.8-B, C).

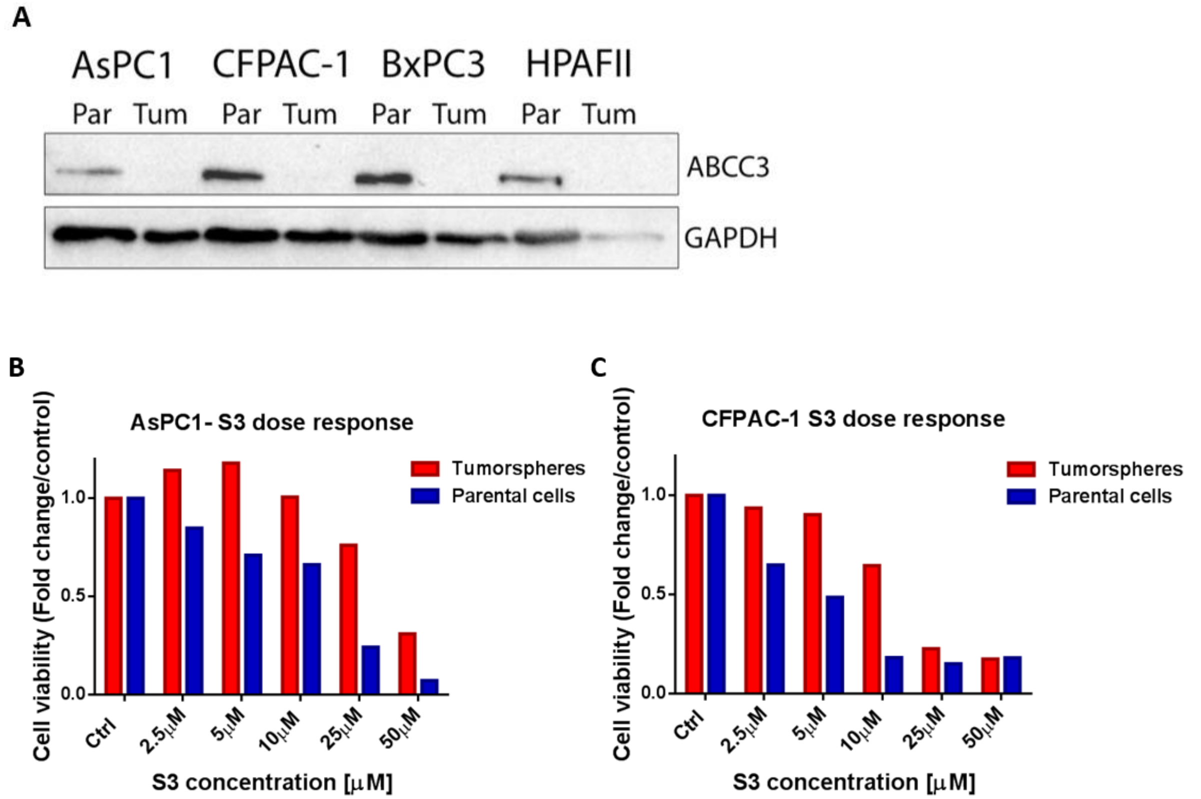


Figure 7.7 ***PDAC tumorspheres do not respond to ABCC3 inhibition.*** *(A) Western blot comparing the expression of ABCC3 in tumorspheres (Tum) and parental counterparts (Par) of AsPC1, CFPAC, BxPC3 and HPAFII cell lines; Comparison of the effectiveness of S3 treatment in reduction of the viability of tumorspheres and parental counterparts in AsPC1 (B) and CFPAC-1 (C) cell lines, N=1. Western blot was performed by Dr Alice Domenichini.*

7.4 Discussion

Pancreatic Ductal Adenocarcinoma (PDAC) is a very aggressive malignancy with very low survival rates. The prognosis for PDAC patients is grim with merely 7-8% having the chance to survival over 5 years. Among many, early metastatic spread and frequent disease relapse and chemoresistant nature have been considered as main reasons for the luck of success in treatment of PDAC patients. It has been discussed that a small sub-population of PDAC cells, called cancer stem cells (CSCs), possessing slow quiescent metabolism allowing it to survive in unfavourable conditions, is responsible for the spreading and relapse of PDAC. Recently, the role of CSCs population on development of chemoresistance has been proposed in several solid cancer types, including PDAC (501). Therefore, it is essential to gain better understanding of the characteristics of pancreatic CSCs to develop more effective therapies targeting these cells. In this chapter, I presented my work on the isolation and characterization of the small population of PDAC cells with stem-like characteristics. My work was part of a bigger project, in which we showed that the isolated population forms tumorspheres, which are characterized by enhanced tumorigenic potential compared to their parental counterparts, as demonstrated by soft agar assay (442). We also showed that PDAC tumorspheres have a unique metabolic profile with reduced metabolic potential compared to whole cell population. Increased resistance of CSCs subpopulation to commonly applied chemotherapeutics, gemcitabine, carboplatin or paclitaxel was also shown, confirming their high chemoresistant nature. Importantly, our results could be also confirmed in the tumorspheres isolated from the primary KPC cell line. Moreover, we showed that tumorspheres derived from different cell lines possess different characteristics that may reflect the heterogeneity of the tumours from which they were derives. As an example, tumorspheres isolated form AsPC1 cell line, derived from highly aggressive tumour, possess increase tumorigenic potential and elevated chemoresistance, compared to other cell lines. These data suggest that isolated tumorspheres may present a novel, more reliable platform for testing new pharmacological interventions in PDAC therapy. Our data suggest that excluding this subpopulation from the analysis of new pharmacological interventions and relaying

on the whole cell population may lead to false-positive results. In fact, primary KPC cell line was shown to be highly responsive to gemcitabine treatment, whereas KPC-derived tumorspheres were unresponsive to the same treatment. This reflects the lack of effectiveness of Gemcitabine treatment in the KPC mouse model (398), as well as marginal effects seen in human therapy. In addition, I showed that the development of pharmacological interventions, targeting molecules overexpressed in CSCs subpopulation might represent a potent strategy in reducing the resistance and relapse of PDAC. I showed that inhibition of GPR55, which expression has been found elevated in PDAC tumorspheres, with Cannabidiol (CBD) strikingly downregulated the viability of the CSCs, compared to parental counterparts. These results may explain the outstanding increase in the survival that we showed for the KPC mice treated with CBD and Gemcitabine (398). Potentially reduced chemoresistance of the tumours, due to CSCs downregulation through GPR55 inhibition, might have enhanced the effectiveness of applied chemotherapy. Similarly, two independent studies demonstrated that selective targeting of CSCs with Berberine or Metformin, increased sensitivity of pancreatic cancer cells to chemotherapy, blocking tumour growth and delaying remission (450, 451, 498).

Collectively, our results demonstrate the importance and potential of the PDAC CSCs subpopulation as a novel tool to test new pharmacological vulnerabilities that may more effectively improve patients' prognosis.

7.5 Whole publication

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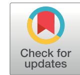
Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential



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ABSTRACT

Cancer stem cells are a population of slow-cycling cells within the tumour bulk, with self-renewal capacity that attracts interest as a therapeutic target. In highly heterogeneous tumours, like pancreatic ductal adenocarcinoma (PDAC) however, the characterisation of cancer stem cells has led to controversial results due to the lack of consensus on specific markers. Here we investigated the characteristics of a population of pancreatic cancer tumorspheres derived from different human pancreatic cancer cell lines and a primary line from a genetically engineered KPC mouse model, using flow cytometry and western blotting to analyse surface and stemness markers. We analysed tumorspheres tumorigenic potential using anchorage-independent soft agar assay as well as their metabolic plasticity and chemoresistance. Pancreatic cancer tumorspheres display a heterogeneous pattern of surface and stemness markers, nevertheless they are characterised by an increased tumorigenic potential and higher chemoresistance. In addition, we have shown that pancreatic cancer tumorspheres have a unique metabolic profile with reduced metabolic potential. Together our results indicate that, despite the heterogeneity characterising pancreatic cancer tumorspheres, we can identify a functional vulnerability that represents a window for pharmacological intervention and development of novel therapeutic strategies.

1. Introduction

In solid malignancies, tumour heterogeneity refers to the complex hierarchical organisation of cancer cells, within the same tumour, with different phenotypical and functional characteristics. According to the intratumoral heterogeneity hypothesis, primary tumours consist of distinct subsets of cells that differ for the expression of surface markers, transcription factors as well as enzymatic activity and metabolic state (Dosch et al., 2015). It has been proposed that, within the bulk of a tumour, a subpopulation of cancer stem-like cells is responsible for tumour initiation, metastatic spread and relapse (Reya et al., 2001).

Cancer stem-like cells (CSCs) or tumour initiating cells (TICs) are generally defined as a subset of slow cycling cancer cells with self-renewal capacity that evades pharmacological treatments, thus contributing to the failure of therapeutic interventions due to the development of chemoresistance as well as tumour relapse and metastatic spread (Shibue and Weinberg, 2017). Evidence of a subset of TICs was first demonstrated in Acute Myeloid Leukaemia (Lapidot et al., 1994) and subsequently they have been identified in different types of solid tumours, including pancreatic cancer (PaCa) (Hermann et al., 2007; Lee et al., 2008; Li et al., 2007).

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Traditionally CSCs, including pancreatic cancer stem-like cells, have been isolated by fluorescent-activated cell sorting (FACS) and flow cytometry using distinct surface markers among which the most common are CD24, CD44, CD133 and epithelial cell adhesion molecule (EpCAM or ESA) (Goodwin et al., 2018). Surface markers have allowed the identification of subpopulations with cancer stem cell-like features and increased tumorigenic potential. Nevertheless, these markers have proven to be heterogeneous and there is currently a lack of consensus on which one is the most reliable to isolate and characterise CSCs (Goodwin et al., 2018; P Nagare et al., 2017). Thus, other that surface markers, other "stemness" markers have been identified that show more consistency. Among these, transcription factors sex determining region Y-box 2 (Sox2) and Octamer-binding transcription factor 4 (OCT4), which are both master regulators of pluripotency in embryonic stem cells (Kim et al., 2009; Zhang and Cui, 2014), have been reported as markers of CSCs (Chou et al., 2015; Hu et al., 2008; Lundberg et al., 2016). C-X-C chemokine receptor type 4 (CXCR4), a G-protein coupled receptor, involved in maintaining the compartment of haematopoietic stem cell (HSCs) (Cheng and Qin, 2012), has been identified as a marker for PaCa CSCs (Heiler et al., 2016). In PaCa, markers that identify a population with cancer stem cells properties also list CD44v6, Tspan8, alpha6beta4, claudin7 and c-Met (Heiler et al., 2016). Hepatocyte growth factor receptor (HGFR) also known as Met (or c-Met) is a Tyrosine kinase transmembrane protein activating signalling pathways involved in proliferation and migration, as well as metastatic spread as it is found overexpressed in numerous malignancies (Organ and Tsao, 2011). Furthermore, STAT proteins are known for maintaining the leukaemia stem cells compartment (Dorritie et al., 2014) and, in Pancreatic Ductal Adenocarcinoma (PDAC), activation of STAT3 by phosphorylation of Tyrosine 705 is known to be an oncogenic driver (Corcoran et al., 2011). The transcription factor STAT3 plays a pivotal role in maintain stem cells self-renewal and promoting cancer cell survival (Corcoran et al., 2011) and thus it may identify a population enriched in CSCs.

From a clinical perspective, cancer stem-like cells attract interest due to their increased resistance to conventional chemo- and radio-therapy, thus leading to tumour relapse and metastatic spread (Abdullah and Chow, 2013; Yu et al., 2012). One of the mechanisms conferring increased therapeutic resistance to CSCs is through the overexpression of ATP-binding cassette (ABC) transporters (Abdullah and Chow, 2013; Adamska et al., 2018; Begicevic and Falasca, 2017; Domenichini et al., 2019). Distinct populations of CSCs have been identified by an increased amount of the fluorescent dye Hoechst 33342 being extruded by a subset of cancer cells (side population SP) overexpressing the ABC transporter ABCG2, also known as breast cancer resistance protein (BCRP) (Ding et al., 2010; Greve et al., 2012; Niess et al., 2015; Van den broeck et al., 2013). A second ABC transporter extruding Hoechst 33342 similarly to ABCG2 and contributing to chemoresistance in cancer stem cells is p-glycoprotein, ABCB1 (or multidrug resistance protein 1, MDR1) (Abdullah and Chow, 2013). Chemoresistance in CSCs is also functionally mediated by the activity of Aldehyde dehydrogenase 1, subfamily A, member 1 (ALDH1A1) which has been extensively investigated as a potential CSCs marker, especially in defining subpopulations with enhanced tumorigenic potential (Abdullah and Chow, 2013; Cheung et al., 2007).

Another important hallmark for the specification of a subpopulation of CSCs is defined by the epithelial-mesenchymal transition (EMT), an evolutionarily conserved process that, in malignant cells, promotes the acquisition of an invasive phenotype responsible for metastatic progression as well as increased chemoresistance (Shibue and Weinberg, 2017). Events of EMT in cancer stem cells are characterised by a decreased expression of epithelial markers, such as E-cadherin. Conversely, the expression of mesenchymal markers like Vimentin and Snail is increased (Dosch et al., 2015; Shibue and Weinberg, 2017).

In addition to phenotypic heterogeneity, the metabolic complexity of the tumour microenvironment is another emerging cancer hallmark showing that CSCs possess unique metabolic profiles compared to the bulk of the tumour. Nevertheless, this is still a relatively unexplored field and the available studies report some discrepancies in their findings (De Francesco et al., 2018; Sancho et al., 2016). Cancer stem cells have been defined as quiescent and with a slower ability to enter the cell cycle, and some authors argue that dormant cancer stem cells may rely on a low glycolytic metabolic rate that allows survival in the highly hypoxic environment characterising some solid tumours (Chen et al., 2016; De Francesco et al., 2018; Takeishi and Nakayama, 2016). Conversely, a study conducted on a highly aggressive subpopulation of pancreatic cancer stem cells has evidenced how CD133^{+} CSCs metabolism mainly relies on oxidative phosphorylation (Sancho et al., 2015), suggesting that, within the same tumour, different subpopulations of CSCs may adopt different metabolic strategies.

In this work, we present the isolation and characterisation of a population of PaCa tumorspheres with the potential of developing a valuable tool to study CSCs metabolic vulnerabilities and to test new pharmacological interventions. Despite marker heterogeneity, we found that tumorspheres were characterised by increased expression of mesenchymal markers, consistent with a higher tumorigenic potential. PaCa tumorspheres also showed reduced metabolic potential and increased chemoresistance. Most importantly results obtained from human pancreatic cancer cell lines were confirmed using primary PaCa cell lines from a clinically relevant mouse model for Pancreatic Ductal Adenocarcinoma (PDAC), the KrasLSL.G12D/+; p53R172H/+; PdxCretg/+ or KPC mouse model. The assays described in this paper, and especially the anchorage-independent colony formation assay, used to determine tumorigenicity of cancer cells, present a reliable platform to investigate new therapeutic targets.

2. Material and methods

2.1. Cell culture

Human PDAC cell lines were cultured as per ATCC® guidelines. AsPC-1 (CRL-1682™) and BxPC-3 (CRL-1687™) required RPMI-1640 as base medium, with the addition of foetal bovine serum (FBS) to a final concentration of 10%. HPAF-II (CRL-1997™) were cultured in Eagle's Minimum Essential Medium (EMEM) with the addition of FBS to a final concentration of 10%, while for CFPAC-1 (CRL-1918™), Iscove's Modified Dulbecco's Medium (IMDM) was used, supplemented with FBS to a final concentration of 10%. All cell lines were cultured in *Mycoplasma*-free conditions, passaged according to the manufacturer's guidelines, and maintained at 37 °C

in an incubator supplemented with 5%CO_2/95% air. To induce the differentiation of a population enriched in tumorspheres, cells that reached 80% confluency were serum starved and cultured for one week in their corresponding base growth medium without FBS. After one week cells were collected, supernatant included, washed with Hank's Balanced Salt Solution (HBSS with the addition of Calcium and Magnesium) and resuspended in a specially formulated tumorspheres medium. Tumorspheres medium consisted of Dulbeccos' Modified Eagle Medium: Ham's F-12 Nutrient Mixture (DMEM/F-12) supplemented with N2-MAX Media Supplement and N21- MAX Media Supplement (both from R&D), recombinant human epidermal growth factor (EGF) to a final concentration of 10 ng/mL and recombinant human fibroblast growth factor 2 (FGF) to a final concentration of 20 ng/mL. Cells, resuspended in tumorspheres medium were then seeded in Corning® Ultra-Low attachment cell culture flasks and passaged weekly. Normal pancreatic epithelial cell line hTERT-HPNE (CRL-4023™) was used for comparison. Before each experiment described below, tumorspheres were disaggregated using Accutase™ (STEMCELL™ Technologies), and, when needed like in the case of BxPC-3, also by forcing the spheroids though a 25G needle several times (Domenichini et al. MethodsX Submitted).

2.2. Primary KPC cells

All animal procedures were approved by Curtin Animal Ethics Committee (Bentley – Western Australia, protocol AEC_2016_40). The KPC (KrasLSL.G12D/+; p53R172H/+; PdxCretg/+) mouse model is a clinically relevant genetically engineered mouse model (GEMM) for PDAC (Hingorani et al., 2005). Mutationally activated KRAS and mutated p53 are responsible for the development of PDAC in this mouse model from around 80 days of age. Upon development of tumour, mice were daily monitored and euthanised as soon as they showed sign of pain and distress. Mice were anesthetised with a mixture of Oxygen/Isoflurane and euthanised according to the animal ethics. Terminal blood collection was performed and tissues were perfused and washed with PBS. Tumour was immediately removed and a section of about 125 mm^3 was minced into small pieces (about 1 mm) in a sterile Petri dish filled with 5 mL of dissociation solution, 5 mg/mL of Collagenase P in Dulbecco Modified Eeagle Medium (DMEM 4.5 g/L of glucose, 2 mM L-Glutamine) supplemented with 10% FBS and with added 1% Penicilin/Streptomycin. Tumour suspension in dissociation solution was transferred into a 25 mL tube and incubated for 90 min at 37 °C on a rocking shaker. After incubations tumour pieces were further dissociated using a 5 mL serological pipette. Cell suspension was passed through a 100 μM first and then a 40 μM cell strainer in order to obtain a single cell suspension. Cells were pelleted by centrifugation at $200 \times g$ (brake off) and washed twice with cold PBS. Cell viability was performed with Trypan blue exclusion and cells were plated in standard cell culture vessels with DMEM supplemented with 10%FBS and added 1% Penicillin/Streptomycin.

2.3. Flow cytometry

For each cell line, cells were cultured so that on the day of the assay the following conditions were analysed at the same time. The first condition was the adherent cells (also named parental, PAR), then the serum starvation stages divided into three time points, SF1 (one day after serum starvation); SF3 (three days after serum starvation) and SF7 (seven days after serum starvation). Finally, the last condition was named TUM and represented tumorspheres after being seeded in tumorspheres medium in low attachment flasks for four days to form spheroid structures. Antibodies used for flow cytometry detection of surface marker and control isotypes were sourced from Miltenyi Biotec, CD133-VioBright-FITC (cat #130-104-273); CD44-PE (cat #130-095-180); CD24-APC (cat #130-095-954); EpCAM (CD326)-PE-Vio770 (cat #130-101-161). Propidium iodide (PI cat #130-093-233) was used to exclude dead cells. Antibodies were titrated to find the optimal concentration for each marker.

CD133-VioBright-FITC	1:200	0.15 μL/sample
CD44-PE	1:200	0.15 μL/sample
CD24-APC	1:400	0.075 μL/sample
EpCAM (CD326)	1:250	0.12 μL/sample
PI	1:2000	0.015 μL/sample

Cells have been collected, washed twice with cold HBSS, and then incubated with 30 μL of the antibody and PI cocktail for 30 min on ice. At least 3×10^5 cells were used per each condition, in addition, unstained controls, isotypes and Fluorescence Minus One (FMO) controls were also analysed. Compensation was performed using capture beads, samples were acquired using BD FACSCanto™II flow cytometer and data were analysed with FlowJo® software.

2.4. Western Blots

Cell lysates from adherent parental cells and their corresponding tumorspheres were prepared in RIPA buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% SDS, 5 mM EDTA) supplemented with protease and phosphatase inhibitor cocktail (Roche). To terminate the reaction, SDS Sample buffer [125 mM Tris- HCl (pH 6.8), 6% SDS, 20% glycerol, and 0.02% bromophenol blue supplemented with 10% β-mercaptoethanol was added and the samples were boiled for 10 min. The proteins were separated on a SDS-PAGE 10% Tris-Glycine Gel and transferred to a nitrocellulose membrane (BioRad). For protein detection the membranes were incubated in 3% BSA in TBS/0.05% Tween-20 blocking buffer for 1 h at room temperature, and incubated overnight, at 4 °C with primary antibodies. Primary antibodies were used against pSTAT3 (Y705) together with total STAT3, ALDH1A1, ABCB1, ABCG2,

Vimentin, E-Cadherin, Snail, Met and Sox2 (all from Cell Signalling Technology, diluted 1:1000 in blocking buffer) CXCR4 and OCT4 (Novus Biologicals, diluted 1:500 in blocking buffer). The day after, HRP-conjugated secondary antibodies (Cell Signalling Technologies) were used at a 1:40000 dilution in 0.75% BSA in TBS/0.05% Tween-20 buffer and incubated for 1 h at room temperature. Signal was detected using the chemiluminescent detection reagent Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare Life Sciences) and imaged using BioRad ChemiDoc XRS+.

2.5. Metabolic flux analysis: seahorse

Extracellular flux analysis has been performed to compare metabolic phenotypes between parental adherent cells and their corresponding tumorspheres. The experimental procedure has required extensive optimization to accommodate a plate-based assay to cells growing in low-adherence conditions. In fact, compared to other cell-types growing in suspension, cancel tumorspheres retain the capability to attach and differentiate back to their parental cell lines when placed in adherent conditions. The assay has been performed using the Agilent Seahorse XFe96 Analyzer according to the following protocol. The day before the assay the Seahorse XF96 Sensor Cartridge has been hydrated and equilibrated overnight at 37 °C in CO_2-free incubator. Then half of a Seahorse XF96 Cell Culture Microplate has been coated using Corning® Cell-Tak™ Cell and Tissue Adhesive according to the manufacturer's guidelines. Cell-Tak™ is a protein matrix formulation used to immobilise non-adherent cells and suggested by the manufacturer to perform Seahorse assays. For normalization purposes, Cell-Tak™ matrix has also been used for parental adherent cells. On half of the plate adherent cells of two different cell lines have been plated at a density of about 3×10^4 cells/well with optimal seeding density being optimised for each cell line and KPC primary cells. Cells have been left to adhere overnight at 37 °C in a 5% CO_2 incubator. On the day of the assay, the rest of the Seahorse XF96 Cell Culture Microplate has been coated with Cell-Tak™. Tumorspheres have been the collected and dissociated to a single-cell suspension using Accutase™ (STEMCELL™ Technologies) and a 25G syringe needle (when needed). Tumorspheres have been seeded on the coated microplate according to the technical overview provided by Agilent Technologies at a seeding density corresponding to their parental adherent cells. A customised Agilent Seahorse XFe96 assay has been developed to measure mitochondrial respiration and glycolytic function in one single experiment by loading sequentially Glucose, Oligomycin, FCCP, Rotenone and Antimycin in the injection ports of the cartridge. The assay has been performed within an hour from seeding the tumorspheres to prevent cells to revert to the adherent phenotype while immobilised with the Cell-Tak™ matrix. Results have been analysed using the Wave Desktop software and reported using Microsoft Excel Seahorse XF Cell Energy Phenotype Report Generator, to automatically compare the Metabolic Potential of parental adherent cells and their corresponding tumorspheres. BCA (bicinchoninic acid) Assay (Thermo Fisher Scientific) for total protein quantification was used for normalization. Assay was repeated at least three times per each cell line.

2.6. Soft agar colony formation

The anchorage-independent soft agar colony formation assay is a well-established protocol to measure the proliferative capacity of cancer cells in non-adherent conditions (Borowicz et al., 2014; Hamburger and Salmon, 1977). In this paper, we use the soft agar assay to compare the tumorigenic potential of pancreatic cancer cell lines and their corresponding tumorspheres in complete growth media (RPMI) and in serum-free media (SF DMEM/F-12). Media formulations have been prepared as 2X concentrated solutions while noble agar (Sigma-Aldrich) has been prepared in two stocks to be diluted in 2X media solutions, 1.2% for the first layer and 1% for the second layer (the cell layer). Six well plates have been coated with a first layer (2 mL) of 1.2% noble agar in either 2X RPMI complete or SF DMEM/F-12 (final 0.6% agar in 1X media). Parental pancreatic cancer cells have been detached and collected and tumorspheres have been collected and dissociated using Accutase™ (STEMCELL™ Technologies) and a 25G syringe needle. Cells have been counted with trypan blue exclusion and about 3×10^4 cells have been resuspended in 5 mL of 0.5% noble agar (1% noble agar in 2X media). For each six well plate the first row (3 wells) has been seeded with parental cells and the second row (3 wells) with tumorspheres. Once the first layer has settled, 1.5 mL of cell suspension containing approximately 1×10^4 cells 0.5% noble agar has been distributed on top of the first layer. The top layer has then been covered with either complete RPMI or SF DMEM/F-12 medium to sustain colony growth and prevent the soft agar from drying and plates have been incubated at 37 °C in a 5% CO_2 incubator for four weeks. Experiment has been repeated three times in RMPI complete and three times in SF DMEM/F-12 for each cell line (parental and tumorspheres). After four weeks media have been removed and discarded and colonies have been fixed for 10 min at room temperature by adding a solution of 10% Methanol/10% Glacial Acetic Acid on top of the agar. After removing the fixative solution, colonies have been stained with 0.05% Crystal Violet solution (Sigma-Aldrich) for 30 min at room temperature on a rocking shaker. Colonies have then been extensively washed with water to de-stain the agar on a rocking shaker. Colonies have been imaged using BioRad ChemiDoc XRS+ and have been counted using ImageJ software.

2.7. Chemoresistance and drug treatments

To assess chemoresistance, adherent cells and their corresponding tumorspheres were treated with a dose response of the standard-of-care treatment for PDAC, gemcitabine. Parental adherent cells were seeded at a density of 25,000 cells/well in 24-well plates. Tumorspheres were collected from low attachment flasks, spun down at $100 \times g$ for 5 min, and resuspended in Accutase™ (STEMCELL™ Technologies) for 10 min at room temperature to dissociate tumour spheroids and obtain a single cell suspension. Tumorsphere cells were seeded in a Corning® Ultra-Low attachment 24-well microplate at a density of 25,000 cells/well. Parental cells and tumorspheres were then treated with gemcitabine at concentrations ranging from 0 to 1000 nM for 5 days. Cell count and

viability was assessed at the end of the experiment using Trypan blue exclusion. Treatment was repeated at least 3 times per each cell line. As reported in a companion paper to the present work (Domenichini et al. MethodsX Submitted), drug testing for chemoresistance was not performed on BxPC-3 as tumorspheres from this cell line are resistant to Accutase dissociation and performing a viability test with counting using Trypan blue exclusion would have been troublesome and, to our opinion, would not have added any more insightful information.

2.8. Statistical analysis

All experiments were performed at least in duplicate and results are representative of at least three independent experiments. Statistical analysis has been performed using GraphPad Prism v.7.04. Statistical significance was considered at a value of $p < 0.05$.

3. Results

3.1. A combination of serum-free and low attachment conditions promotes the formation of tumour spheroids in pancreatic cancer cells

When cultured in serum-free media, adherent cells from pancreatic cancer cell lines (Fig. 1Ai) slowly start to undergo a series of visible changes, and the most noticeable is that some of them will start to detach and aggregate to form some proto-spheroids cell suspensions (Fig. 1Aii). After seven days in serum free media, all the remaining adherent cells are detached via trypsinization and then maintained in low adherence flasks and medium supplemented with FGF and EGF (Fig. 1Aiii). Cells in these conditions form spheroid-like structures that we name pancreatic cancer tumorspheres and display a distinct morphology of aggregates where individual cells cannot be distinguished as they appear to be fused together, similarly to what has been previously reported (Johnson et al., 2013). Interestingly, tumorspheres derived from different cell lines show a unique conformation reflecting the heterogeneity of the parental, adherent cells they differentiate from. While cancer tumorspheres grow at a slow rate consistent with being a slow cycling subset of tumour cells, normal non-cancerous cell line hTERT-HPNE fails to form spheroids and tends to lose the characteristics of viable cells when cultured in low adherence conditions (Fig. 1B).

3.2. Cell surface and stemness markers in pancreatic cancer tumorspheres reflect the heterogeneity of their parental adherent counterparts

Cell surface markers have been widely used to identify subsets of cancer stem cells (CSCs) or tumour-initiating cells (TICs) with different tumorigenic potential. For this purpose, flow cytometry was used to detect and compare cell-surface markers in adherent cells and in their corresponding tumorspheres, as well as analysing intermediate stages during serum starvation, in a panel of human pancreatic cancer cell lines. The gating strategy was set as described in Domenichini et al. (MethodsX Submitted): a time vs side

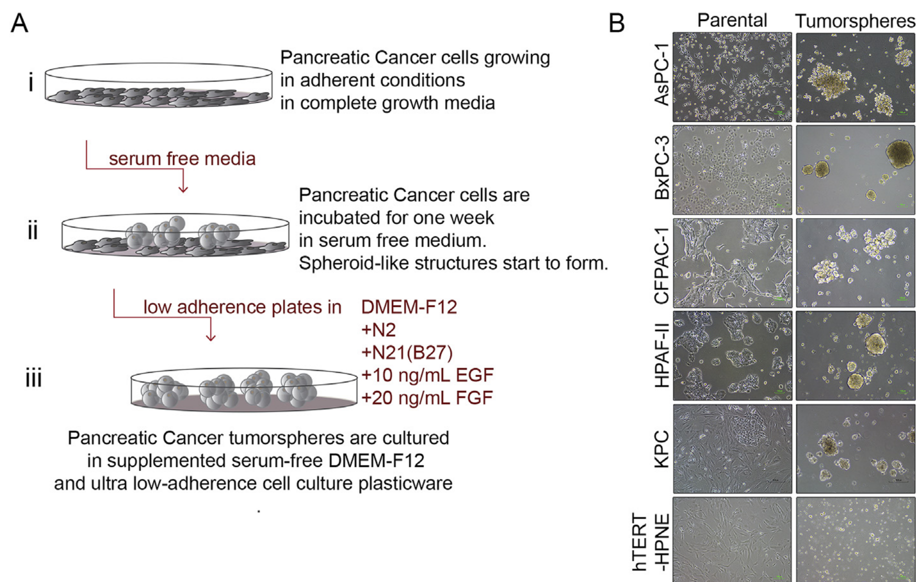

Fig. 1. (A) Schematic representation of the protocol described in this paper to isolate pancreatic cancer tumorspheres (iii) from adherent parental cells (i) after serum starvation (ii). (B) Panel of four human pancreatic cancer cell lines (AsPC-1, BxPC-3, CFPAC-1 and HPAF-II) and a murine cell line (KPC) derived from primary pancreatic tumours in KPC transgenic mice. For each cell lines adherent cells (Parental) pre-serum starvation are shown and their corresponding tumour spheroids (Tumorspheres) in low-attachment condition and stem cell medium. For comparison, a normal pancreatic epithelial cell line (hTERT-HPNE) is shown. Normal epithelial cells fail to form spheroids and do not grow in low-adherence plasticware. Scale bar 100 μM.

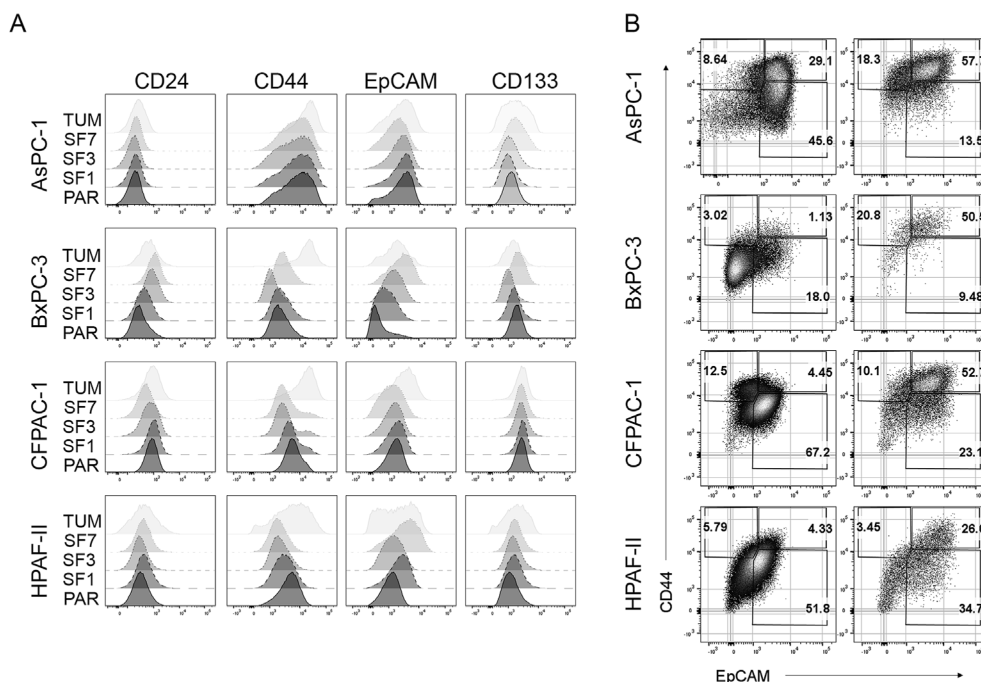

Fig. 2. (A) Histogram representation of surface marker expression, CD24, CD44, EpCAM and CD133 across the tumorspheres development time-course for four pancreatic cell lines; AsPC-1, BxPC-3, CFPAC-1 and HPAF-II. For each cell line parental cells (PAR) were analysed, as well as three days staged during serum starvation (SF1, SF3, SF7) and tumorspheres (TUM). (B) Bivariate density plots of EpCAM versus CD44 expression highlight heterogeneity between parental adherent cell line and the tumoursphere time points; PAR and TUM respectively, showing a consistent increase in $CD44^{+}/EpCAM^{+}$ cells in tumorspheres. Results are representative of 4 independent experiments.

scatter height (SSC-H) gate was used to assess sample quality during acquisition. Pancreatic cancer cell lines, and especially tumorspheres, tend to form aggregates hence the forward scatter (FSC) Area vs Height (FSC-A vs FSC-H) gate was used to only select single events. Propidium Iodide was used to exclude dead cells from the analysis as in the tumorspheres isolation process, during serum starvation, only slow cycling cells that will contribute to form the tumour spheroids will survive. All the remaining events were considered and analysed. Results were analysed taking into account antibody specificity and isotypes were used to exclude non-specific binding of antibodies, and fluorescence minus one (FMO) staining were also used in all cell lines to exclude signal spill-over and define gating boundaries (Domenichini et al. MethodsX Submitted). Results (Fig. 2A) show that AsPC-1 parental cells (PAR) and their corresponding tumorspheres (TUM) do not express CD24 and serum starvation (SF-SF3-SF7) does not induce the expression of the marker in this cell line. Conversely, in BxPc3 we appreciate an increase of CD24 expression in tumorspheres (TUM) compared to their parental counterparts (PAR) and throughout the serum starvation stages (SF1-SF3-SF7). HPAF-II cell line is CD24 positive but the expression of the marker does not change in tumorspheres (TUM) compared to their parental counterparts (PAR). CFPAC cells show the highest levels of CD24 but the expression of the marker does not increase in tumorspheres (TUM) compared to their parental counterparts (PAR). All analysed human pancreatic cancer cell lines express CD44 and, in all cell lines, there is an increased CD44 expression in tumorspheres (TUM) compared to their parental counterparts (PAR). The same increase is detected for the epithelial cell adhesion molecule EpCAM. Interestingly, BxPC-3 parental cells (PAR) show a very low expression of EpCAM that gradually increased during serum starvation stages (SF1-SF3-SF7) and in tumorspheres (TUM). CD133 expression only slightly increased in AsPC-1 and HPAF-II tumorspheres, although it does not show any difference in BxPC-3 and CFPAC-1 tumorspheres compared to their parental counterparts. Results highlight heterogeneity between the different human pancreatic cancer cell lines, although they evidenced a common pattern. In all analysed cell lines, we observed that in tumorspheres (TUM) there was an increased proportion of CD44/EpCAM double positive cells compared to their parental counterparts (PAR) (Fig. 2B).

In addition, in order to characterise our population of tumorspheres we have compared the expression levels of an array of markers previously discussed in literature (Heiler et al., 2016) in pancreatic cancer tumorspheres (TUM) and in their parental adherent counterparts (PAR), including intermediate stages in serum starvation (Fig. 3). Expression of these markers appears to be highly heterogeneous between the different cell lines and when comparing different time points during the procedure we used to isolate the tumorspheres population from their adherent counterparts. Aldehyde dehydrogenase 1, subfamily A, member 1 (ALDH1A1) is more expressed in tumorspheres compared to parental cells in AsPC-1, BxPC-3 and CFPAC-1, where it also shows an increased expression during serum starvation stages, in particular day three (SF3) and seven (SF7) in serum-free media. Conversely, ALDH1A1 expression gradually decreases from parental adherent cells to tumorspheres, through serum free stages in HPAF-II and KPC cell lines.

Octamer-binding transcription factor 4 (OCT4), expression is higher in tumorspheres of all the analysed human pancreatic cancer

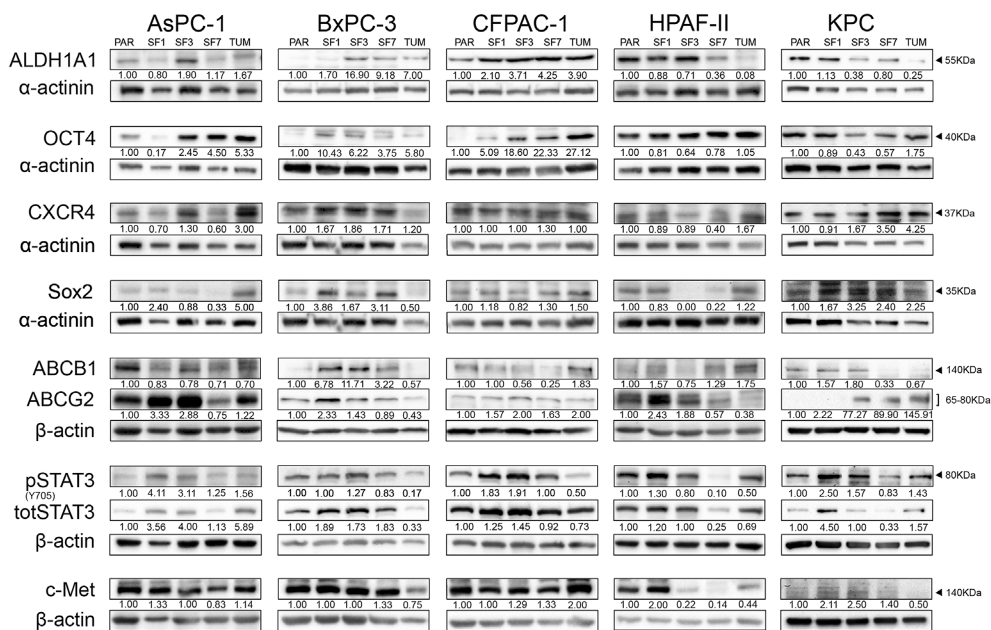

Fig. 3. Western Blots summarising the expression of different stemness ALDH1A1, OCT4, CXCR4, Sox2, ABCB1, ABCG2, pSTAT3, STAT3, c-Met across the tumorspheres development time-course for four human pancreatic cell lines; AsPC-1, BxPC-3, CFPAC-1 and HPAF-II, and murine primary pancreatic cancer line KPC. For each marker a quantitative value normalised for the corresponding loading control is indicated.

cell lines and in the primary KPC line from murine PDAC. Notably, AsPC-1 and CFPAC-1 tumorspheres show the greatest increase in OCT4 expression through a steady increase during serum starvation stages.

CXCR4 expression is markedly increased in tumorspheres compared to their parental counterparts in AsPC-1, HPAF-II and in the murine KPC. In BxPC-3, the expression of CXCR4 increases during serum free stages, when intensity of the bands is normalised to the loading control (α-actinin). In CFPAC-1 no difference in CXCR4 expression can be appreciated between parental cells, tumorspheres and serum free stages.

Together with OCT4, the transcription factor sex determining region Y-box 2 (Sox2) is responsible for maintaining the compartment of pluripotent and neural stem cells during embryonic development (Zhang and Cui, 2014). When normalised to the loading control (α-actinin), Sox2 expression appears increased in tumorspheres, compared to their parental counterparts of AsPC-1, CFPAC-1, HPAF-II and KPC cell lines. In BxPC-3, Sox2 expression appears to be reduced in tumorspheres, although it shows a peak in serum free stages, especially SF1 and SF7.

P-glycoprotein ABCB1, also known as multidrug resistance protein MDR1, is found overexpressed in CFPAC-1 and HPAF-II tumorspheres. In BxPC-3 cells, ABCB1 expression peaks in serum free conditions SF1 and SF3, while in AsPC-1 and KPC cell lines expression of MDR1 is higher in parental cells, gradually decreasing during serum starvation and in tumorspheres.

ABC transporter, subfamily G, member 2 (ABCG2), also known as breast cancer resistance protein (BCRP), shows a marked increase of expression from parental cells to tumorspheres through the serum starvation stages in cells derived from primary tumour of KPC mice. When normalised to the loading control, ABCG2 expression is higher in tumorspheres also in the CFPAC-1 cell line. Interestingly, in AsPC-1, BxPC3 and HPAF-II the expression of ABCG2 peaks after the first day in serum free media (SF1). Elevated ABCG2 expression in these cell lines is maintained at the stage SF3 and then gradually decreases so that in tumorspheres, the expression of this marker is either similar (AsPC-1) or lower than in parental cells (BxPC-3 and HPAF-II).

Activation of STAT3 by phosphorylation of Tyrosine 705 is known to be an oncogenic factor in many cancer types including pancreatic cancer (Corcoran et al., 2011). Increased phosphorylation of STAT3 was found in AsPC-1 and KPC tumorspheres, while in all other cell lines pSTAT3 seems to be relatively higher in serum free stages SF1 and SF3. Interestingly the variability in the activation of STAT3 by phosphorylation at the Tyr 705 site also corresponds to a similar fluctuation in the level of total STAT3.

Finally, when normalised to the loading control, c-Met showed an increased expression in CFPAC-1. In AsPC-1 expression of Met appears consistent through all the analysed stages, while in BxPC-3 and HPAF-II it decreases in tumorspheres. Similarly, in KPC tumorspheres Met expression is lower compared to their adherent counterparts, but it is more intense during serum starvation.

3.3. Pancreatic cancer tumorspheres show increased expression of mesenchymal markers

Pancreatic cancer stem cells are recognised as important mediators of therapy resistance and tumour relapse. In addition, pancreatic cancer CSCs play an important role in tumour metastatic spread to distant sites. The ability of cancer stem cells to migrate from the primary tumour depends on the activation of the epithelial to mesenchymal transition (EMT). With the expression of EMT markers, cancer stem cells acquire properties of increased survival and decreased proliferative ability, together with an increased

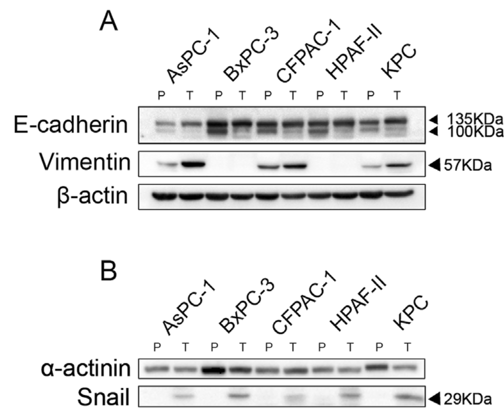

Fig. 4. Expression of epithelial to mesenchymal transition (EMT) markers. (A) For each of the four human pancreatic cell lines; AsPC-1, BxPC-3, CFPAC-1 and HPAF-II, and murine primary pancreatic cancer line KPC, expression of E-cadherin and Vimentin were compared in parental cells (P) and tumorspheres (T). In parental cells the epithelial marker E-cadherin is present in two forms, as a precursor (135 kDa) and as a mature protein (100 kDa). Tumorspheres only show a strong expression of the E-cadherin precursor, lacking the mature protein. Expression of the mesenchymal marker Vimentin is clearly increased in tumorspheres on AsPC-1, CFPAC-1 and KPC. As previously reported in literature (REF) (Buonato et al., 2015; Karnevi et al., 2016) BxPC-3 and HPAF-II are Vimentin-negative cell lines. (B) An additional mesenchymal marker has been analysed. Snail is expressed exclusively in tumorspheres (T) of all analysed cell lines.

invasive phenotype (Zhou et al., 2017). In this paper, we have verified the expression of mesenchymal markers Vimentin and Snail (Fig. 4) in parental adherent cells (P) and their corresponding tumorspheres (T). Results show that the expression of mesenchymal maker Vimentin is increased in tumorspheres compared to their parental counterparts in AsPC-1, CFPAC-1 and primary cell line derived from pancreatic tumours in KPC mice (Fig. 4A). BxPC-3 and HPAF-II showed no expression of Vimentin, in line with data already present in the literature (Buonato et al., 2015; Karnevi et al., 2016). We have then analysed the expression of another mesenchymal marker, Snail, and results show that negligible expression of Snail is detected in parental cells (P) from all the analysed cell lines (Fig. 4B). On the contrary, a higher expression of snail is detected in tumorspheres (T) in all the cell lines. E-cadherin (Fig. 4A), used as epithelial marker, shows a different expression pattern in parental cells and tumorspheres. In all cell lines, parental cells show two bands corresponding to the E-cadherin precursor at 135 kDa and the mature protein at around 100–120 kDa (Beavon, 2000). Conversely, tumorspheres in all cell lines are characterised by a marked reduction of the band corresponding to the mature E-cadherin (100 kDa), despite still showing an intense band corresponding to the E-cadherin precursor (135 kDa).

3.4. Pancreatic cancer tumorspheres are metabolically more quiescent and have a reduced metabolic potential

To further characterise pancreatic cancer tumorspheres, we modified a metabolic assay in order to measure at the same time both the mitochondrial respiration and the glycolytic function of pancreatic cancer tumorspheres compared to their parental counterparts. We determined the oxygen consumption rate (OCR) and the extracellular acidification rate (ECAR) using the $XF^{e}96$ Agilent Seahorse Extracellular Flux Analyzer. Results show that, when normalised to the total protein content, tumorspheres from human pancreatic cancer cell lines AsPC-1 (Fig. 5A), BxPC3 (Fig. 5D) and HPAF-II (Fig. 5J) have a reduced baseline OCR compared to their parental counterparts. This evidence is confirmed when analysing the primary murine PDAC cell line KPC; in fact baseline OCR in KPC tumorspheres is lower compared to their parental counterparts (Fig. 5M). Similarly, AsPC-1 (Fig. 5B), BxPC-3 (Fig. 5E) and HPAF-II (Fig. 5K) tumorspheres, as well as KPC tumorspheres (Fig. 5N), in basal condition have a lower ECAR compared to their parental counterparts. This indicates that the basal glycolytic activity of the population of pancreatic cancer tumorspheres is reduced compared to the bulk of adherent tumour cells.

When exposed to stressors that require a higher energy demand, human PDAC cell lines AsPC-1, BxPC-3, HPFAF-II and murine KPC cell line, undergo an increase of their metabolic activity. Parental cells respond to the presence of inhibitors of mitochondrial function by elevating their glycolytic rate, measured as an at least 4-fold increase in the extracellular acidification rate (ECAR, Fig. 5B-E-K-N blue bars). AsPC-1 (Fig. 5B red bar), BxPC-3 (Fig. 5E red bar), HPAF-II (Fig. 5K red bar) and KPC (Fig. 5N red bar) tumorspheres also respond to the presence of mitochondrial stressors by increasing their ECAR and glycolytic rate. Nevertheless, in tumorspheres AsPC-1, BxPC −3, HPFAF-II and murine KPC cell line, the ECAR in stressed conditions is lower compared to their parental counterparts.

In the presence of inhibitors of mitochondrial function parental AsPC-1 (Fig. 5A blue bar), BxPC-3 (Fig. 5D blue bar), HPAF-II (Fig. 5J blue bar) and KPC (Figure 5M blue bar) cells also increase their oxygen consumption rate (OCR). Conversely, in tumorspheres, when exposed to stressors, OCR shows no significant difference between basal and stressed conditions (Fig. 5A-D-J-M red bars).

The difference between the basal metabolism and the metabolic activity measured under stressed conditions reveals a high metabolic potential for parental cells (Fig. 5C-F-L-O blue line). On the contrary, tumorspheres have a more quiescent basal metabolism and under stressed conditions, their metabolic activity only undergoes a minor shift (Fig. 5C-F-L-O red line), indicating a much

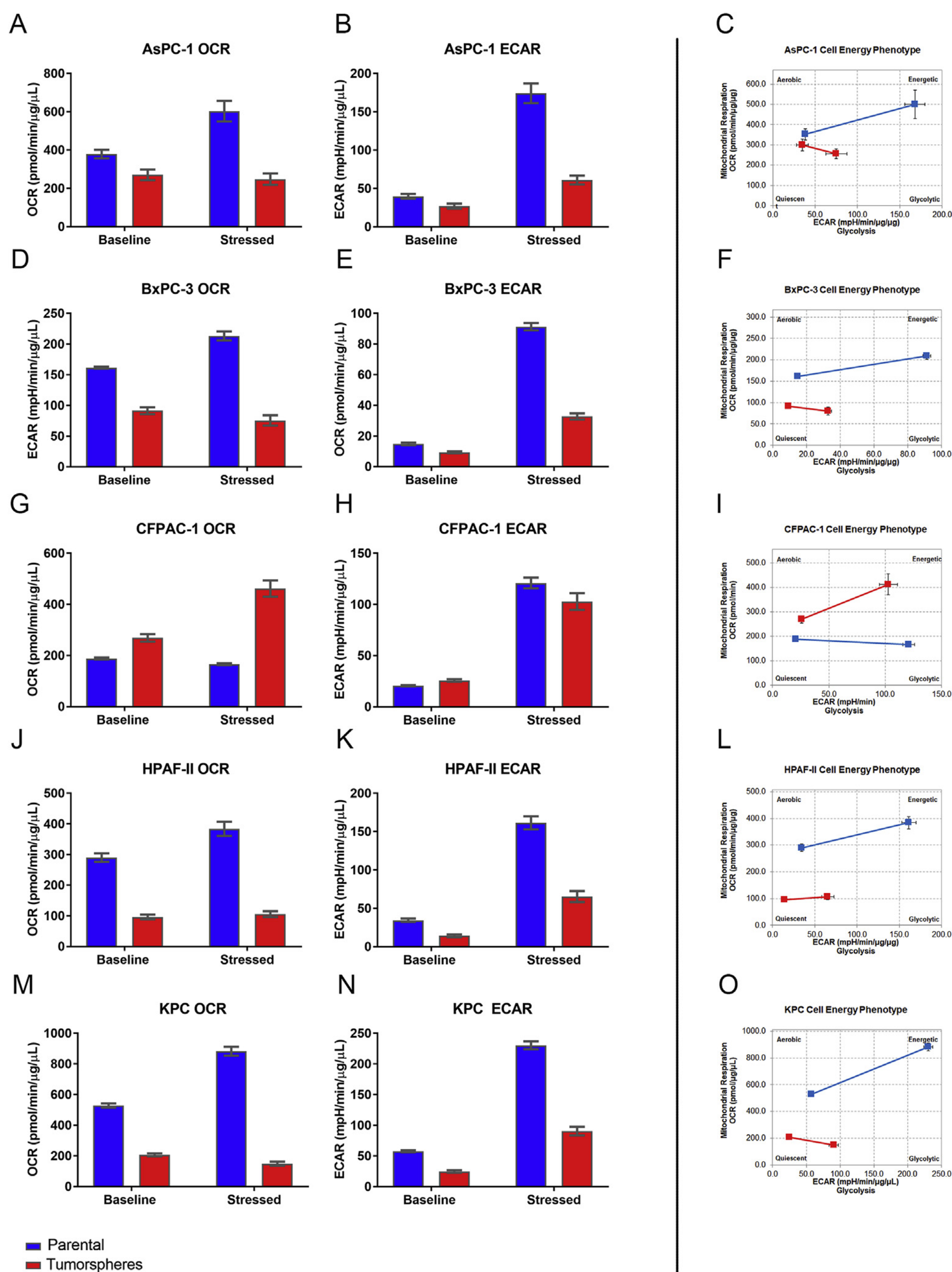

Fig. 5. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the XF96 Agilent Seahorse Extracellular Flux. For each of the four human pancreatic cell lines; AsPC-1 (A–B), BxPC-3 (D–E), CFPAC-1 (G–H) and HPAF-II (J–K), and murine primary pancreatic cancer line KPC (M–N) differentiated parental cells (blue bars) and tumorspheres were compared (red bars). For each cell line a cell energy phenotype diagram was generated, AsPC-1 (C), BxPC-3 (F), CFPAC-1 (I), HPAF-II (L) and KPC (O) showing the comparison between metabolic plasticity of parental cells (blue) versus tumorspheres (red).

lower metabolic potential compared to their parental counterparts.

The only exception is represented by pancreatic cancer cell line CFPAC-1 where tumorspheres show a higher OCR compared to their parental counterparts both at a basal level and when exposed to stressors (Fig. 5G). CFPAC-1 parental cells and tumorspheres have a similar baseline ECAR and, when responding to inhibitors of mitochondrial function, they both increase it to a similar extent (Fig. 5H). CFPAC-1 tumorspheres activate a more energetic metabolism when under stressed conditions, increasing both OCR and ECAR compared to their parental counterparts which, instead, only increase their glycolytic rate (Fig. 5I).

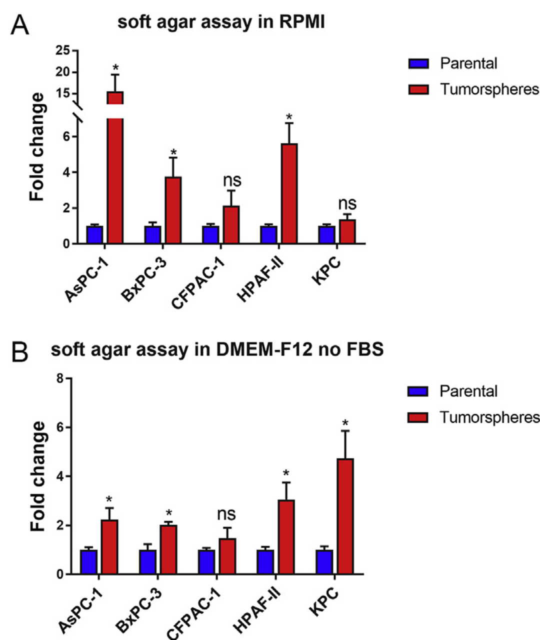

Fig. 6. The ability of forming colonies in anchorage-independent conditions using the soft agar assay was measured. Assay was performed in RPMI (complete growth medium for parental cells) and RPMI (base serum-free medium used for culturing tumorspheres). Colonies were counted using ImageJ software and, the ability of forming colonies was measured as fold change increase in clonogenic potential of tumorspheres (red bars) versus the parental control (blue bars) which value is, as a convention, set at 1. (*) indicates statistical significance with $p < 0.05$; (ns) indicates no statistical significance.

3.5. Pancreatic cancer tumorspheres have an enhanced tumorigenic potential

To assess the ability of pancreatic cancer tumorspheres to grow in anchorage-independent conditions, one of the hallmarks of cancer cells that correlates with tumorigenicity, we have performed a soft agar colony assay for each cell line. For this purpose, we used both serum enriched cell growth medium (RPMI) and serum-free medium (DMEM/F-12 no FBS) and we compared anchorage-independent growth of tumorspheres and their parental counterparts.

As a result, in all cell lines tumorspheres showed a higher clonogenicity, calculated as fold increase in the ability to form colonies, compared to their parental counterparts.

Strikingly, in AsPC-1 cell line, tumorspheres have higher clonogenic ability when growing in complete RPMI compared to DMEM/F-12 without FBS. Interestingly, AsPC-1 is a human cell line isolated from a very aggressive and highly metastatic form of adenocarcinoma in the head of the pancreas (Deer et al., 2010). In RPMI complete growth medium (Fig. 6A), AsPC-1 tumorspheres show on average a 15-fold increase in their ability to grow colonies in anchorage-independent conditions compared to their parental counterparts ($t_{16} = 3.691$, $p = 0.002$). In DMEM/F-12 medium without FBS (Fig. 6B), AsPC-1 tumorspheres are twice more clonogenic in anchorage independent conditions compared to their parental counterparts ($t_{15} = 2.37$, $p = 0.316$).

In BxPC-3, tumorspheres also show a statistically significant increase in their anchorage-independent growth both in RPMI complete ($t_{10} = 2.533$, $p = 0.029$, Fig. 6A) and DMEM/F-12 without FBS ($t_{10} = 3.911$, $p = 0.003$, Fig. 6B). Similarly, HPAF-II tumorspheres have a higher proliferative potential is soft agar assay in RPMI ($t_{14} = 4.055$, $p = 0.001$, Fig. 6A) as well as in serum free conditions in DMEM/F-12 ($t_{16} = 2.934$, $p = 0.010$, Fig. 6B).

Primary cells isolated from pancreatic tumours in KPC mice (KPC) show an opposite pattern. Interestingly, in DMEM/F-12 medium without FBS (Fig. 6B), despite less colonies were observed compared to RPMI, KPC tumorspheres showed an increased colony-formation ability compared to their parental counterparts ($t_{16} = 3.279$, $p = 0.005$), while in RPMI complete (Fig. 6A) the difference between parental cells and tumorspheres is non-significant ($t_{16} = 1.211$, $p = 0.243$).

Finally in CFPAC-1 cell line, tumorspheres show a clear trend of increased growth rate compared to their parental counterparts in RPMI (Fig. 6A), although not statistically significant ($t_{16} = 1.372$, $p = 0.188$). Similarly, when considering the clonogenic growth in serum free conditions in DMEM/F-12, CFPAC-1 tumorspheres show a non-significant trend of increased colony formation ability compared to their parental counterparts ($t_{16} = 1.13$, $p = 0.275$).

3.6. Pancreatic cancer tumorspheres have increased chemoresistance to gemcitabine

To assess whether pancreatic cancer tumorspheres could be used as a valuable tool to test new pharmacological interventions we have analysed their drug response to Gemcitabine, compared to their parental counterparts. Using Trypan blue exclusion cell

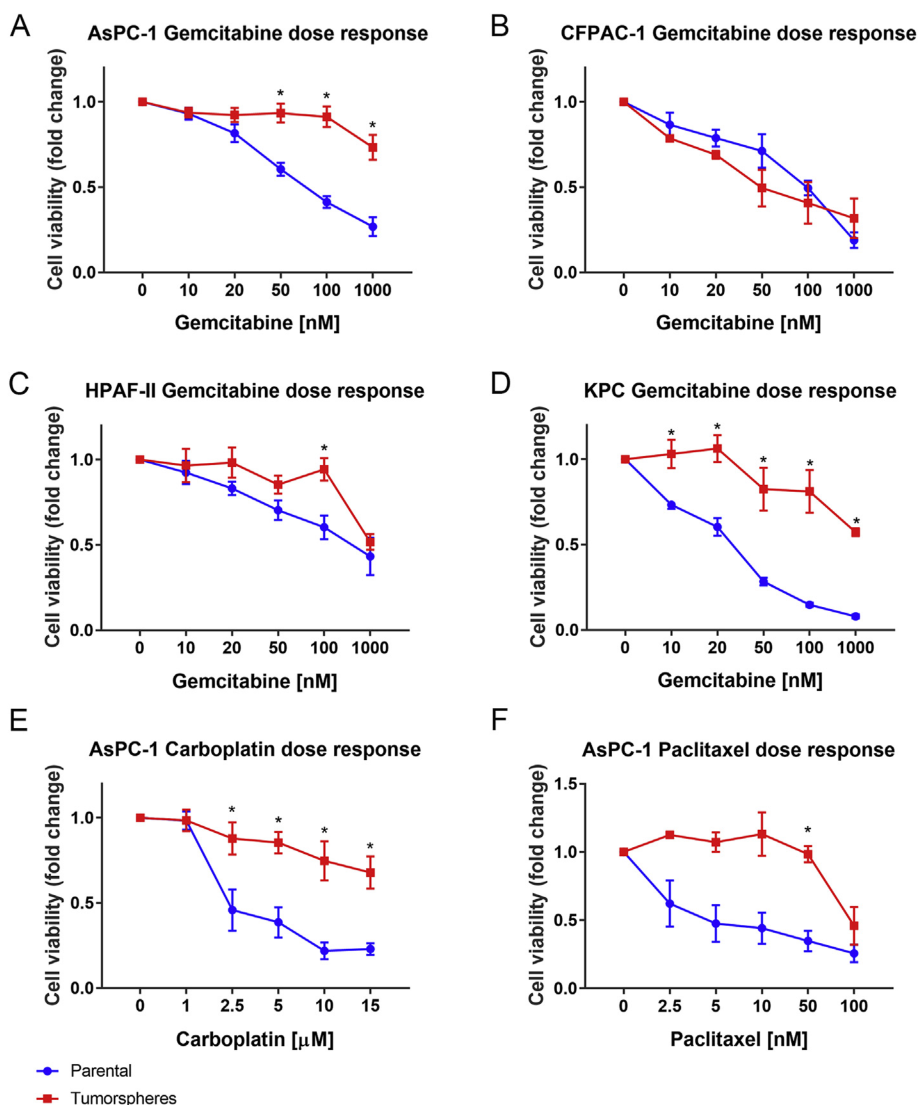

Fig. 7. Response of human pancreatic cancer cell lines AsPC-1 (A), CFPAC-1 (B) and HPAF-II (C) and murine primary pancreatic cancer line KPC (D) to increasing doses of common therapeutic drug, Gemcitabine. Dose response in parental cells (blue) was compared to their corresponding tumorspheres red). (E) AsPC-1 dose response to carboplatin indicates that in this cell line tumorspheres (red) are resistant to the chemotherapy drug, compared to their parental counterparts (blue). (F) AsPC-1 dose response to paclitaxel indicates that in this cell lines tumorspheres (red) have a tendency to be more resistant than their parental counterparts (blue). Star (*) indicates statistical significance where $p < 0.05$.

counting we have found that AsPC-1 tumorspheres viability is significantly unaffected by Gemcitabine treatment at the highest dose (Fig. 7A) compared to their parental counterparts (at 50 nM, $t_6 = 4.897$, $p = 0.003$; at 100 nM, $t_6 = 7.238$, $p < 0.001$ and at 1000 nM, $t_6 = 5.093$, $p = 0.002$). This evidence is strongly supported by performing Gemcitabine dose response in KPC tumorspheres (Fig. 7D). Primary cells isolated from murine PDAC of KPC mice are very sensitive to Gemcitabine treatment and at the highest doses viability is less than 20% (less than 10% at 1000 nM). Conversely, KPC tumorspheres are significantly more resistant to Gemcitabine treatment already at 10 nM ($t_5 = 2.982$, $p = 0.030$; at 20 nM, $t_5 = 4.474$, $p = 0.006$; at 50 nM, $t_5 = 3.612$, $p = 0.015$; at 100 nM, $t_5 = 4.465$, $p = 0.004$ and at 1000 nM, $t_5 = 17.09$, $p < 0.0001$).

HPAF-II tumorspheres (Fig. 7C) are significantly more resistant to their parental counterparts only at 100 nM ($t_5 = 3.495$, $p = 0.017$). Interestingly, HPAF-II parental cells appear to be on average more resistant compared to AsPC-1 parental cells ($p = 0.042$ at 100 nM) and especially to KPC parental ($p = 0.025$ at 20 nM, $p = 0.002$ at 50 nM, $p = 0.003$ at 100 nM and $p = 0.032$ at 1000 nM).

CFPAC-1 cell line shows a different response to Gemcitabine treatment as both parental cells and tumorspheres are equally sensitive to the chemotherapy dose-response treatment (One-Way ANOVA for parental cells, $F_{(5, 18)} = 23.55$ $p < 0.0001$ and for tumorspheres, $F_{(5, 18)} = 9.5$ $p = 0.0001$). Moreover, the difference in the dose response between CFPAC-1 tumorspheres and their parental counterparts is not statistically significant (Fig. 7B).

With the aim of exploring in the future the potential of tumorspheres to test chemoresistance and to exploit new pharmacological vulnerabilities, we have also analysed the response of AsPC-1 tumorspheres to other drugs used in the treatment of pancreatic cancer, like Carboplatin and Paclitaxel. Results show that AsPC-1 tumorspheres are significantly more resistant to Carboplatin than their parental counterparts (Fig. 7E) from the concentration of 2.5 μM ($t_6 = 2.725$, $p = 0.034$; at 5 μM $t_6 = 4.296$, $p = 0.005$; at 10Mm, $t_6 = 4.225$, $p = 0.005$ and at 15 μM $t_6 = 4.438$, $p = 0.004$). Furthermore, although only significant at 50 nM ($t_2 = 6.737$, $p = 0.021$), AsPC-1 tumorspheres show a trend towards a higher resistance to Paclitaxel treatment compared to parental cells (Fig. 7F).

4. Discussion

In this work, we have described a new strategy to isolate tumorspheres from pancreatic cancer cells. We have optimised a novel *in vitro* platform that can be used to predict patients' response to therapies and to investigate new pharmacological interventions targeting cancer stem cells (CSCs) metabolism.

Our results indicate that the tumorspheres model represents a good predictive tool to study new metabolic vulnerabilities and test novel therapies that could be applied to patient-derived material. We have shown that pancreatic cancer tumorspheres are enriched in cancer stem cells and we believe this platform may be an efficient alternative strategy when patient-derived material is limited and isolating different subpopulations of cancer stem cells representing less than 1% of the whole tumour (Fitzgerald and McCubrey, 2014; Lee et al., 2008) might not be representative of tumour heterogeneity.

Previous studies focussed on isolating distinct subpopulations of cancer stem cells based on the expression of surface markers (Visvader and Lindeman, 2008). In pancreatic cancer, different CSCs subpopulations with increased tumorigenic potential have been isolated according to the expression of markers like CD44, CD24, and EpCAM (Li et al., 2007), CD44 and c-Met (Li et al., 2011), CD133 (Hermann et al., 2007; Sancho et al., 2015). Nevertheless, the list of putative stemness markers for cancer stem cells is continuously being revised and updated depending on the specificity of the assay and the variability between tumour samples, in addition to intra-tumoral heterogeneity.

We have found that the pancreatic cancer tumorspheres described in the present work have a higher expression of $CD44^+/EpCAM^+$ cells. These two markers, together with CD24, represent the most commonly reported surface markers for pancreatic cancer stem cells (Au - Rasheed et al., 2010; Goodwin et al., 2018; Heiler et al., 2016; Li et al., 2007) and they correlate with a more aggressive and chemoresistant phenotype. However, it has not yet been identified a unique surface marker to isolate and define cancer stem cells (Dalla Pozza et al., 2015) and other "stemness" markers have been investigated (Goodwin et al., 2018; Heiler et al., 2016). In the present work we have performed a screening of several of these stemness markers and found that, despite remarkable heterogeneity of the different analysed cell lines, some proteins show a more consistent overexpression in tumorspheres compared to their parental counterparts. OCT4, CXCR4 and Sox2, in fact, are overexpressed in pancreatic cancer tumorspheres in four out of five of the cell lines we have analysed. Transcription factors Sox2 and OCT4 play a pivotal role in maintaining the pluripotent compartment of embryonic stem cells (Kim et al., 2009; Zhang and Cui, 2014) while G-protein coupled receptor CXCR4, involved in maintaining the compartment of haematopoietic stem cell (Cheng and Qin, 2012), has been linked to aggressiveness and metastasis in pancreatic cancer (Goodwin et al., 2018). In addition, it has been proposed that cancer stem cells are responsible for metastatic spread of solid tumours and their ability to migrate to distant sites to establish new metastatic niches is dependent upon the activation of epithelial to mesenchymal transition (EMT) (Brabletz, 2012; Mani et al., 2008; Singh and Settleman, 2010; Zhou et al., 2017). Indeed, we have found that, despite the phenotypical heterogeneity, pancreatic cancer tumorspheres analysed in this work show an increased expression of mesenchymal markers Vimentin and Snail, compared to their parental adherent counterparts. These evidences further support our hypothesis that pancreatic cancer tumorspheres we have described are enriched in cancer stem cells that have the potential to colonise new metastatic niches.

Nevertheless, it has been questioned whether surface markers alone are sufficient to define and investigate cancer stem cells. For this reason, a marker-independent way to characterise CSCs (P Nagare et al., 2017) has been proposed in alternative to the more traditional marker-dependent approach to define cancer stem cells. In addition to phenotypical heterogeneity, this marker-independent approach addresses functional heterogeneity within the tumour, to isolate spheroid-like structures that are indeed enriched in cancer stem cells, as previously described for colorectal carcinomas (Shaheen et al., 2016). Our results successfully confirm that tumorspheres can be isolated from primary pancreatic tumours and validate the hypothesis that tumor spheroids represent a reliable *in vitro* platform to study properties and vulnerabilities of CSCs (P Nagare et al., 2017). From a functional point of view, stem cells and pancreatic cancer stem cells appear to have distinct metabolic profiles compared to the bulk of the tumour cells. Even though only a limited number of studies have investigated CSCs metabolism, it is clear that this could represent an effective way to identity and target cancer stem cells (Dando et al., 2015; De Francesco et al., 2018). An interesting study has shown that drugs like metformin, which induce metabolic reprogramming, can target chemoresistant breast cancer stem cells and, in combination with chemotherapy, effectively suppress tumour progression (Hirsch et al., 2009). More recently, Candido et al. (2018) have proposed a similar mechanism for pancreatic cancer stem cells. Pancreatic cancer cell lines differ for their ability of forming tumorspheres enriched in cancer stem cells. These differences could reflect into different metabolic vulnerabilities and responses to metformin, as well as other drugs targeting cell metabolism which, in turn, could enhance cancer stem cells sensitivity to chemotherapy (Candido et al., 2018). Another intriguing study has identified a population of highly tumorigenic and chemoresistant CD133-positive pancreatic cancer stem cells predominantly relying on oxidative phosphorylation and characterised by a limited metabolic plasticity (Sancho et al., 2015). Nevertheless, it has been observed that cancer stem cells are quiescent and slow cycling (Chen et al., 2016; De Francesco et al., 2018; Takeishi and Nakayama, 2016) and thus maintaining a low glycolytic metabolic rate is important to preserve the quiescent state. In addition, a preference for the glycolytic pathway as a way to produce energy while reducing the production of

toxic reactive oxygen species (ROS) is an effective strategy to survive in an highly hypoxic environment characterising certain solid tumours, including pancreatic cancer (De Francesco et al., 2018). In the present research, we have demonstrated that, the metabolic profile of pancreatic cancer tumorspheres represents a more reliable tool to identify and analyse cancer stem cells. In fact, here we report that tumorspheres isolated from pancreatic cancer cell lines maintain a more quiescent metabolic state in response to metabolic stressors. In stressed conditions, pancreatic cancer tumorspheres tend to maintain a low metabolic potential, with a low oxygen consumption rate (OCR) while only slightly increasing their glycolytic metabolism. In contrast, parental cells respond to external stressors by elevating both their glycolytic rate and oxygen consumption (mitochondrial respiration). In parental cells, the increase in oxygen consumption rate represents a metabolic vulnerability as it negatively affects cell survival in the highly hypoxic environment characterising pancreatic cancer. Nevertherless, in tumorspheres the reduced metabolic potential could also represent a metabolic vulnerability, indicating a more limited capacity for tumorspheres to respond to extremely unfavourable conditions. The only exception is represented by CFPAC-1 cell line, where both parental cells and tumorspheres elevate their glycolytic rate when exposed to stressors, and tumorspheres elevate their mitochondrial respiration. Functional differences in CFPAC-1 line might be due to the fact that this pancreatic cancer cell line may have been differentiated from unique molecular events and therefore it does not recapitulate the full heterogeneity of the primary tumour (Deer et al., 2010).

Our results, despite considering a population of tumorspheres enriched in cancer stem cells, rather than a distinct subpopulation, further validate previous findings indicating that pancreatic stem cells possess a limited metabolic plasticity (Sancho, 2016; Sancho et al., 2015, 2016). In addition, our work supports the importance of identifying new metabolic vulnerabilities to target cancer stem cells.

Pancreatic cancer is an aggressive malignancy with a five-year survival rate of only 5–10% due to development of resistance to the current therapies and a rapid metastatic spread (Abrams et al., 2018, 2019). It has been discussed that tumour metastatic progression and the ability to relapse after chemo- and radio-therapy may be due to a subpopulation of quiescent, slow-cycling cells endowed with multilineage potential called cancer stem cells (Fitzgerald and McCubrey, 2014; McCubrey and Cocco, 2014). In the present work, we have isolated a population of pancreatic cancer tumorspheres enriched in cancer stem cells that show increased tumorigenic potential compared to their parental counterparts as demonstrated by their enhanced clonogenic growth in soft agar assay. Furthermore, we have extended our investigation to some of the most commonly applied therapies for pancreatic cancer (Adamska et al., 2018), and observed that pancreatic cancer tumorspheres are indeed more resistant. We have analysed tumorspheres of different pancreatic cancer cell lines and identified a variability that may reflect the heterogeneity of the tumours they have been isolated from (Deer et al., 2010). Functional assays, like the anchorage-independent growth in soft agar and the chemoresistance, showed that cell lines isolated from more aggressive carcinomas (AsPC-1) have higher tumorigenic potential and are more chemoresistant. When we have tested the KPC line isolated from murine primary pancreatic cancer, we have shown that they are metabolically similar to AsPC-1. Our results suggest that excluding tumorspheres from studies aimed at finding new and more effective therapies might be misleading. In fact, if we only base our analysis on KPC parental cells, we may find them very sensitive to Gemcitabine, yet when KPC mice are treated with Gemcitabine their survival rate does not increase (Ferro et al., 2018). We show in this paper that this failure is likely to be due to the presence of resistance cells and clearly show that tumorspheres isolated from KPC mice are resistant to Gemcitabine. Interestingly, a recent study by Abrams et al. (2019) has elucidated the potential of a common nutraceutical, Berberine, as an effective agent to reduce chemoresistant in solid malignancies, including pancreatic cancer. Berberine could be potentially exploited to target pancreatic cancer stem cells as it can play a role in blocking EMT as well as tumour progression and invasion (Abrams et al., 2019). Together these results support the hypothesis that tumorspheres represent a good model to test chemoresistance and validate new therapeutic drugs on tumorspheres isolated from patient-derived material. Soft agar assay using cancer tumorspheres could in fact be a cheaper and faster alternative strategy than patient-derived xenografts (PDX) to test and validate new drugs.

In conclusion, we have shown that pancreatic cancer tumorspheres isolated in this work are enriched in cancer stem cells. Nevertheless, we believe that, while focussing on the most common universal surface markers and stemness properties might be misleading, functional characteristics of tumorspheres like a reduced metabolic plasticity, higher colony formation potential and chemoresistance represent a more reliable approach. We propose tumorspheres as a novel *in vitro* predictive model to study patient-derived material for drug testing in order to study new metabolic vulnerabilities in pancreatic cancer.

Conflicts of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbior.2019.02.001.

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8. Final discussion and future plans

Pancreatic cancer is a very aggressive and devastating disease. Although its incidence remains considerably low compared to other solid malignancies, pancreatic cancer is considered the fourth most common cancer-related cause of death. Despite the extensive research in the last few decades, the survival of PDAC patients remains low, with 5-year survival around 8%.

The high mortality is a multifactor consequence of aggressive phenotype, lack of distinctive symptoms leading to late diagnosis and early metastatic spread. Additionally lack of an effective therapeutic approach adds to the dismal prognosis of PDAC patients. The only potentially curative option for PDAC patients is the surgical intervention. However, only 15-20% of the patients are suitable for surgery, with the rest of them presenting with locally advanced or metastatic disease at the time of the diagnosis. Therefore, chemotherapy treatment, although providing minimal effects, is the main therapeutic option for PDAC patients. Up to recently, gemcitabine was considered as a gold standard in PDAC therapy. Currently, Abraxane (albumin-bound paclitaxel) used in combination with gemcitabine or FOLFIRINOX are first line treatments for pancreatic cancer patients. However, their increased efficacy is accompanied by increased frequency of severe side effects and lowered quality of life, thus restricting the application to the patients with good performance status.

One of the factors responsible for the lack of effectiveness of the therapies is high chemoresistance of PDAC tumours. Cancer cells develop a variety of mechanisms both, intrinsic or acquired, that allow them to decrease the efficacy of the applied therapies. Thus, the ability to overcome that resistance could substantially improve the obtained outcomes. An additional factor contributing to the lack of effective therapies is the high heterogeneity of PDAC tumours. Not only different tumours present with different oncogenic mutation, but also within the same tumours a heterogeneous genetic landscape leads to the dysregulation of plethora of signalling pathways. In addition, changes in metabolism and existence of different cell populations add to the complex nature of the disease. This, consequently, impedes

the development of targeted therapies that could be applied to a broad group of patients. Several clinical trials have been designed to test compounds targeting the main pathways dysregulated in PDAC tumorigenesis. However, either applied alone, or in combination with standard-of-care chemotherapy, targeted therapies have not provided the expected breakthrough in PDAC treatment, with only few of them being accepted for the clinical use. One of the reasons for the unsuccessful outcome of the clinical trials in PDAC was the lack proper stratification of patients into the cohorts. Screening of the patients and application of the targeted therapies based on their mutational landscape should be taken in consideration during all phases of the pre-clinical and clinical validation as well as during therapy. Therefore, therapies aiming at those molecules, which mutations or expression are observed in the prevailing percentage of PDAC tumours, would potentially result in better outcome. However, attempts made so far in inhibiting of KRas protein, which is mutated in 95% PDAC cases, did not provide positive results. Nevertheless, the positive outcome of targeted therapies in other cancer types supports a further evaluation of the potential of personalised medicine in PDAC. It is therefore pivotal to identify new therapeutic targets that could be explored clinically.

During my PhD studies, I aimed to develop novel therapeutic strategies to counteract the progression of pancreatic cancer. The main goal of my project was to identify new therapeutic targets in PDAC, explore their role in PDAC tumorigenesis and validate their clinical potential. Based on previous studies performed in our group, we hypothesized that both G protein coupled receptors and ABC transporters might play a role in PDAC by forming a loop, which activation perpetuates cancer progression. Thus, my PhD project was divided in 3 parts, in which I investigated the potential of G Protein-coupled receptor GPR55 and ABC transporter ABCC3 as potential therapeutic targets in PDAC. In the last chapter, I explored the role of pancreatic cancer stem cells (CSCs) in the resistance of pancreatic cancer and investigated their potential as a novel tool to predict the efficacy of new therapeutic vulnerabilities.

In the first part of my project, I finalised and complemented the work commenced by Dr Riccardo Ferro at The Queen Mary University of London, UK. In this project, the role and the pharmacological potential of G protein-coupled receptor GPR55 was

investigated. The involvement of GPR55 in development and progression of several malignancies has been previously demonstrated and its correlation between tumour aggressiveness and migration was shown in several solid cancers. However, no studies investigated the potential of GPR55 as a therapeutic target in PDAC. In this study, we demonstrated that GPR55 is overexpressed in PDAC samples, both human and murine. Moreover, using several approaches we demonstrated that GPR55 activity is crucial for PDAC cell proliferation and clonal expansion *in vitro* and disease progression *in vivo*. Genetic ablation of the receptor in the KPC transgenic model of PDAC resulted in a significant prolongation of the survival compared to the KPC mice, highlighting the importance of GPR55 in PDAC progression. Furthermore, activation of MAPK signalling and regulation of cell cycle and autophagy were demonstrated as some of the main mechanisms,] involved in GPR55-mediated regulation of PDAC progression. Having confirmed the key role of GPR55 in PDAC, its pharmacological potential was explored with the aim to identify novel and potent therapeutic option PDAC patients. Cannabinoids have been studied for several years as potential anti-tumorigenic agents. Since for many years GPR55 was classified as a receptor belonging to endocannabinoid system, we screened several cannabinoids and demonstrated strong inhibition of GPR55-induced stimuli caused by cannabidiol (CBD) treatment. CBD treatment mirrored the effects of GPR55 silencing, decreasing PDAC cell proliferation and clonal expansion through inhibition of ERK1/2 activation and regulation of G1/S transition of cell cycle. Although no apoptosis could be detected after GPR55 inhibition, my study demonstrated the induction of autophagic cell death in the cells treated with CBD. These results support previously reported results showing that the cannabinoids treatment or the inhibition of ERK signalling induce autophagy and demonstrate for the first time that the observed effects might be mediated through GPR55 modulation. More importantly, GPR55 inhibition with CBD proved to be an efficacious and safe approach in the KPC mouse model, the most clinically relevant mouse model of pancreatic cancer. CBD treatment slightly increased the survival of the KPC mice (7 days) compared to the vehicle-treated animals and, its effects were comparable with the standard chemotherapy gemcitabine (9 days) although both treatments were statistically non-significant. Strikingly, the combination of CBD and gemcitabine significantly increased the mice

lifespan, prolonging the survival almost three times compared to vehicle. I speculate that one of the reasons for the observed enhancement of the activity of each drug alone might be due to the CBD-mediated increase in autophagy, leading to an increased sensitivity of the cells to the chemotherapy treatment. In addition, the potential inhibition of the epithelial to mesenchymal transition (EMT) induced by CBD treatment could slow down the metastatic spread of the primary tumours, further contributing to the increase in survival. Importantly, a significant reduction in the viability of PDAC tumorspheres enriched in cancer stem cells was demonstrated after cell treatment with CBD. The significantly increased effect of the treatment, compared to the parental population, is consistent with enhanced expression of GPR55 in PDAC tumorspheres. These data suggest that selective targeting of the cancer stem-like cell population, which resistance to gemcitabine was also demonstrated, might be another reason for the increased efficacy of CBD/gemcitabine combination observed in the KPC mice. However, more investigations are necessary to fully elucidate the mechanisms behind these effects.

It is now well established that GPR55 is the main receptor of lysophosphatidylinositol, LPI. LPI is a bioactive lipid, which mitogenic functions have been reported in several cancers. Therefore, one of the mechanisms involved in the upregulation of PDAC cell growth that we observe might be the effect of the LPI-mediated stimulation of GPR55 activity. Indeed, we showed that blocking the LPI-induced signalling and activity mediated by GPR55 remarkably slowed down PDAC progression. Therefore, the inhibition of LPI functions by targeting its endogenous receptor GPR55, proved to be an effective therapeutic strategy. However, other than GPR55, more receptors for LPI exist (e.g. GPR119) and their potential activation by LPI cannot be excluded. Therefore, I speculated that blocking the synthesis or release of LPI could substantially improve PDAC survival and provide a more potent therapeutic approach. It was established in our group that ATP-binding cassette transporters and G-protein-coupled receptors could define an autocrine loop involving LPI in cancer cells. A study in my group, demonstrated that in prostate and ovarian cancer LPI is released into the extracellular media by the ABCC1 transporter where then it could bind and activate GPR55, regulating cell growth. The preliminary data obtained by Dr

Riccardo Ferro indicated that in pancreatic cancer ABCC3 transporter might be involved in the release of LPI into the extracellular matrix. Therefore, in the main part of my project, I investigated the role of ABCC3 in LPI release and in PDAC progression; furthermore, I also validated the pharmacological potential of targeting ABCC3 in PDAC therapy.

The role of ABC transporters in cancers has been widely explored in terms of their involvement in development of chemoresistance. Acting as active pumps, ABC transporters are known to export xenobiotics as well as active drugs from cancer cells, decreasing their efficiency. Therefore, studies so far focused on the multi-drug resistance aspect of ABC transporters and the majority of developed therapies aimed at its reversal. A few studies investigated the role of ABC transporter in cancer progression, beyond chemoresistance, and their potential as main pharmacological targets in counteracting PDAC progression has not been fully explored. In this project, I demonstrated that ABCC3 is highly overexpressed in PDAC cells and tissues and importantly, the overexpression of ABCC3 significantly correlates with low overall survival of the patients. Both *in vitro* and *in vivo* analysis showed the importance of ABCC3 in PDAC cell proliferation and clonal expansion and in tumour growth in the xenograft mouse model. The role of ABCC3 in the regulation of HIF1α and STAT3 signalling was also demonstrated. It is known that hypoxia is one of the main events influencing PDAC progression, invasion and chemoresistance through a plethora of mechanisms. In addition, hypoxia is an important feature of the PDAC microenvironment, characterized by the presence of a dense desmoplastic reaction that promotes, and is modulated by, hypoxia. Importantly, a correlation between hypoxia, PDAC progression and patients' outcome has been documented. Similarly, STAT3 activation is one of the earliest events in PDAC development and it considered as one of the main oncogenic pathways in PDAC, responsible for regulation of cell survival, stem cell self-renewal and inflammation. Thus, the demonstrated data introduces a novel mechanism regulating PDAC progression, mainly through the indirect control of STAT3 signalling and hypoxia. Importantly, I could confirm that the observed regulation of PDAC progression involves ABCC3-mediated LPI release. My data demonstrated that LPI is a mitogenic factor in PDAC and that it stimulates the

activity of STAT3 and HIF1α signalling. On the other hand, the reduction of cellular LPI release through ABCC3 knockdown or the blockage of LPI functions by GPR55 inhibition substantially reduced the activity of STAT3 and HIF1α. These data confirmed the existence of an ABCC3-LPI-GPR55 loop in PDAC, which activation drives PDAC progression through activation of STAT3 signalling and hypoxia.

Having demonstrated the significant role that the ABCC3-LPI-GPR55 axis plays in PDAC progression, I verified the potential of blocking LPI release through the pharmacological inhibition of ABCC3. A specific inhibitor of ABCC3, called S3, was designed, as a derivative of the clinically approved drug sulindac. A higher efficacy in ABCC3 inhibition, together with lack of COX inhibition and a better pharmacokinetic profile was demonstrated for S3, supporting its further evaluation. *In vitro* studies showed high potency of S3 in reducing the proliferation and clonal expansion of PDAC cells with elevated expression of ABCC3. Importantly, the obtained results mimicked the effects of ABCC3 genetic silencing, decreasing levels of activated STAT3 and HIF1α, which confirms the specificity of S3 towards ABCC3. Using several assays, the increase in apoptosis was also demonstrated after modulation of ABCC3 activity, presenting another mechanism of ABCC3-mediated regulation of PDAC cell proliferation.

To determine whether targeting ABCC3 holds clinical potential in PDAC therapy, the efficacy of S3 treatment was evaluated i*n vivo*. Three mouse models were used for the evaluation of the safety and efficacy of S3. Significant results were observed in the xenograft mouse model, in which S3 treatment significantly prolonged the survival of the mice, with 30% of treated mice showing complete tumour remission. Interestingly, the effects of ABCC3 inhibition were maintained even after the conclusion of the treatment. More importantly, high efficacy of S3 was also demonstrated in the KPC transgenic mouse model, in which S3 treatment caused two-fold extension of the survival, compared to vehicle-treated mice. Moreover, the higher efficacy of S3 treatment compared to Abraxane, standard-of-care chemotherapy was demonstrated which in combination with the lack of evident side effects makes ABCC3-targeting a potent and promising therapeutic approach.

Several clinical trials tested the efficacy of targeted therapies in PDAC. Mainly, the combinations of targeted therapies with standard chemotherapy were verified in terms of increased efficacy. However, so far only EGFR inhibitor, Erlotinib, got FDA approval to be used in combination with gemcitabine in PDAC treatment, although it gives a very marginal improvement. Moreover, there have been very limited cases in which a tested compound showed efficacy as single agent in a very aggressive disease such as pancreatic cancer. My results demonstrated that targeting ABCC3 with S3 as a single agent is an efficacious therapeutic approach in PDAC treatment. Pharmacological inhibition of ABC transporters has been so far investigated in terms of chemoresistance reversal. Therefore, the effectiveness of ABC transporter-targeting molecules has been mostly tested in combination with standard chemotherapy. Nevertheless, although successful in increasing the efficacy of co-applied therapeutics, the studied combinations were characterized by elevated toxicity, restraining their further clinical evaluation. Therefore, considering high toxicity of previously developed ABC transporter inhibitors, the specificity and safety profile of S3 makes it a good candidate for clinical trials. Higher efficacy than standard chemotherapy in prolonging mice survival and lack of adverse events that frequently accompany chemotherapy treatment further support the clinical exploration of S3 as a single drug therapy in PDAC. In addition, oral bioavailability of S3 and the fact that sulindac, based on which S3 was developed is a clinically approved therapeutic, suggest a potentially fast implementation of S3 into the clinics. Moreover, the promising *in vitro* evaluation of the S3 and CBD combination and the safety profile of both drugs in the animal models of PDAC makes the combination a good candidate for further pre-clinical and clinical evaluation. Provided the importance of LPI in PDAC progression, dual inhibition of its release and activity could substantially improve PDAC patients' perspectives.

Interestingly, ABCC3 expression in PDAC stroma was demonstrated and its inhibition with S3 reduced the levels of stromal markers. In particular, vimentin, a fibroblast marker of cells with a mesenchymal phenotype was remarkably decreased in both cell lines and tumour tissues of the KPC mice. My data show that ABCC3 targeting may act both, on the primary tumour and PDAC stroma, decreasing their viability and

reducing tumour favouring stromal functions. Pancreatic stroma has been extensively studied as a crucial driver of PDAC progression and the indispensability of desmoplasia for PDAC progression has been well established, suggesting it as a potential therapeutic target. However, the results obtained so far through targeting the stromal components in clinical trials, such as the Hedgehog signalling, were not encouraging. One of the major drawbacks in the previous attempts to deplete the dense tumour environment was the observed higher metastatic rates that resulted in a setback for the applicability of these strategies. In my study, no evident increase in metastatic rates could be observed in the mice in the S3 treatment group. Furthermore, histopathological analysis of the murine livers demonstrated considerably lower number of metastatic lesions and fibrotic phenotype in the S3-treated animals. Significantly reduced vimentin levels were detected in the livers of the same animals. Similar observations were obtained in regional lymph nodes. Early metastatic spread of the primary tumours is one of the main factors contributing to the dismal prognosis of PDAC patients. It not only accelerates the disease progression, but also restrains the therapeutic options for PDAC patients. So far, only complete resection of the tumour has been proven effective. However, only 15-20% of diagnosed patients undergo potentially curative surgery, whereas the majority of the patients when diagnosed already presents widespread metastases. The possibility to manage the onset and the rates of metastatic spread could substantially improve the perspectives for PDAC patients. Although only preliminary, these data might suggest that ABCC3 targeting with a small molecule inhibitors like S3 might prolong the survival of pancreatic cancer patients through targeting both the tumour bulk and the surrounding stroma and simultaneously slowing down the metastatic spread. Thus, S3 treatment could not only substantially prolong PDAC patients' survival but also potentially increase the percentage of the patients that might benefit from potentially curative surgery. However, these data need more exploration.

Finally, I could demonstrate that the expression of both, GPR55 and ABCC3 is regulated by *TP53*, tumour suppressor gene, which mutations have been observed in around 75% of PDAC cases. High expression of GPR55 and ABCC3 was observed in the samples with mutated *TP53*, whereas presence of WT *TP53* correlated with low

expression of both proteins, suggesting the importance of GPR55 and ABCC3 in *TP53* mutated PDAC. I could also demonstrate that the p53-mediated regulation of GPR55 and ABCC3 expression involves the modulation of the activity of miR34 that belongs to a family of micro-RNAs, which role in PDAC has been previously documented. Consequently, I showed for the first time that p53 mutations could boost PDAC progression through the activation of GPR55 and ABCC3. These results have several consequences for PDAC research. First, they confirm the importance of both ABCC3 and GPR55 in pancreatic cancers characterized by the presence of mutated p53. Second, they allow the selection of a cohort of patients that might benefit from GPR55 and ABCC3-targeted therapies. Considering the high prevalence of the PDAC tumours that bear TP53 mutations, the demonstrated pharmacological potential of GPR55 and ABCC3 is of a high value for PDAC clinical research.

In addition, a novel platform for screening new therapeutic vulnerabilities was developed during my project. We demonstrated that PDAC cell-derived tumorspheres are enriched in pancreatic cancer stem cells and are characterized by increased resistance to conventional therapies. Therefore, we propose that testing of new therapeutic interventions should not exclude this small but important sub-population of cancer cells and that the identification of a pharmacological approach selectively targeting PADC tumorspheres could substantially increase the efficacy of available therapies.

Overall, my results confirmed the existence of an ABCC3-LPI-GPR55 loop in pancreatic cancer, which activity regulates progression of the disease. My data revealed a novel dependence of PDAC on p53-mediated regulation of GPR55 and ABCC3 expression that in turn activates the MAPK/ERK and STAT3 signalling, driving cancer progression. My results demonstrate that the inhibition of LPI activity through the ABCC3 targeting with specific drugs, such as S3, or GPR55 inhibition with CBD is an effective and safe therapeutic approach that could be progressed to clinical trial. Collectively, my findings hold important implications for pancreatic cancer treatment, providing a valuable pre-clinical validation for GPR55 and ABCC3 targting in pancreatic cancer and paving the way for potential clinical trials.

Although the data that I obtained during my PhD project show promising therapeutic strategy for PDAC patients, there are still several points that needs further clarification and questions that have arisen during the study. To complement the obtained data and address these points, the following studies should be additionally performed:

I demonstrated that ABCC3 inhibition with S3 influences liver status and regional lymph metastasis in the transgenic mouse model of PDAC. More in depth analysis of the observed phenomenon and the mechanisms involved should be explored.

- I demonstrated that ABCC3 is involved in LPI release from the cells and that inhibition of this process blocks the LPI-mediated signalling, regulating PDAC progression *in vitro*. *In vivo* lipidomic analysis of LPI levels in the plasma of KPC mice treated with vehicle or ABCC3 inhibitor would provide *in vivo* confirmation of ABCC3-mediated LPI release in PDAC and support the hypothesis of the existence of ABCC3-LPI-GPR55 loop in PDAC.
- I have demonstrated the mitogenic role of LPI in PDAC and showed that the inhibition of its activity either by blocking of its release or LPI-mediated activation of GPR55 remarkably slows down PDAC progression. Taking into consideration the involvement of EGFR activity in the stimulation of LPI synthesis, combination of EGFR inhibition with blocking of either ABCC3 or GPR55 could potentiate the effects of each inhibitor alone. Thus, the effects of combination of S3 or CBD with EGFR inhibition on PDAC cell number and anchorage-independent growth should be investigated.
- In the initial *in vitro* screening, I showed that the horizontal inhibition of ABCC3 and GPR55 with S3 and CBD respectively synergistically increased the effectiveness of each drug alone in decreasing the number of viable PDAC cells. Considering the efficacy and safety profile demonstrated for these drugs as single agent *in vivo*, the evaluation of their combination in mouse model of PDAC should be performed.
- I demonstrated that the modulation of ABCC3 activity regulates the cell cycle in PDAC cell lines. However, we observed a major difference between the ABCC3 silencing and its pharmacological inhibition with a cell cycle arrest in G1/S phase in the first case and a G2/M block in the second. The mechanisms responsible for the observed

discrepancies should be further investigated to fully understand the mechanisms of ABCC3 inhibition with S3.

- Data available in the literature showed that ERK inhibition in PDAC cells increases autophagy in the cells, sensitizing them to autophagy inhibition. Therefore, dual inhibition of autophagy and ERK was proposed for PDAC therapy. Similarly, gemcitabine treatment induced autophagy in PDAC cell lines. Our data demonstrated that GPR55 inhibition by CBD decreased ERK activity and induced autophagy in PDAC cells. Combination treatment coupling GPR55 and autophagy inhibition with or without gemcitabine addition should be evaluated both *in vitro* and *in vivo*.

9. Appendix

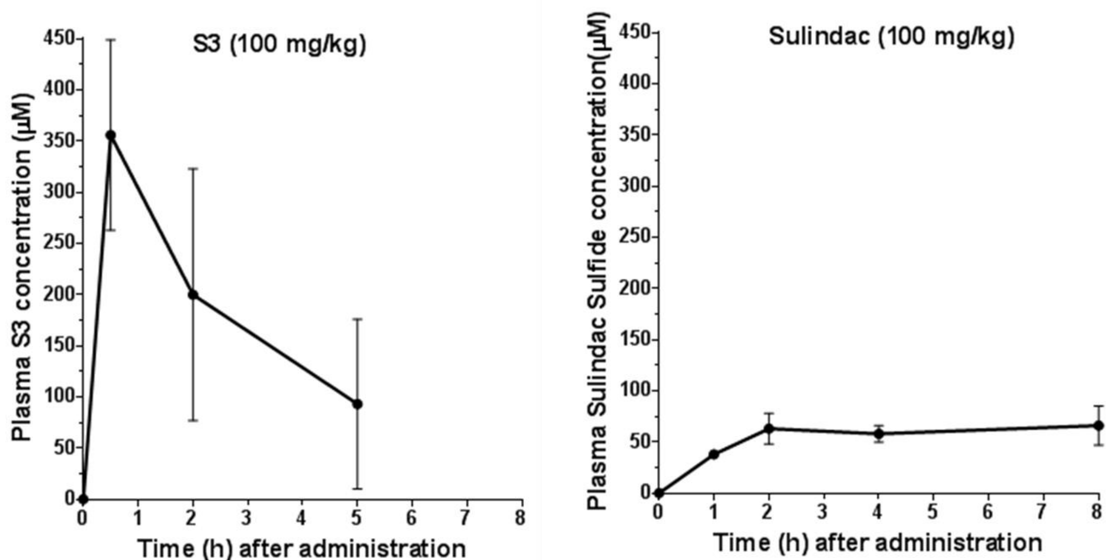


Figure 1 ***S3 is more stable than sulindac in vivo.*** *Pharmacokinetic profile of S3 and sulindac sulfide in mice presented as plasma concentrations of S3 and sulindac sulfide, respectively, at various times after mice treatment with 100 mg/kg by oral gavage. Results are presented as mean ± SD of 3 independent experiments. Prof Gary Piazza at University of South Alabama performed these experiments.*

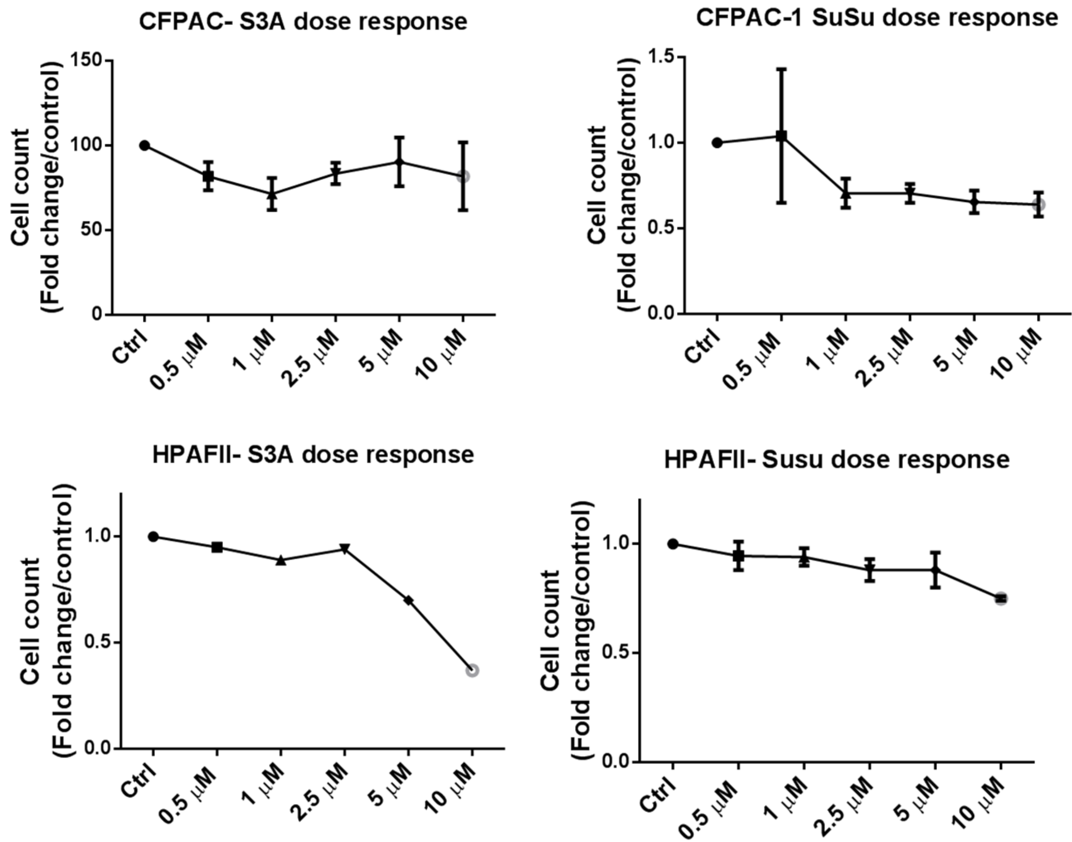
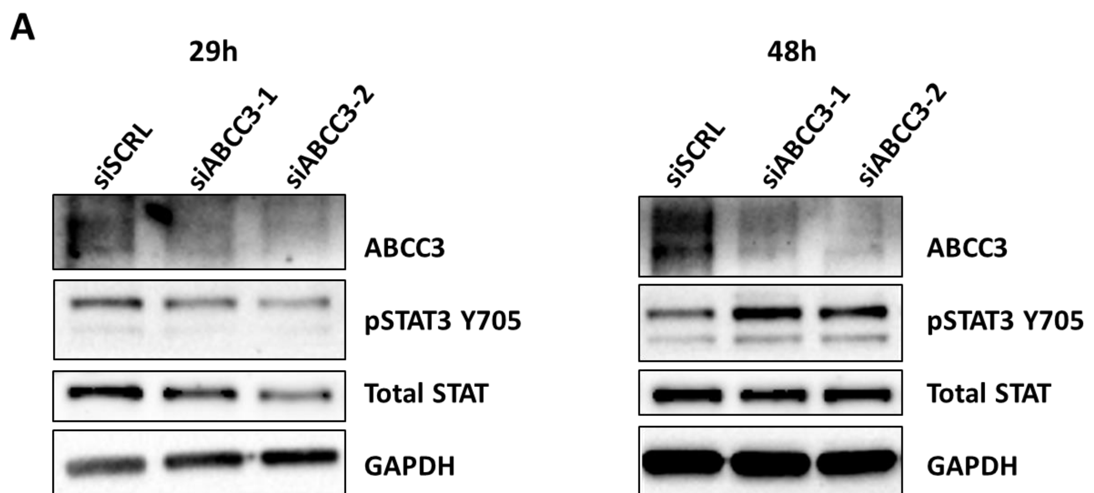


Figure 2 ***Sulindac sulphide derivative, S3, is more potent than other sulindac derivatives.*** *The effects of CFPAC-1 (A) and HPAFII (B) treatment with S3A and SuSu on the cell number measured after 72h. Results are presented as mean ± SEM of 2 (CFPAC-1, HPAFII-SuSu) and 1 (HPAFII-S3A) independent experiments.*



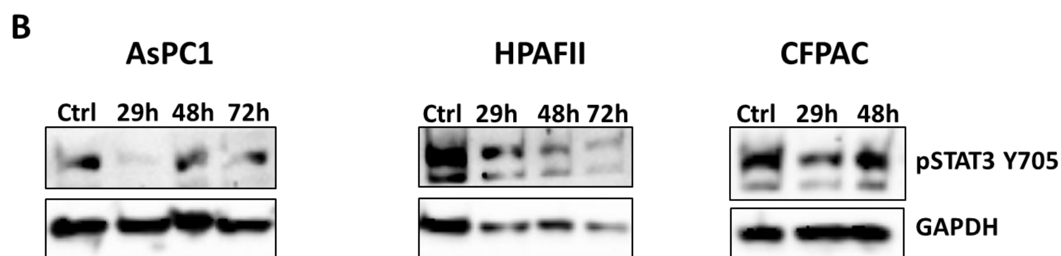


Figure 3 ***ABCC3-mediated regulation of STAT3 is time-dependent.*** *(A) Representative Western blot images showing the effects of ABCC3 knockdown in CFPAC-1 cell line on the expression of activated STAT3 (pSTAT3 Y705) after short (29h) and long (48h) time of transfection; (B) Representative Western blot images showing the time optimization of the ABCC3 knockdown with sequence siABCC3-1 on the expression of activated STAT3 (pSTAT3 Y705) 29, 48 or 72h of transfection.*

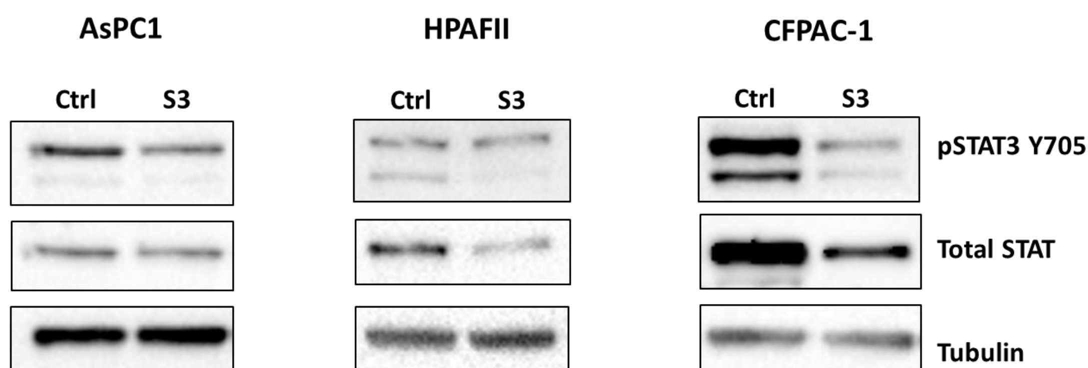


Figure 4 ***ABCC3 inhibition downregulates STAT3 signalling.*** *Representative Western blot images showing the effects of S3 treatment on the expression of total and activated STAT3 in the AsPC1, HPAFII and CFPAC-1 cell lines.*

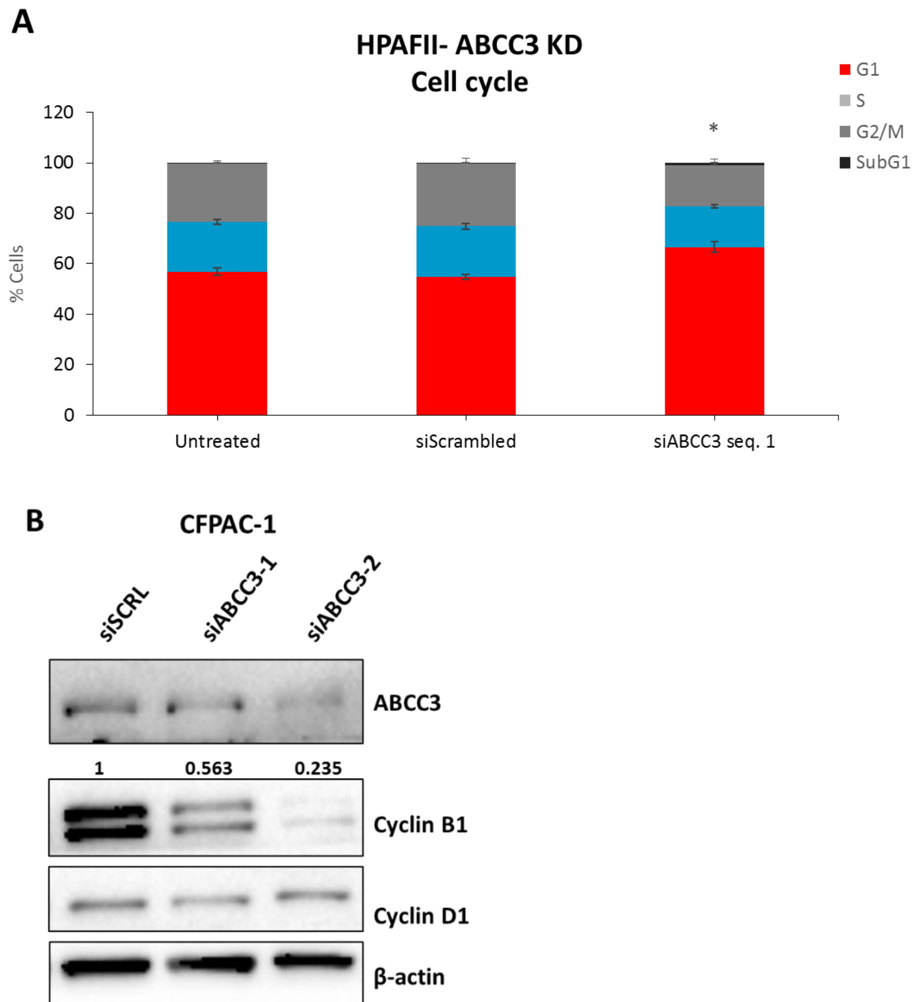


Figure 5 ***ABCC3 regulates cell cycle in PDAC.*** *(A) Cell cycle analysis of the effects of ABCC3 silencing (siABCC3 seq.1) in HPFAII cell line, results are presented as mean ± SEM of 3 independent experiments; Performed by Dr Riccardo Ferro, QMUL; (B) Representative Western blot image showing the effects of ABCC3 knockdown on the expression of Cyclin B1 and Cyclin D1 in CFPAC-1 cell line. The quantitative analysis is presented as mean of 2 independent experiments*

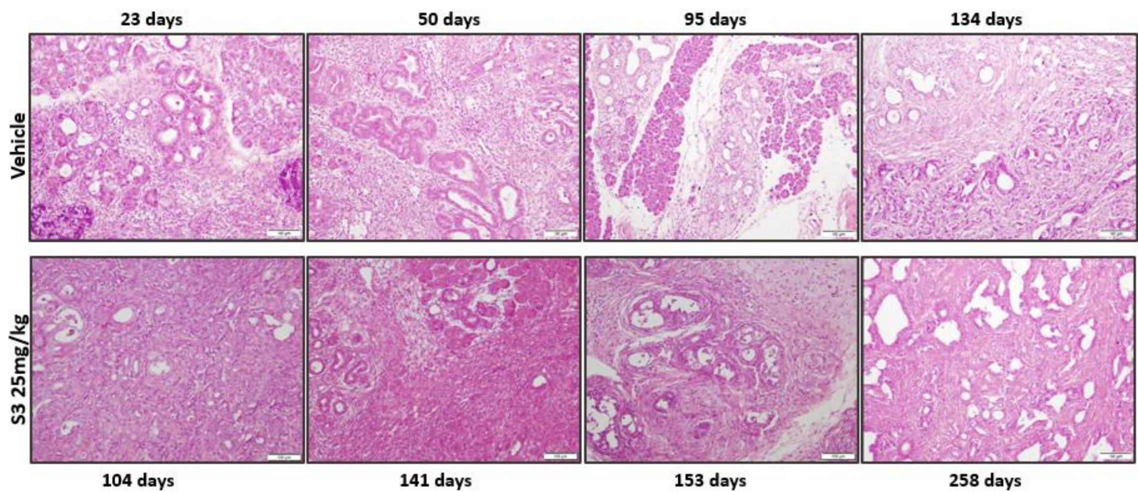


Figure 6 ***KPC mice develop fully invasive tumours.*** *Histopathological analysis of the pancreatic tumours resected at the end point of the experiment (duration of the treatment shown) from the KPC mice treated with vehicle or S. Scale bar: 100µM*

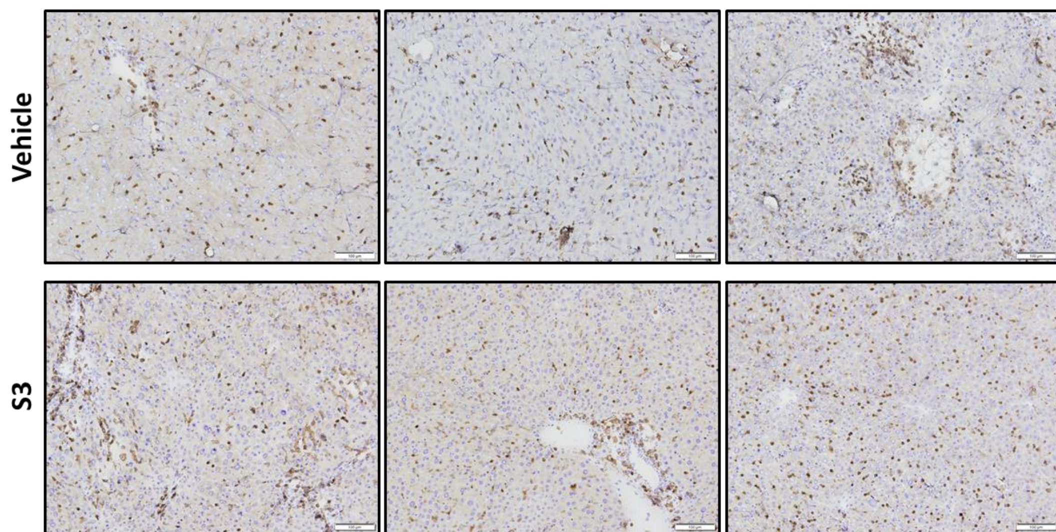


Figure 7 ***ABCC3 inhibition does not affect CD11b expression in KPC mice.*** *Representative IHC images showing the expression of CD11b in the FFPE liver tissues from three vehicle-treated and three S3-terated KPC mice. Scale bar: 100µM*

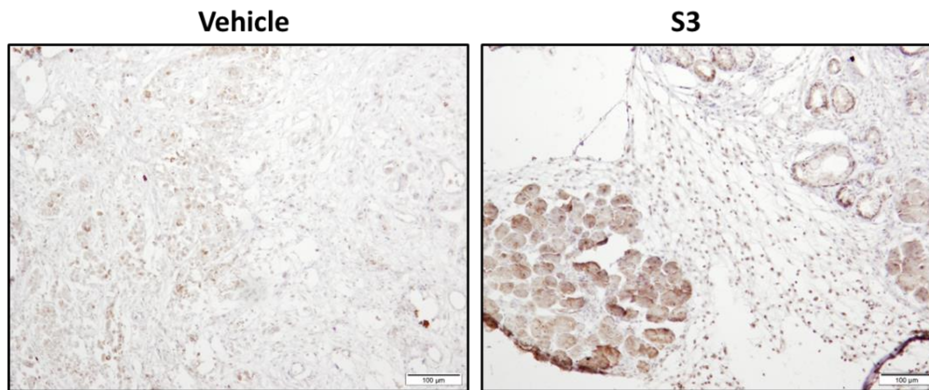


Figure 8 ***ABCC3 inhibition increases apoptosis in PDAC.*** *Representative IHC staining of cleaved caspase 3 in the PDAC tumour resected form the KPC mice treated with vehicle or S3. Scale bar: 100µM*

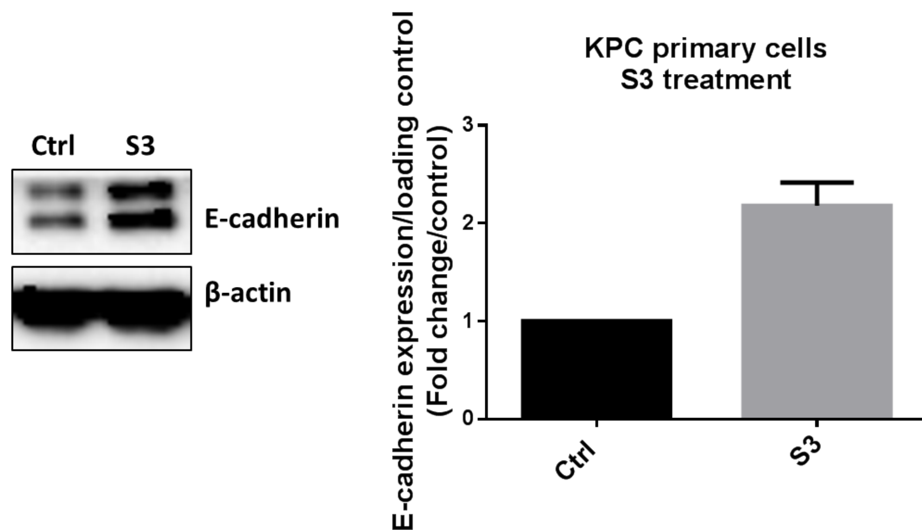


Figure 9 ***S3 treatment influences EMT in PDAC.*** *Representative Western blot image and quantification of the effects of S3 treatment on E-cadherin expression in the KPC primary cell line. Quantitative analysis is presented as mean ± SEM of 2 independent experiments.*

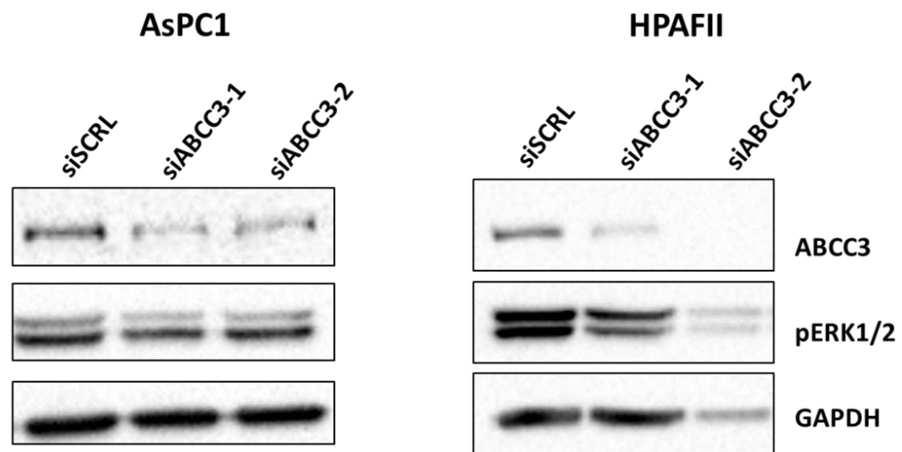


Figure 10 Downregulation of ABCC3 reduces the activity of MAPK pathway. *Representative Western blot images showing the effects of ABCC3 silencing on the activity pERK1/2 in AsPC1 and HPAFII cell lines*

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Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies

Aleksandra Adamska, Alice Domenichini, and Marco Falasca*

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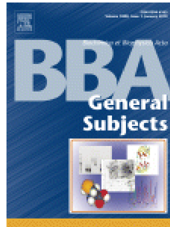

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ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward?

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I, Professor Marco Falasca, as the senior corresponding author and senior investigator, endorse that this level of contribution by the candidate indicated above is appropriate.

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- **Adamska A**, Ferro R, Lattanzio R, Capone E, Domenichini A, Damiani V, Chiorino G, Akkaya BG, Linton KJ, De Laurenzi V, Sala G, Falasca M. ABCC3 is a novel target for the treatment of pancreatic cancer. Adv Biol Regul 2019 Apr 24. pii: S2212-4926(19)30036-3. doi: 10.1016/j.jbior.2019.04.004.

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Title: ABCC3 is a novel target for the treatment of pancreatic cancer

Author: Aleksandra Adamska,Riccardo Ferro,Rossano Lattanzio,Emily Capone,Alice Domenichini,Verena Damiani,Giovanna Chiorino,Begum Gokcen Akkaya,Kenneth J. Linton,Vincenzo De Laurenzi,Gianluca Sala,Marco Falasca

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To Whom It May Concern:

I, Aleksandra Adamska, contributed as a first author, to the conception, design, performance and analysis of experiments, writing and manuscript preparation for publication entitled: **Adamska A**, Ferro R, Lattanzio R, Capone E, Domenichini A, Damiani V, Chiorino G, Akkaya BG, Linton KJ, De Laurenzi V, Sala G, Falasca M. *ABCC3 is a novel target for the treatment of pancreatic cancer.* Adv Biol Regul 2019 Apr 24. pii: S2212-4926(19)30036-3. doi: 10.1016/j.jbior.2019.04.004.

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Signature___________________ Date___________________

- Domenichini A, Edmands JS, **Adamska A**, Begicevic RR, Paternoster S, Falasca M. Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential. Adv Biol Regul. 2019 Feb; 72: 63-77; doi: 10.1016/j.jbior.2019.02.001

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Title: Pancreatic cancer tumorspheres are cancer stem-like cells with increased chemoresistance and reduced metabolic potential

Author: Alice Domenichini,Jeanne S. Edmands,Aleksandra Adamska,Romana-Rea Begicevic,Silvano Paternoster,Marco Falasca

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I, Dr Alice Domenichini, as the first author, endorse that this level of contribution by the candidate indicated above is appropriate.

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I, Aleksandra Adamska, contributed as a joined first author to the conception, design, writing and manuscript preparation for publication entitled: **Adamska A**, Elaskalani O, Emmanouilidi A, Kim M, Abdol Razak NB, Metharom P, Falasca M. *Molecular and cellular mechanisms of chemoresistance in pancreatic cancer.* Adv Biol Regul, 2018 May; 68: 77-87; doi: 10.1016/j.jbior.2017.11.007

I, Omar Elaskalani, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

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I, Professor Marco Falasca, as the senior corresponding author and senior investigator, endorse that this level of contribution by the candidate indicated above is appropriate.

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12. Attached publications

International Journal of
Molecular Sciences

MDPI

Review

Pancreatic Ductal Adenocarcinoma: Current and Evolving Therapies

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Abstract: Pancreatic ductal adenocarcinoma (PDAC), which constitutes 90% of pancreatic cancers, is the fourth leading cause of cancer-related deaths in the world. Due to the broad heterogeneity of genetic mutations and dense stromal environment, PDAC belongs to one of the most chemoresistant cancers. Most of the available treatments are palliative, with the objective of relieving disease-related symptoms and prolonging survival. Currently, available therapeutic options are surgery, radiation, chemotherapy, immunotherapy, and use of targeted drugs. However, thus far, therapies targeting cancer-associated molecular pathways have not given satisfactory results; this is due in part to the rapid upregulation of compensatory alternative pathways as well as dense desmoplastic reaction. In this review, we summarize currently available therapies and clinical trials, directed towards a plethora of pathways and components dysregulated during PDAC carcinogenesis. Emerging trends towards targeted therapies as the most promising approach will also be discussed.

Keywords: PDAC; chemotherapy; gemcitabine; Abraxane; FOLFIRINOX; combination therapies; targeted therapies

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive solid malignancies. Despite quite a low incidence, it remains the fourth leading cause of cancer-related deaths in the modern world, mainly because of dismal diagnosis [1]. In the last decades, significant improvements have been achieved in the screening and therapy of different solid cancers, highly incrementing patients' chance for cure. Nevertheless, despite the advancement in pancreatic cancer research, the mortality to incidence ratio has not experienced significant revision over the last few decades. The five-year survival rate remains just around 5–7% and one-year survival is achieved in less than 20% of cases [2]. This grim prognosis is mainly caused by the lack of visible and distinctive symptoms and reliable biomarkers for early diagnosis as well as aggressive metastatic spread leading to poor response to treatments [3]. In fact, around 50% of diagnosed patients present with metastatic disease. Furthermore, tumour heterogeneity and plasticity cause PDAC to develop chemoresistance. Progression of the disease through consecutive stages is accompanied by accumulating morphological and genetic alterations. Consequently, aberrations in signalling pathways are observed in PDAC progression. Over-activation of many signalling pathways involved in growth and proliferation, as well as altered expression of tumour suppressor genes are regularly detected in PDAC, influencing cell proliferation, survival and invasion. The broad repertoire of genetic and metabolic remodelling allows PDAC to survive under harsh conditions and increases proliferative ability. Furthermore, recent analysis of gene expression and activity allowed for classification of observed mutations into four distinct phenotypic subtypes defined as squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX) [4]. Each of the subtypes is characterized by different mutational landscape,

tumour histopathological features and correlates with different prognosis. Classification of diagnosed patients into one of these four subtypes may provide substantial prognostic value and be of great therapeutic relevance, allowing for more personalized treatments. Additionally, a dense, diffuse stroma called desmoplasia, is formed around the tumour, contributing to its resistance and influencing tumour progression and invasion [5–7]. All described events make pancreatic cancer resistant to currently applied therapies, demanding for novel, broader approaches to improve PDAC patients' perspectives. Conventional cytotoxic treatments, such as chemotherapy and radiotherapy, have been rather unsuccessful in improving patients' chances for survival, offering marginal benefits. Single agent gemcitabine, as well as its combinations, failed to provide expected results, prolonging life expectancy only moderately. Similarly, disappointing effects were achieved with multidrug regimens (e.g., folinic acid-fluorouracil-irinotecan-oxaliplatin also known as FOLFIRINOX) and targeted therapies. Therefore, there is a pivotal need for development of novel, effective strategies aiming to advance current therapeutic possibilities. Improvement in the field of targeted, more personalized therapies is of high importance. Multiple preclinical and clinical studies are being developed in order to address these points; however, because most of them are in early phases, it is still too soon to draw any conclusion. In this review, we provide a broad description of the development of PDAC therapy, and introduce currently available therapies and strategies that are presently being undertaken to improve PDAC patients' perspectives.

2. Disease Staging-Essential Factor in Pancreatic Ductal Adenocarcinoma (PDAC) Therapy

Treatment options for pancreatic ductal adenocarcinoma are rather limited and highly depend on the disease's stage. Therefore, proper diagnosis and accurate staging allow for better prognosis and highly influence treatment choice and patients' chance of survival. Multi-detector computed tomography (CT) scan accompanied by three-dimensional (3D) reconstruction is currently the first choice imaging option for preoperative staging of PDAC [8,9]. However, due to poor sensitivity in distinguishing between peritoneal and small hepatic metastasis, CT scan is not suitable to accurately predict resectability [10]. Endoscopic ultrasound, magnetic resonance imaging and laparoscopy are also used to properly classify diagnosed tumours and, with the latter, exclude intraperitoneal metastases [11,12]. Other than imaging techniques, CA19-9 levels evaluation, despite its limitations, is recommended to correctly stage PDAC, once diagnosed, as well as to assess the response to therapy [13]. From a surgical point of view, PDAC is classified based on the tumour node metastasis (TNM) system, in which primary tumour size (TX, T0–T4), regional lymph nodes (NX and N0–N1) and distant metastasis (M0–M1) are assessed [14,15]. Based on the combination of assessed TNM values, diagnosed tumours are staged due to anatomic state and divided into different prognostic groups (0–II resectable; III locally advanced, unresectable; IV metastatic unresectable). For clinical management, PDAC is divided into 4 main categories depending on the tumour extension: resectable, borderline resectable, which exhibit venous involvement of superior mesenteric vein/portal-vein (SMV/PV) and gastroduodenal artery encasement, locally advanced and metastatic. Currently, surgical resection of the pancreas with microscopically free margins remains the only realistic and potentially curative option for pancreatic cancer patients, however it is restricted to earlier disease stages. Unfortunately, at the time of diagnosis, less than 20% of patients have a resectable tumour [16]. The remaining patients frequently present tumours and metastases, which are already too widespread to be surgically removed. At this stage of the disease retroperitoneal and perineural infiltration, haematogenic dissemination and angioinvasion are observed. In particular, cancers of the body and tail of the pancreas are often detected at the late stage and they usually present major vessels involvement, such as hepatic artery or celiac axis [17]. Therefore, even despite the lack of metastasis, they are usually classified as unresectable.

3. Therapy for Metastatic Cancer

Once metastasized, pancreatic cancer prognosis is poor. Chemotherapy treatment remains the main option for patients with advanced and metastatic tumours. Radiation, in combination with chemotherapy, is another option for unresectable, metastatic cancer [18]. Nonetheless, the effects achieved by both approaches are mainly a mildly increased survival rate and lowered cancer-related symptoms. Moreover, due to elevated toxicity, combination chemotherapy, which is associated with slightly better outcomes, is limited only to patients with a good performance status (PS). Therefore, depending on the PS, PDAC patients may be subjected to combination or single-agent treatment. Multidrug regimens would potentially increase the patient anti-tumour response. However, they are associated with higher toxicity and greater incidence of adverse effects [19]. Nevertheless, in all therapeutic regimens, some general side effects are expected, including complications associated with a reduction in blood cell counts, vomiting and nausea, diarrhoea, constipation, mouth ulcers, poor appetite, hair loss, nervous system changes, and infertility. It has been considered that some of these adverse effects, especially blood clotting and weight loss, may be one of the reasons for the ineffectiveness of current therapies, forcing their early termination. Therefore, learning how to manage these adverse symptoms could significantly improve patients' outcomes. Current clinical trials and available therapies are listed in Tables 1 and 2.

3.1. Gemcitabine

In the earliest decades of pancreatic cancer treatment, despite considerable toxicity, 5-fluorouracil (5-FU), its analogues, as well as their combinations have been used with moderate efficacy in improving patients' life [20,21]. Since 1997, gemcitabine has been accepted as a reference first-line therapy drug for patients with a good performance status [22]. Its advantage over 5-FU has been reported in different individual studies. In a comparative phase III study (n = 126) of single agent gemcitabine and 5-FU, a clinical benefit response was experienced by 23.8% of gemcitabine-treated patients compared to 4.8% of 5-FU-treated patients [22]. The median survival time was 5.6 and 4.4 months for gemcitabine and 5-FU-treated patients respectively, and the one-year survival rate was 18% for gemcitabine patients and 2% for 5-FU patients. All the results were statistically significant. Gemcitabine was also shown to substantially improve patients' disease-related symptoms. Other phase II/III trials also reported a positive or partial positive response to gemcitabine, in the range of 5.4% to 12% [23,24] and median overall survival time ranging from 5 to 7.2 months [25]. One-year survival of 18% and median survival time of 6.2 months were reported in the successive study [26]. Importantly, besides grade 3 and 4 myelosuppression that was observed in around 30% of patients [26], lower systemic toxicity was attributed to gemcitabine treatment. Recently, CO-101, a lipid-drug conjugate of gemcitabine has been developed. The drug was designed to enter cancer cells independently of the human equilibrative nucleoside transporter 1 (hENT1) and therefore to overcome cancer resistance to gemcitabine; however, no significant difference in the efficiency of CO-101 and gemcitabine has been observed [27]. A modified version of gemcitabine (Acelarin) is currently under investigation in a phase III trial, with the aim to delay cancer cells' resistance [28]. The addition of a phosphoramidate motif to gemcitabine was expected to diminish resistance acquired by PDAC cells after gemcitabine treatment. The data obtained so far showed that this modification increases the intracellular concentration of gemcitabine, mainly by ensuring its activity independently of nucleotide transporters.

3.2. Combination Therapies: Gemcitabine-Based Therapies

Following the positive results obtained with gemcitabine treatments, studies on more intensive and effective combination therapies composed of gemcitabine and different cytotoxic and biological agents have been developed. As previously mentioned, despite an acceptable toxicity profile and increased response rates, significant improvement in overall survival (OS) over single-agent gemcitabine was rarely observed [29–33]. However, when groups of patients were restricted to good

performance status only, a survival benefit of combination treatment could be noticed [34–36]. In 2005, a combination of cisplatin, epirubicin, fluorouracil, and gemcitabine (PEFG) was tested for treatment of advanced PDAC patients [37]. A clear benefit in all efficacy parameters, together with moderately increased incidence of haematological adverse events, was observed. However, the small sample size diminished the value of these studies. In another study, 5-FU and fluoropyrimidine combination (S-1) showed a clinical benefit of the same efficiency as gemcitabine in metastatic patients [38]. Moreover, a combination of S-1 with gemcitabine showed improvement in most of the efficacy parameters and, despite the increased incidence of haematological toxicities such as neutropenia or thrombocytopenia, S-1/gemcitabine combination has become another viable option for a first line PDAC therapy, according to the results obtained from various randomised controlled trials in Asia [39]. It has been previously demonstrated that epidermal growth factor receptor (EGFR) is one of the molecules overexpressed in pancreatic cancer, playing an important role in carcinogenesis [40,41]. Moreover, its expression has been correlated with poor prognosis, metastasis, and sensitivity to chemo- and radiotherapy. Therefore, targeting this family of receptors presents a promising perspective for novel PDAC therapies and has been explored in a plethora of clinical trials. A Phase III trial examining the combination of gemcitabine and erlotinib (EGFR inhibitor) for the treatment of advanced and metastatic cancers showed moderate, but statistically significant improvement in both median survival rates (23% vs. 17%) and overall survival (6.2 vs. 5.9 months) [42]. Based on these results, gemcitabine/erlotinib combination received Food and Drug Administration (FDA) approval and became a preferred option for treatment of advanced, unresectable pancreatic tumours. Surprisingly, no correlation between EGFR expression and treatment efficiency has been noted ($p = 0.4784$) [43]. On the other hand, rash incidence, one of the adverse effects experienced by treated patients, seemed to correlate with patients' positive response. Another gemcitabine-based combination, involving capecitabine, elicited significant prolongation of survival and became, together with erlotinib, one of the systemic treatment alternatives. Nevertheless, only patients with good PS responded positively to this treatment [34,44]. The effectiveness of capecitabine/gemcitabine combination applied as an adjuvant treatment was recently demonstrated in the European Study Group for Pancreatic Cancer (ESPAC)-4 trial. Combination of capecitabine with oxaliplatin (Cape-Ox) [45] as well as gemcitabine, docetaxel, and capecitabine (GTX) [46] are also used and restricted to good PS patients. Moreover, capecitabine's superiority over gemcitabine as a radiosensitiser has been proposed in the selective chemoradiation in advanced localised pancreatic cancer (SCALOP) trial [47]. Other studies investigating combination therapy with gemcitabine showed very moderate or no significant improvement. Therapy using gemcitabine and platinum analogues (cisplatin or oxaliplatin) did not give clear results [30,32]. In some trials, the addition of cisplatin to gemcitabine had no effect on pancreatic cancer patients, whereas other studies showed an increase in median OS time (7.5 vs. 6 months) [32,33,48]. Table 1 lists former and current gemcitabine-based and combination therapies.

3.3. Abraxane and FOLFIRINOX: New Hope or Defeat?

Taxanes, such as docetaxel or paclitaxel, have been also considered for PDAC therapy. However, due to their poor solubility and consequently unsatisfactory delivery, their effectiveness was highly reduced. Nevertheless, a significant response to a combination of gemcitabine and albumin-bound paclitaxel (nab-paclitaxel, Abraxane) was observed in patients with advanced pancreatic cancer [49,50]. A synergistic effect of the drug combination was attributed to the improvement in the intratumoral delivery of both gemcitabine and paclitaxel, facilitated by fused albumin [51]. The effects of this combination treatment, in a phase III trial (n = 861), significantly surpassed the single-agent gemcitabine therapy in all tested parameters. The median OS time of 8.5 and 6.7 months was noted in Abraxane-gemcitabine and gemcitabine groups, respectively. A similar advantage was observed for progression-free survival (5.5 vs. 3.7 months) and one-year survival (35% vs. 22%). Unfortunately, the positive response to this therapy was accompanied by a considerable increase in occurrence of adverse events, including grade 3 or 4 neutropenia, leukopenia, neuropathy, febrile neutropenia,

or fatigue [52]. Nevertheless, the increase in patients survival rates, at all time points, was a base for FDA approval and establishment of Abraxane-gemcitabine as the first-line therapy option for patients with advanced and metastatic pancreatic cancer. Its applicability for treatment of stage IV metastatic PDAC was also recently demonstrated in a case study, with increased quality of life and clinical response in a patient with a poor PS [53]. Interestingly, modification of the Abraxane administration regimen was proposed to improve its toxicity profile [54]. Recently, based on the proven advantageous and synergistic activity of its particular components [55–58], a multidrug combination (irinotecan, oxaliplatin, fluorouracil, and leucovorin) called FOLFIRINOX has been shown to be an effective first line therapy, especially for patients with metastatic pancreatic cancer. The anti-tumour effect in patients with advanced cancer was shown in a phase I trial [59] and confirmed in a phase II–III study, which explored patients' response to FOLFIRINOX and single-agent gemcitabine [60]. The superiority of FOLFIRINOX over gemcitabine was recognised in all efficacy parameters, including OS (11.1 vs. 6.8 months), progression-free survival (PFS) (6.4 vs. 3.3 months), and one-year survival rate (48.4% vs. 20.6%), which presented statistically significant improvement. Unfortunately, the safety profile of FOLFIRINOX treatments was not favourable. The study showed increased incidence of grade 3 or 4 thrombocytopenia, neutropenia, febrile neutropenia, and diarrhoea, or grade 2 alopecia [60]. On the contrary, a significant reduction in the deterioration of quality of life was observed in patients treated with FOLFIRINOX compared to gemcitabine [61]. The positive response to FOLFIRINOX was also noted by a separate study conducted in India [62]. Despite its considerable toxicity, FOLFIRINOX is considered as a first-line option for patients with advanced and metastatic pancreatic cancer. However, its use is constrained to patients under the age of 75 and with good PS. To improve patients' tolerance to the drug, modifications of FOLFIRINOX (e.g., mFOLFOX-folinic acid, fluorouracil, oxaliplatin- or FOLFIRI-folinic acid, fluorouracil, irinotecan) are currently being assessed [63,64].

Table 1. Gemcitabine-based combination therapies.

Treatment	Phase	*n*	OS (Months)/ Response Rate (%)	Outcome	*p*	Reference
Gem vs. 5-FU	R FL III	126	5.65 vs. 4.4	FDA approved	0.0025	[22]
Gem-5FU vs. gem	FL III	322	6.7 vs. 5.6	No statistically significant improvement in OS	0.09	[65]
FOLFIRINOX	R II/III	342	11.1 vs. 6.8	FDA approved	<0.001	[60]
Abraxane	R III	861	8.5 vs. 6.7	FDA approved	<0.001	[49]
Erlotinib + gem/gem	R III	569	6.2 vs. 5.9	FDA approved	0.038	[42]
Gem + cisplatin/gem	R III	195	7.5 vs. 6.0	Improved survival, but not statistically significant	0.15	[33]
	R III	400	7.2 vs. 8.3	Failed to demonstrate improvement	0.38	[32]
PEFG vs. gem	III	99	38.5% vs. 8.5%	Little sample size	0.0008	[37]
Gem + oxaliplatin	III	313	9.0 vs. 7.1	Significant improvement in response rate and PFS, but not statistically significant OS	0.13	[30]
Gem + capecitabine vs. gem	III	319	8.4 vs. 7.2	Not statistically significant improvement in OS	0.234	[44]
	III	533	7.1 vs. 6.2	Alternative treatment for patients with good PS	0.08	[34]
S-1 + gem/gem	III	834	9.7 vs. 8.8	Not inferior to gemcitabine. Approved in Japan as alternative	<0.001	[39]
Gem + irinotecan	III	360	6.3 vs. 6.6	Good tumour response but no improvement in OS	0.789	[29]

FDA, Food and Drug Administration; R, randomized; PS, performance status; OS, overall survival; PFS, progression-free survival; gem, gemcitabine; PEFG, cisplatin, epirubicin, fluorouracil, and gemcitabine combination.

Despite elevated adverse effects, the introduction of FOLFIRINOX and Abraxane to PDAC therapeutic repertoire brought new hope for patients and investigators. Considering that patients' PS is one of the most important predictive factors, learning how to manage the toxicity of these multidrug regimens may further improve their feasibility. In addition, the failure of most of the gemcitabine-based combination treatments and the establishment of Abraxane as a new drug of reference in PDAC therapy makes it tempting to assume that the design of new clinical studies investigating Abraxane and FOLFIRINOX-based combination therapies might be a breakthrough in the improvement of the present grim perspective for PDAC patients.

4. Surgery—The Cornerstone of PDAC Therapy

Considering the lack of definite survival benefit presented by conventional chemotherapy, complete resection followed by adjuvant treatment remains the only realistic curative option for PDAC patients. In general, the operability status is dictated mainly by the extent of venous involvement. However, the choice of surgery and its extent is imposed not only by the tumour localization and extension, but also by the surgeon's expertise and by the patient's performance status (PS), which is one of the major prognostic factors. For patients that are eligible for resection (resectable, borderline resectable), available surgical options are: pancreaticoduodenectomy (head/body of the pancreas and nearby organs are removed), distal pancreatectomy (tail, body and spleen), total pancreatectomy (whole pancreas and nearby organs) or palliative surgery (stent or bypass), which may alleviate symptoms of biliary and gastric outlet obstruction [66]. Pancreaticoduodenectomy, introduced by Whipple and Kausch at the beginning of 20th century, is a three-step procedure of exploration, resection and reconstruction. It is currently a safe procedure and results in low mortality and morbidity [67]. Significantly worse postoperative recovery and outcome has been demonstrated after total pancreatectomy, which is reserved for few indications, mainly because of metabolic imbalance [67]. The extent of the resection has been widely discussed over the last years; however, none of the procedures showed significant superiority over the standard pancreaticoduodenectomy. One of the most important factors for prognosis of postoperative survival and surgery success is R0 resection, in which histologically free margins are detected [68]. In R1 and R2 resections, microscopic and macroscopic tumours are still visible at the margins and correlate with reduced survival [16]. In borderline resectable and locally advanced tumours, vascular resection and reconstruction of superior mesenteric vein/portal-vein (SMV/PV) should be considered. It has been confirmed in a series of studies that SMV/PV resection and, in some cases, arterial resection should be performed in order to achieve R0 resection without reducing patient's survival compared to standard PD and so achieving similar outcomes for all resectable patients [69,70]. For patients with tail and body cancers with venous encasement, extended distal pancreatectomy with resection of celiac artery has been proposed, however the small number of studies conducted on this procedure limits the determination of its survival benefits [71–73]. In some cases, splenectomy must be performed as well; however, there is still controversy over splenic preservation and its impact on patient's overall outcome [67]. Tumour size is one of the most important independent prognostic factors [74]. It has been demonstrated that larger tumours can be associated with higher venous involvement and thus with high probability of microscopically positive resections (R1) [70]. Higher blood loss during surgery, which is another prognostic factor, has been also reported during resection of larger tumours [75]. Unfortunately, only in 2% of diagnosed patients, tumours smaller than 2 cm in diameter, which is the statistical cut-off, are detected. Another survival factor is the ratio between examined and negative lymph nodes, described as lymph node ratio (LNR), which may give more insight into the extent of the metastatic disease [15]. There are some discrepancies on whether extended lymphadenectomy has any benefit in terms of survival over standard lymphadenectomy [76]. Nevertheless, it has been shown that it considerably increases both R0 resection rate and survival, which highly depends on the number of resected and negative lymph nodes [77,78]. However, the jury is still out on the minimal number of lymph nodes that should be resected and examined to properly assess the prognosis. Despite the low percentage of

patients undergoing surgery, the chance of survival for surgical patients has significantly increased in the last few decades. Regardless of considerably high postoperative complications, the mortality rates do not exceed 5% [79]. The effectiveness of surgery and patients' long-term survival depends partially on lymph-node infiltration but also on the surgeon's expertise and the number of operations performed by the hospital. Unfortunately however, even after successful resection the median survival time is 20 months, with 25% five-year survival rate [74]. The majority of resected patients suffers from tumour recurrence (~40%) within 6–24 months post-surgery [80], highlighting the necessity for preoperative/postoperative therapies in order to achieve more effective treatments. Therapeutic regimen options for PDAC patients are presented in Figure 1.

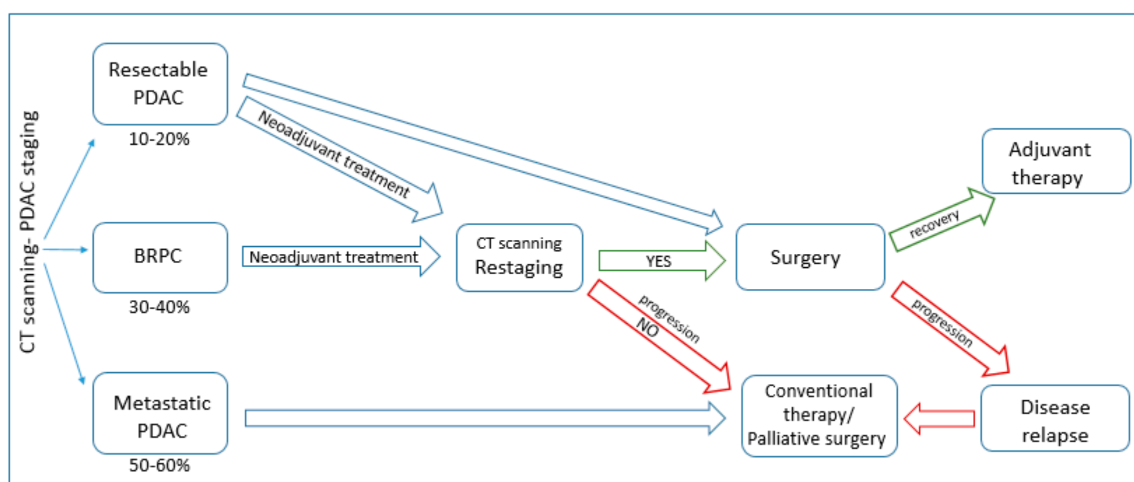

Figure 1. Schematic model of therapeutic strategies for diagnosed pancreatic ductal adenocarcinoma green—successful procedure; red—failed procedure.

5. Neoadjuvant and Adjuvant Therapies

Surgery followed by adjuvant therapy has been shown to provide slight, but significant survival benefit for non-metastatic patients in several phase III studies. Thus far, gemcitabine and 5-FU-based postoperative chemoradiation has been considered as standard of care, improving the median OS time for 2–5 months [81,82]. However, adjuvant therapy remains a controversial field, with results obtained in clinical trials ranging from definite survival benefit [83] to negative impact on patients' OS [82]. In addition, almost 60% of resected patients present early tumour progression or prolonged recovery, disabling planned postoperative treatment. Therefore, if no distant metastasis has been detected during cancer diagnosis and staging, the recommended first line treatment is neoadjuvant chemotherapy. This therapy aims to enhance drug delivery and tumour oxygenation and minimise tumour burden, which may result in downstaging and more definite surgical resection [84] and reduce the risk of tumour implantation during pancreatectomy [85]. Preoperative treatment might also avoid the delay between the diagnosis and the start of postoperative treatment, usually caused by patient's prolonged recovery, and enable treatment of early micrometastases [86]. In addition, higher number of patients may benefit from this treatment compared to patients profiting from adjuvant therapies, which sometimes need to be postponed or cancelled due to postoperative complications, prolonged recovery or early recurrence [87,88]. Cancer chemo-sensitivity might be also determined. However, neoadjuvant treatment also raises several concerns, such as disease progression during preoperative treatment or possible increase in surgical complications. Nevertheless, no difference in morbidity or mortality has been observed so far between patients subjected or not to preoperative treatment [89]. On the other hand, neoadjuvant therapy may avoid unnecessary surgery in patients, whose disease progressed during the treatment, therefore selecting a subpopulation of patients that might benefit from further therapy [90,91]. However, this aspect remains debatable. Different approaches for preoperative

therapy, such as chemoradiation, chemotherapy followed by radiotherapy or chemotherapy alone have been considered, even though none of the strategies showed considerable superiority. The optimal therapy, its duration and the most adequate time for its initiation still need to be determined. Thus far, there has also been no consensus on the advantage of preoperative over postoperative treatment, making the use of this therapy controversial.

5.1. Neoadjuvant Therapy in Resectable Patients

Application of neoadjuvant therapy for localized resectable tumours remains controversial since it delays the surgery and may result in disease progression. On the contrary, preoperative therapy of resectable patients reduces the risk of intraoperative tumour spillage, sterilizes lymph nodes and may improve patients' response to postoperative treatment. It has been shown in different independent studies that, in patients who showed no progression after applied therapy, higher rate of R0 resections, lower recurrence and better survival are observed [92–95]. As for the choice of therapeutic regimen, different strategies have been studied, most of them being gemcitabine-based. Twenty-three-month survival and 66% R0 resection rate have been shown after gemcitabine-based radiotherapy, demonstrating feasibility of neoadjuvant therapy in resectable tumours [96]; however, the single arm design of this study impairs its proper evaluation. Gemcitabine combined with cisplatin presented significant increase in the resection rate (70%) compared to gemcitabine alone (38%) when applied in preoperative settings [97]. Phase II trial of gemcitabine and cisplatin is currently ongoing. Another study on the same combination proved 71% R0 resection rates and 26.5 months survival for resected patients [98]. Gemcitabine applied with oxaliplatin and radiation therapy resulted in overall survival (OS) of 18 months after successful surgery [99]. Considering modest improvement with gemcitabine/cisplatin combination in resectable tumours, the same modality followed by gemcitabine-based chemoradiation has been tasted in phase II trial. However, 66% resectability and OS of 17.4 months did not give ground to continue the study [100]. Neoadjuvant vs. post-operative radiation for resectable patients has been evaluated in a large retrospective study, showing slight benefit for the preoperative group (23 vs. 17 months) and definite advantage over untreated patients (12 months) [101]. However, the results of meta-analysis in several studies contradicted these results, showing no benefit in any parameter [102]. Interestingly, radiochemotherapy was claimed to be superior to monotherapy treatment by Gillen and colleagues [103]; however, only marginal benefit of preoperative gemcitabine with or without radiation has been reported in another study. 5-FU based chemoradiation has been widely applied as neoadjuvant treatment for resectable tumours [104]; anyhow, its efficacy is far from being optimal. It also showed considerable toxicity, with 32% of patients requiring hospitalization in one of the conducted studies [92]. Modification of applied treatment schedule (30 Gy instead of 50.4) helped to reduce the toxicity, however obtained results were not promising [105]. Similarly, detrimental effect (61% resection rate) has been reported in retrospective meta-analysis of 5-FU and cisplatin combination. Paclitaxel and radiotherapy have been also evaluated; however, the results (34% R0 resection) did not show any advantage over gemcitabine or 5-FU-based treatments [106]. Taken together the results show that no consensus can be reached on the applicability of neoadjuvant treatment for resectable tumours and no difference between preoperative and postoperative therapy has been reported in terms of survival benefit. In addition, limited number of cases and mostly retrospective studies restrain proper analysis of conducted studies. However, available data and theoretical reasoning justifies its use.

5.2. Neoadjuvant Therapy in Borderline Resectable and Locally Advanced PDAC

There is considerable inconsistence in the definition of borderline resectable pancreatic cancer (BRPC), hampering proper design of studies. BRPC is a "marginally" resectable lesion with no distant metastasis, however showing perivascular fat plane absent over 180 degrees of SMN and PV [107,108]. Callary and colleagues summarized this criteria and additionally stated that no CT evidence of vascular encasement should be seen in resectable tumours [109]. Recently, this concept was extended

by the MD Anderson Cancer Center (MDACC), including tumour biology, anatomic relationships and patient's condition to specifically classify diagnosed disease [8]. Therefore, borderline resectable tumours have been divided into three categories: (A) anatomically defined BRPC; (B) possibility, but not diagnostic staging for micrometastatic disease; and (C) marginal PS, but susceptible for surgery. Definition of BRPC has developed over the years; however, its determination in clinical practice is still challenging. There are some difficulties to demarcate BRPC patients and those with locally advanced tumours, which highly influence the response and therefore, provide inconsistent data. Nevertheless, posing the risk of incomplete, margin-positive resection, BRPC patients seem ideal candidates for neoadjuvant therapy in order to complete successful margin-negative resection [8,89]. However, inconsistencies in proper tumour classification, as well as difficulties in determining downstaging effects before resection, caused by dense stroma and the presence of pancreatitis, influence the clinical interpretation of results and therefore have an impact on the proper assessment of its efficacy. As an example, it has been reported by Hoffman and colleagues that a response rate of less than 10% has been detected by CT scan after preoperative treatment, whereas tumour response of 71% has been stated after surgery [110]. Similarly, White and colleagues suggested that dependence on CT imaging for the determination of tumour resectability may deprive around 12% of patients of the chance for R0 resection [111]. Moreover, in another study, despite only 0.8% of patients presenting a radiologically restaged disease after the preoperative treatment, 80% of the studied population underwent surgery with 95% of R0 resections, proving the inapplicability of radiographic imaging for the evaluation of post-treatment outcomes [112]. The inaccurate assessment of treatment efficacy may result from fibrotic scar changes caused by neoadjuvant chemoradiotherapy (neoCRT), which may cause false negative results. Aforementioned results demonstrated that CT scanning is of little clinical value in assessment of tumour response to therapy and proposed that, provided good PS and lack of metastasis, tumour resection should be performed regardless of radiographic evaluation of preoperative treatment efficacy. Margin status should be considered as a more appropriate indicator of the response to neoadjuvant treatment.

Thus far, there have been very few prospective studies assessing the advantage of neoadjuvant therapy in borderline resectable PDAC. In the first one, carried out in 2001 on 15 patients, 5-FU and radiation were applied in order to downstage the tumour and allow surgery. Sixty per cent of patients were able to have a complete resection with negative margins, leading to the conclusion that chemoradiation may successfully improve resectability in "marginally resectable" patients [113]. Another, multi-centre prospective study compared different neoadjuvant modalities in 21 borderline patients. Ten of them received gemcitabine-based chemoradiation and the remaining 11 were subjected to induction chemotherapy (gemcitabine/cisplatin/5-FU) followed by 5-FU based radiation. Regrettably, the study had to be terminated due to lack of significant improvement; however, it showed that both regimens are well tolerated and have similar effectiveness [114]. Unfortunately, the majority of studies are of retrospective nature, most of them showing good tolerability and modest efficacy in resectability improvement. Different strategies for neoadjuvant therapies of BRPC have been applied. Gemcitabine/capecitabine, gemcitabine/oxaliplatin [115] and 5-FU based treatments have been tested and showed modest improvement in resectability; e.g., 40% of gemcitabine/capecitabine-treated patients was able to receive surgery, with 82.3% R0 resections [116]. Gemcitabine and S-1 combination has been also evaluated for both: resectable and BRPC tumours, achieving 74% R0 resection rate. A two-year survival for almost 46% of patients and median OS time of 35 months after completion of surgery appear to be encouraging [117]. Gemcitabine and docetaxel combination (NeoGemTax) applied in neoadjuvant settings allowed for R0 resection in 87% of patients, initially stated as borderline resectable or unresectable [115]. Radiotherapy (RT) has provided slightly better results in terms of surgery rates (74% vs. 61%) and decreased recurrence [87], however no superiority over chemotherapy has been observed in terms of survival. Concurrent chemoradiation has brought considerably encouraging results. Several studies have tested full dose chemotherapy (e.g., gemcitabine, S-1 or capecitabine) combined with full dose radiotherapy [118,119]. A Japanese retrospective study on

gemcitabine-based radiation therapy demonstrated 92% of R0 resections that could be completed for patients receiving treatment, compared to 52% of untreated patients. Consequently, higher long term survival has been reported (59.4% two-year survival) [120]. Evans and colleagues proved the superiority of gemcitabine over 5-FU-based RT and a median OS time of 34 months for patients subjected to successful surgery [96]. Gemcitabine-based therapies, combined with radiation, were explored in multiple studies showing promising results. Taken together, data showed that the resectability rate ranged between 24–100%; however, the vast majority of those patients (67–100%) were able to receive the R0 resections, demonstrating the efficacy of neoadjuvant therapy [121]. Chemotherapy (e.g., gemcitabine, 5-FU or gemcitabine/cisplatin) followed by radiotherapy has been also considered, with increased possibility to perform pancreatoduodenectomy in patients subjected to preoperative treatments [121]. In a more recent study review, 57 borderline resectable patents were treated with Gemzar, Taxotere and Xeloda, followed by radiation. The results of this study showed that 56% of patients were able to receive surgery, with almost 97% of them having R0 resection [122]. In general, after the analysis of available data, it can be observed that around 30% of borderline resectable patients subjected to neoadjuvant treatment may undergo surgery and, in these cases, mostly complete R0 resections are performed. This shows promising perspectives; however, the retrospective character of most of the studies and the low number of patients highly hinder drawing proper conclusions.

Although less promising and challenging, preoperative treatment followed by surgery has also been considered for locally advanced pancreatic cancer (LAPC). It has been demonstrated in several studies that preoperative treatment applied to LAPC patients decreased lymph node involvement, which may be considered as a positive predictor of survival benefits [118]. Improvement in OS for LAPC patients has been demonstrated in few studies [123], however most of the reports showed contradictory results. One of the meta-analysis of patients with advanced PDAC tumours demonstrated that an average 33.2% of analysed patients underwent surgery, with 79.2% of R0 resections after completion of preoperative treatment. Results were comparable with these of initially resectable patients, whose R0 resection rates were 82.1% [103], demonstrating the feasibility of this approach and underlining the potential of neoadjuvant treatment to increase resectability, even for locally advanced tumours. Gemcitabine-based combinations, such as gemcitabine and oxaliplatin (NeoGemOx) [115], gemcitabine and capecitabine [47,116], PEFG/PEXG (cisplatin, epirubicin, 5-FU/capecitabine and gemcitabine) or PDXG (docetaxel replacing epirubicin) [124] showed promising results in converting tumours to resectability and increasing the number of patients subjected to surgery, demonstrating the feasibility of subjection of LAPC to neoadjuvant therapy. FOLFIRINOX-based regimens recently emerged as new restaging strategy, significantly increasing resection rates [123,125,126]; however, more prospective studies are necessary to fully evaluate their efficacy. Additionally, a case study of a patient with locally advanced PDAC demonstrated that the nab-paclitaxel/gemcitabine combination followed by FOLFIRINOX resulted in tumour remission and completion of R0 resection [127]. No disease progression was observed 18 months following the completion of the therapy. This case demonstrates the potential benefit of neoadjuvant therapies for locally advanced tumours and makes it worth considering further exploration.

Overall, it has been shown in a recent meta-analysis that the median survival time of 18–20.5 months could be achieved in initially unresectable patients [8]. Importantly, it has also been demonstrated that neoCRT significantly reduced local recurrence compared to adjuvant treatments (34% vs. 5%) [93]. Unfortunately, another large retrospective analysis demonstrated that around 65% of preoperatively treated and resected patients had tumour recurrence, with 40% of them developing distant metastasis [81]. Therefore, down-staging neoadjuvant therapy combined with treatment targeting micrometastasis, undetectable at the time of diagnosis, could improve this grim prognosis. Following the success of nab-paclitaxel and FOLFIRINOX in metastatic disease, new strategies involving combination treatments based on these drugs have also been considered in neoadjuvant/adjuvant

settings [128,129]. Currently, FOLFIRINOX-based preoperative therapy is being tested for improved efficacy, mainly in down-staging tumour burden, as well as targeting micrometastasis.

5.3. Adjuvant Therapy in PDAC

Once completing successful surgery, post-resection adjuvant therapy is subsequently applied to reduce the risk of relapse. 5-FU-based chemoradiation has been claimed to improve survival up to 10 months in the Gastrointestinal Tumour Study Group (GITSG) trial [130]. However, the same combination failed to demonstrate any benefit in the subsequent European Organisation for Research and Treatment of Cancer (EORTC) trial [131], which was attributed to suboptimal RT dose (40 Gy instead of 50) and 20% of patients failing to receive full chemotherapy treatment. Gemcitabine-based adjuvant therapy has been explored in the Charité Onkologie CONKO-001 trial [81]. A significant improvement in all evaluated parameters (PFS, OS) could be observed, providing evidence of the benefit of gemcitabine-based post-operative treatments. In addition, the durable effect of investigated regimen has been demonstrated in a follow-up study (10-year survival of 14.3% compared to 5.8% for non-treated group) [132]. Chemoradiation with the use of gemcitabine/docetaxel/capecitabine (GTX) followed by 5-FU/RT is also considered [133]. Disappointing results were delivered by the ESPAC-3 trial, comparing 5-FU and gemcitabine-based treatments, which did not prove any benefit for the gemcitabine arm [133]. However, median survival times in both arms surpassing 20 months confirmed efficacy of both regimens. Similar results were provided in the radiation therapy oncology group RTOG 9704 trial, in which the improvement in the gemcitabine arm did not show statistical significance [134]. S-1 has been also compared with gemcitabine for adjuvant treatment in the Japan Adjuvant Study Group of Pancreatic Cancer JASPAC-01, showing an encouraging five-year survival rate of 44.1% in the S-1 group compared to 24.4% for the gemcitabine treated patients [135]. Based on this data, S-1 should be considered as another standard of care; however, the analysis was restricted to Asian population. No survival benefit has been demonstrated with combination of erlotinib and gemcitabine in adjuvat setting (CONKO-005). No difference in PFS (11.6 months for both groups) or OS (24.6 months for erlotinib/gemcitabine and 26.5 months in gemcitabine arm) was observed. However, an estimated long-term effect in favour of the erlotinib group (five-year survival of 28% vs. 19% for gemcitabine) gives ground for further exploration of this approach [136]. Phase II and phase III trial assessing the role of erlotinib in addition to gemcitabine in adjuvant setting and determining the efficacy and safety of concurrent fluoropyrimidine after gemcitabine-based or non-gemcitabine based adjuvant therapy is currently conducted by the Radiation Therapy Oncology Group (RTOG) foundation [137]. Several trials are also exploring the use of FOLFIRINOX and Abraxane following surgery. Studies comparing adjuvant gemcitabine and mFOLFIRINOX (NCT01526135), adjuvant gemcitabine and neoadjuvant and adjuvant FOLFIRINOX (NCT02172976), as well as pre-operative and adjuvant FOLFIRINOX (NCT01660711) are currently ongoing. Similarly, phase II study of the nab-paclitaxel and FOLFIRINOX is currently ongoing (NEOLAP; NCT02125136). The Adjuvant Pancreatic Adenocarcinoma Clinical Trial (APACT) trial (NCT01964430) is also evaluating nab-paclitaxel and gemcitabine vs. gemcitabine alone to treat resected patients.

Taken together, discrepancies exist in the evaluation of the efficacy of neoadjuvant and adjuvant therapies. Their benefit has been claimed in a retrospective study (1999–2006), in which preoperative chemotherapy followed by radiation resulted in 78% of patients completing restaging, 53% resection rate and overall better clinical outcome [103]. However, another retrospective analysis of PDAC resections (1992–2011) showed no difference in resection margins between untreated patients and those subjected to neoadjuvant treatment [138], undermining the concept of neoadjuvant therapies. This lack of consensus is mostly due to no unequivocal definition of borderline resectable cancers, small collection of cases and limited number of prospective studies, impeding proper evaluation and interpretation of the results.

Nevertheless, many clinical trials are still ongoing in order to combine the best neoadjuvant agents with postoperative adjuvant therapies, hoping to obtain more prominent improvements in

the survival of patients with resectable or borderline resectable tumours. Neoadjuvant FOLFIRINOX and postoperative gemcitabine [139] are presently under investigation in a multi-institutional Alliance trial (NCT01821612). Thus far, no severe adverse events, precluding from completion of surgery, have been reported. Sixty-eight per cent of patients underwent surgery, with 93% R0 resection rate. At the time of the initial evaluation, 82% of patients were still alive, with median post-treatment survival time of 10 months. Nab-Paclitaxel/gemcitabine combination has been recently explored in the context of preoperative therapy for both borderline resectable and locally advanced tumours. The NEONAX (NCT02047513) and the GAIN-1 (NCT02210559) studies are currently under investigation. Immunotherapy (GVAX vaccine, CD40 antagonists), neoadjuvant capecitabine (CAPOXIRI; NCT01760252) or studies of different FOLFIRINOX regimens are currently ongoing [140].

6. Targeted Therapies—A New Prospect for PDAC Treatment?

As aforementioned, pancreatic cancer presents high heterogeneity in terms of mutational landscape of crucial signalling pathways. Most of pancreatic tumours (around 95%) carry *RAS* mutations. The most frequent among them are *KRAS* alterations (85%), which mainly consist of substitution of G12, resulting in a constitutively active protein [141]. *KRAS* mutations have been recognized as the earliest event in PDAC initiation (PanIN1); however, this is not a sufficient requisite for cancer onset and its progression [142]. During tumour development, *KRAS* alterations accumulate, together with other mutations that pile up progressively. Other common mutations include inactivation of cyclin-dependent kinase inhibitor 2 (*CDKN2*) (in around 90% of PDAC cases) and mothers against decapentaplegic homolog 4 (*SMAD4/DPC4*) (~55%), *BRCA2*, MutL homolog 1 (*MLH1*) or protease, serine 1 (*PRSS1*) alteration. Furthermore, 50–70% of PDAC cases carry mutation in the tumour protein 53 (*TP53*) gene, which occur at later stages of PanIN, contributing to the malignant progression of PDAC rather than its initiation [143]. Such variety of accumulating mutations results in the dysregulation of a plethora of signalling pathways playing a vital role in many crucial processes including apoptosis, cell proliferation and differentiation. Overall, around 60 mutations in 12 different signalling pathways accompany the development of aberrant ducts in PDAC [144]. Among many, changes in Hedgehog, Notch, Wnt, transforming growth factor beta (TGF-β) and RAS/MAPK/PI3K, JAK-STAT pathways, which are normally responsible for the correct development of the pancreas, are recognized as main contributors in PDAC progression [145–147]. In addition, crucial molecules and pathways from both the tumour itself and the surrounding stroma, such as EGFR-mediated pathways, proangiogenic or embryonic pathways influence PDAC resistance to therapy and correlate with poor prognosis. Considering the wide variety of signalling pathways dysregulated in pancreatic cancer and triggering its progression, targeted therapies have emerged as a possibility to augment available therapeutic strategies (Figure 2). This approach has been successfully implemented in the treatment of different solid tumours, with imatinib mesylate (Gleevec) being the first FDA approved targeted treatment of metastatic gastrointestinal tumours in 2002 [148]. Since then this therapeutic approach has been widely used and many targeted drugs for e.g., colorectal, melanoma or non-small lung cancer have been approved [149,150]. However, due to the heterogeneous nature of pancreatic cancer and complex stromal interactions, most of the targeted therapies failed to exhibit any clinical benefit compared to standard treatment. The only exception was erlotinib, an epidermal growth factor receptor (EFGR) inhibitor that, in combination with gemcitabine, showed a moderate but statistically significant (two weeks) improvement in patients' survival [42]. Although many of the studies on targeted PDAC therapies showed promising results in preclinical or clinical settings, most of them failed during phase II/III trials (Table 2). Nevertheless, numerous phase I/Ib studies are still ongoing with many of them showing encouraging results, enabling to move on to phase II/III trials.

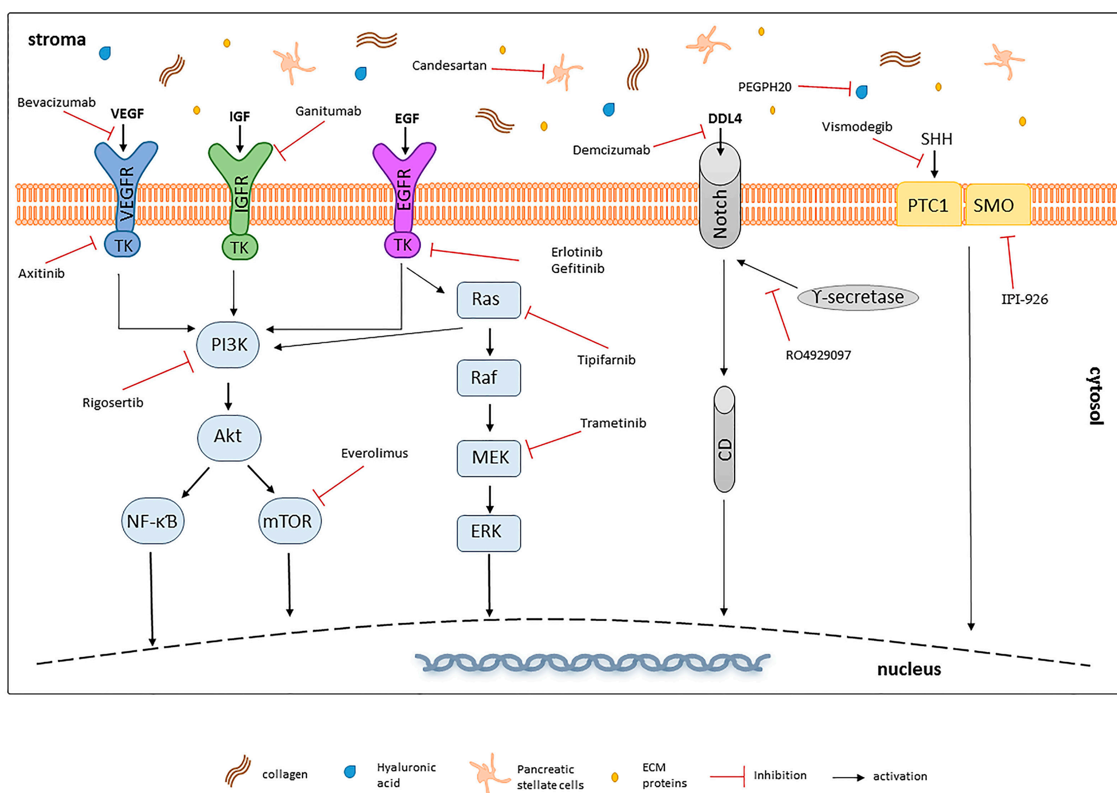

Figure 2. Comparison of selected targeted therapies in as anti-pancreatic ducal adenocarcinoma (PDAC) approach.

6.1. Targeting Growth Factor Receptors

EGFR belongs to ErbB family of receptors, containing a tyrosine kinase domain, which activation is involved in regulation of key processes such as cell cycle, cell survival and differentiation through activation of multiple downstream signalling pathways, including RAS/PI3K/Akt or MAPK/ERK. EGFR pathways are over-activated in PDAC as a consequence of high receptor density, overexpression of ligands or EGFR activating mutations [151]. Considering the high prevalence of EFGR mutations in pancreatic cancer patients and the success of erlotinib, an adenosine triphosphate (ATP) competitor for binding to tyrosine kinase (TK) domain, as a PDAC therapeutic, other molecules targeting this pathway have been intensely tested. Both antibodies blocking EGFR activation and inhibitors of tyrosine kinase domain of the receptor have been evaluated. However, most of them failed to show any improvement over the standard treatment. As an example, cetuximab, an EGFR-binding monoclonal antibody, showed promising phase I results in combination with capecitabine but revealed no statistical significance in survival benefit in further studies [152]. Gefitinib treatment combined with gemcitabine was also evaluated and resulted in 1 year survival rate of 27% and median survival time of 7.3 months [153]. Although encouraging, gefitinib has been considered not as promising as erlotinib. Therapies designed for patients harbouring human epidermal growth factor receptor 2 (*HER-2*) mutations emerged as another possibility. It has been demonstrated that more than 10% of PDAC patients overexpress HER-2 protein and its expression has been correlated with patients' poor survival [154]. Therapy of HER-2 positive patients with capecitabine and trastuzumab, though promising, was unsuccessful in phase II clinical trials [155]. The main drawback of this study was the small number of patients harbouring *HER-2* alterations. After getting FDA approval for chemotherapy, lapatitib has been also tested in combination with gemcitabine for pancreatic cancer patients; however, the results showed only moderate improvement, with a median survival time of four months [156]. Lapatinib/capecitabine combination has been also tested as second-line therapy

for pancreatic cancer. Although the treatment was well tolerated and provided improvement for a subset of patients, the limited number of participants impairs evaluation of its clinical benefit [157]. Nimotuzumab (anti-EGFR monoclonal antibody) [158] and afatinib (TK inhibitor) [159] also showed encouraging results in preclinical or clinical studies and their therapeutic application is currently under evaluation. Insulin-like growth factor 1 receptor (IGF1R) is also highly overexpressed in pancreatic cancer and its excessive activation leads to boosted stimulation of downstream pathways, increasing cell proliferation and survival [160]. Several drugs targeting these molecules, especially monoclonal antibodies ganitumab and cixutumumab have been evaluated; however, no statistically significant improvement of survival was observed [161]. Similarly, a study on the combination of ganitumab and gemcitabine failed to show significant benefit over the single agent gemcitabine in phase III clinical trial causing the closure of the study [162].

6.2. KRAS Pathways Inhibition

KRAS mutations are widespread in pancreatic cancer, with more than 90% of diagnosed patients having mutated *KRAS* gene. Membrane-bound guanosine triphosphate hydrolase (GTP-ase) protein encoded by this gene is activated by the family of EGFRs and induces signalling involved in a plethora of cellular functions. When mutated, KRAS gains oncogenic activity and is maintained in a constitutively active state, continuously inducing downstream signalling pathways (MAPK/ERK, PI3K/Akt) contributing to increased proliferative signals, invasiveness and inhibition of cell apoptosis. Although the idea of a KRAS inhibition raised a lot of hope, its direct targeting did not bring the expected results. Therefore, strategies targeting proteins along the RAS signal transduction pathway have been widely explored. For example, tipifarnib, an inhibitor of farnesyl-transferase (an upstream effector of RAS, essential for its activation) was studied in combination with gemcitabine but unfortunately, showed no superiority over standard therapy in phase III trial [163]. Another strategy is blocking KRAS downstream signalling molecules, such as MAPK pathway, which activation is observed in later stages of pancreatic cancer and favours cancer development. However, MEK targeting, with selumetinib combined with capecitabine [164] or trametinib/gemcitabine combination [165], was not able to increase OS or provide statistically significant results. Nevertheless, taking account of the promising results obtained in preclinical studies, ERK inhibition is still explored as a potential pancreatic cancer treatment. A combination of trametinib and GSK2256098 (focal adhesion kinase, FAK inhibitor) is planned to be tested and a proposed study is currently recruiting participants (NCT02428270). Ulixertinib BVD-523, an ERK inhibitor, is also currently tested in combination with gemcitabine/nab-paclitaxel in phase Ib trial [166]. Another crucial pathway in pancreatic cancer is PI3K signalling, that is activated in response to EGFR induction, and in turn, triggers activation of several downstream targets such as Akt, pS6 or mTOR, influencing cell survival, metabolism and proliferation [167]. Therefore, PI3K signalling inhibition represents another possibility for PDAC therapy. A combination of gemcitabine and rigosertib, a Ras mimetic and small molecule inhibitor of PI3K, has been evaluated; however, it failed to enhance patients' response when combined with gemcitabine [168]. Data from everolimus and sunitinib (mTOR inhibitors) studies suggested promising results, incrementing the progression-free survival time (from ~5 to 11 months) [169,170], potentially improving prognosis for a selected groups of patients. A combination of everolimus and capecitabine has also been tested resulting in 8.9 months OS [171]. Being a single arm study, the impact of everolimus on patients' response is hard to determine. Nevertheless, considering previous results of capecitabine monotherapy showing 5.9 months survival, the achievement of 8.9 months seems encouraging. However, the differences in the study's design and patients' population make this assumption arguable. Likewise, disappointing results were obtained in other phase II studies, in which everolimus or temsirolimus were used to inhibit PI3K/Akt/mTOR pathways [172,173]. Another mTOR inhibitor, PBI-05204 (NCT02329717), is currently tested for patients with stage IV pancreatic cancer. Moreover, it is considered that combining PI3K and MEK inhibitors may have a potential synergic activity [174].

6.3. Targeting Angiogenesis

Angiogenesis is a pivotal process required for tumour growth and metastasis. Therefore targeting the mechanisms regulating this process seems to be a tempting strategy to reduce cancer progression. Among many factors, vascular endothelial growth factor (VEGF) and its receptor have been mostly studied in the context of the abovementioned process [151]. It is claimed that therapy against those molecules, although not effective in terms of modulation of cancer cell proliferation in vitro, may reduce proliferation of endothelial cells, decrease infiltration and metastasis in vivo. However, studies investigating the anti-angiogenic agents axitinib (inhibitor of VEGFR, mast/stem cell growth factor receptor SCF) and platelet-derived growth factor receptor PDGFR tyrosine kinases) [175,176] or Avastin (bevacizumab, a VEGF-A inhibitor) [177] did not exhibit positive and statistically significant results. Due to unmet primary endpoint of OS, Pfizer had to discontinue its study on axitinib combined with gemcitabine [178]. Likewise, phase II study or sorafenib (Raf kinase, VEGF-R2/R3 and PDGFR-β oral inhibitor) alone or in combination with gemcitabine did not exhibit promising activity in metastatic patients [179]. Similarly, addition of aflibercept (a recombinant protein targeting VEGF signalling) to gemcitabine, although promising in pre-clinical studies, did not improve patients' OS and resulted in an increase of the incidence of adverse effects [180]. Likewise, a study on necuparanib and nab-paclitaxel/gemcitabine, although initially promising, had to be terminated due to lack of expected efficacy [181]. At present, phase II trial of the novel anti-angiogenic agent TL-118 (NCT01509911) is being assessed.

6.4. Other Targets

One of the most encouraging results so far has been obtained from JAK-STAT pathways inhibition studies, especially in tumours with an inflammatory microenvironment. The role of JAK-STAT pathway in cell proliferation migration and apoptosis has been widely elucidated. Increased expression of the members of these two pathways in PDAC has been shown by gene-expression analysis [182] and they have been shown to directly contribute to the initiation and progression of pancreatic cancer. JAK1 and JAK2 inhibition with a capecitabine and ruxolitinib combination did not show significant benefits in the survival of untreated patients. However, in patients resistant to gemcitabine, the combination showed improvements in performance status and pain management [183] and phase III studies of this combination are currently ongoing [184]. A phase III study evaluating the Janus kinase inhibitor momelotinib in combination with nab-paclitaxel/gemcitabine has just terminated (NCT02101021) and the results are expected to be published. The importance of Notch pathway in PDAC is also well known, and its role in chemoresistance was highlighted in various reports [185,186]. It has been shown that its inhibition, i.e., through anti-DDL4 antibodies (tarextumab or demcizumab) combined with gemcitabine, exhibited anti-tumour activity and indicated a possible reversal of chemoresistance, mainly by targeting pancreatic cancer stem cells [187] and therefore showing a therapeutic potential. However, although after a promising phase I outcomes, the Yosemite trial, evaluating the combination of demcizumab and gemcitabine/Abraxane had to be discontinued due to unmet primary endpoint of PFS [188]. Moreover, an interim OS analysis failed to show any benefit over the Abraxane arm. Recent exciting results have been obtained with gemcitabine and MK-0752 (an inhibitor of γ-secretase, the cleaving enzyme in Notch-mediated cascade), although further studies are needed [189]. Another γ-secretase inhibitor, RO4929097, has been tested in phase II studies, in which good tolerance and moderate OS response was reported; however, the limited cohort of 18 patients limits proper assessment of this study [190]. Interestingly, it has been suggested that combined targeting of both JAK and Notch pathways surpasses their individual inhibition, however the effect of that approach on patients' outcome is still to be determined.

Poly ADP-Ribose pathway (PARP) presents another possibility for targeting PDAC. These enzymes are activated in response to DNA damage and it has been shown that patients with a defective DNA recombination pathway may positively respond to PARP inhibitors [191]. Moreover, BRCA mutations, impairing DNA repair, might be also targeted by those compounds. Therefore, many clinical

trials targeting this pathway are currently ongoing. Olaparib is an oral poly (ADP-ribose) polymerase inhibitor, which has shown promising activity in different cancers bearing BRCA mutations [192]. Olaparib is currently being tested in a phase III trial for patients with BRCA mutated pancreatic cancer (NCT02184195) and combination of gemcitabine/cisplatin with another PARP inhibitor, veliparib, is also being evaluated [193,194]. Tumour suppressor *TP53* is another gene highly mutated in PDAC progression. Its normal activity is essential for cell apoptosis, cell metabolism and DNA damage repair, therefore its deactivation highly contributes to the development of a plethora of malignancies [143]. Study of p53 targeting molecule, SynerGene Therapeutics 53 (SGT-53), is being currently tested in combination with nab-paclitaxel/gemcitabine (NCT02340117).

6.5. Targeting Tumour–Stroma Interactions

One of the reasons for the dismal prognosis of PDAC is a high chemoresistance caused by the huge genetic heterogeneity and plasticity of PDAC tissues. An additional factor contributing to cancer resistance is the formation of a dense, diffuse stroma called desmoplasia [5]. Pancreatic stellate cells (PSCs), fibroblasts, blood vessels and proteins form a dense environment through the expression of multiple molecules (e.g., chemokines, EGFs, Cox-2) and interact with cancer cells, influencing tumour progression and invasion [7]. Other than forming a dense barrier around the tumour, the desmoplasia is also responsible for poor vascularisation of tumours and consequently, causes nutrient depletion as well as impairs drug delivery to cancer cells [6]. Therefore, it has been shown that, by formation of a cancer promoting environment, cancer stromal cells influence PDAC development. The cross-talk between cancer and stroma cells allows for formation of a feed-forward loop, perpetuating cancer progression. Thus, the tumour microenvironment is an important factor in cancer development, and tumour stroma is another attractive target for PDAC treatment, potentially increasing the efficacy of chemotherapy. However, results from conducted studies are not clear cut. One of the first pieces of evidence of the potential benefits of targeting the stroma comes from nab-paclitaxel/gemcitabine studies, which showed a significant increase in the intracellular gemcitabine concentration due to decreased cancer-associated fibroblasts and stroma disruption facilitated by nab-paclitaxel [49]. As mentioned above, targeting multiple receptor tyrosine kinases, e.g., blocking of VEGFR and PDGFR with dovitinib, showed an improvement in therapeutic efficacy in mouse models, and clinical trials are currently ongoing [195,196]. Hedgehog pathway plays a pivotal role in cell survival and proliferation during development. Typically, it is repressed in mature pancreas; however, its activation has been observed during carcinogenesis. In addition, sonic hedgehog (SHH) and its downstream effectors take part in the formation of desmoplasia, contributing to decreased drug delivery [197,198]. Therefore, the Hedgehog pathway inhibition raised a lot of interest in terms of its potential to decrease the proliferation and invasion of PDAC cells [199]; however, its inhibition showed contradictory results. Very encouraging and promising results of the Hedgehog inhibition (via Smoothened) with an infinity pharmaceuticals inhibitor of sonic hedgehog (IPI-926) agent were obtained by Olive et al. [200], demonstrating a potent anti-tumour activity of the compound in a series of preclinical studies. Combined with gemcitabine or nab-paclitaxel, IPI-926 significantly increased drug delivery, reduced metastases and prolonged mice survival. Infinity pharmaceuticals conducted clinical trials of the compound in combination with gemcitabine and, despite the initials promising phase I/II results, the study needed to be discontinued due to decreased survival rate in the IPI-926/gemcitabine group compared to the gemcitabine alone group [201]. Interestingly, failure of Hedgehog targeting has been attributed to emerging evidence of the release of tumour restraining caused by the inhibition of this pathway. Currently, there are no FDA-approved Hedgehog inhibitors, nevertheless, clinical trials of chemotherapeutics and Hedgehog inhibitors are ongoing. Vismodegib (GDC-0049), an inhibitor of Hedgehog signalling pathway via inhibition of Smoothened, is under evaluation in combination with gemcitabine or gemcitabine and nab-paclitaxel for advanced and metastatic patients [202]. Its application as a sole agent has been also considered for neoadjuvant therapy [203]. Another molecule identified as possible target in the inhibition of cancer stroma is connective tissue growth factor (CTGF). Its overexpression in PDAC

tissues has been confirmed, together with its ability to induce PSCs proliferation, migration and fibrogenesis mediated by chemokines activation [204]. SB225002, a Cxcr2 receptor inhibitor, prolonged survival of mice in in vivo studies [205]. Similarly, targeting the same receptor with a monoclonal antibody FG-3019 combined with gemcitabine showed a significant increase in gemcitabine efficiency in KPC mouse model [206], presenting a promising strategy for novel PDAC therapeutics. It is also known that pancreatic stellate cells (PSCs) and extracellular matrix (ECM) proteins actively participate in the formation of the tumour stroma [207] and in the activation of a plethora of cancer-promoting pathways leading to an increased tumorigenicity and chemoresistance by enhancing cancer stem-like phenotype [208,209]. Therefore, there are many strategies aiming to inhibit PSCs activation and ECM production. Among different agents, angiotensin II type 1 receptor blockers (ARBs) showed the most promising results. Candesartan, one of ARBs, was able to suppress PSCs activation as well as prolong patients' survival for more than 6 months when combined with ACEIs (angiotensin I converting enzyme inhibitors) [210]. Another member of ARBs, losartan, apart from inhibiting PSCs activation, decreased levels of hyaluronan and collagen in the stroma, remodelling tumour microenvironment and increasing blood perfusion [211]. Matrix metalloproteinase inhibitors (e.g., marimastat) have also been tested, although no evidence of their superiority over gemcitabine has been provided [212]. Targeting of non-cellular stroma compartments, such as hyaluronic acid (HA), showed promising preliminary data. HA is a matrix component, which depletion might facilitate drug delivery by overcoming barriers caused by dense stroma. After promising results from a clinical trial of PEGPH20 (a PEGylated recombinant hyaluronidase which can deplete accumulated HA in tumours) and gemcitabine [213], PEGPH20 with Abraxane [214] combination is currently in progress. Overall, targeting the stroma and its particular components seems to be a promising and novel approach. Considering the significant contribution of dense tumour microenvironment in chemoresistance, agents aiming at releasing stroma may considerably improve tumour vasculature and drug delivery. However, there is some controversy regarding the safety of this strategy. Few studies have suggested that excessive relaxation of surrounding stroma may facilitate release of tumour cells, contributing to cancer dissemination [215]. Therefore, this aspect should be considered during design of pre-clinical and clinical studies.

Table 2. Selected targeted therapies and immunotherapies for PDAC.

Drug Target	Treatment	Phase	*n*	OS	Comment	*p*	Reference
KRas pathway inhibitors							
KRAS (farnesyl transferase)	Tipifarnib + gem vs. gem	R III	688	193 vs. 182 (days)	Acceptable toxicity profile, but no statistically significant differences in survival parameters	0.75	[163]
MAPK	Selumetinib + erlotinib 2nd line	SA II	46	7.5	Modest antitumor activity. Specific molecular subtypes may provide greatest benefit	–	[216]
MAPK	Trametinib + gem vs. gem	R II	160	8.4 vs. 6.7	No statistical difference in OS, PFS and response rate was observed	0.453	[165]
MAPK	Selumetinib + cape vs. cape 2nd line	R II	70	5.4 vs. 5.0	No improvement in OS	0.92	[164]
MAPK	Sorafenib + gem vs. gem		104	9.2 vs. 8.0	No statistical significance was achieved in all parameters studied	0.231	[217]
mTOR	Everolimus + erlotinib	SA II	16	2.9	Disease progression observed in 15 patients. Study stopped due to impossibility to reach preplanned OS of 6 months	–	[173]
PI3K	Rigosertib + gem vs. gem	R II/III	160	6.1 vs. 6.4	Study was discontinued due to no significant difference in survival	NR	[168]
Growth factor receptors inhibitors							
EGFR	Erlotinib + gem vs. gem	R III	569	6.2 vs. 5.9	FDA approved	0.038	[42]
EGFR	Cetixumab + gem vs. gem		743	6.3 vs. 5.9	Combination arm did not achieve significance in improvement of OS	0.19	[152]

Table 2. *Cont.*

Drug Target	Treatment	Phase	*n*	OS	Comment	*p*	Reference
EGFR/IGFR	Cixutumumab + erlotinib + gem vs. erlotinib + gem	R Ib/II	116	7.0 vs. 6.7	Dual inhibition of EGFR and IGFR did not improve OS or PFS	0.64	[161]
EGFR	Gefitinib + gem	SA II	53	7.3	Promising results, especially in patients with PTEN expression.	–	[153]
HER-2	Trastuzumab + cape	SA II	17	6.9	No improvement in mOS or PFS; low number of patients and HER2 expression	NR	[155]
TK	Dasatinib	SA II	51	4.7	No activity of single agent dosatinib in metastatic PDAC, no improvement in OS and PFS	–	[216]
TK	Lapatinib + gem	SA II	29	4	No improvement in survival, small case sample	–	[156]
IGFR	Ganitumab + gem vs. gem	R III	800	7.0 vs. 7.2	No improvement in all assessed parameters	0.494	[162]
Angiogenesis inhibitors							
VEGFR	Axitinib + gem vs. gem	R III	632	8.5 vs. 8.3	No significant survival benefit compared to single agent gem	0.544	[176]
VEGF-A	Bevacizumab + gem + erlotinib vs. gem + erlotinib	R III	301	7.1 vs. 6.0	Despite improvement in PFS could be observed ($p = 0.0002$), no statistically significant difference in OS was achieved	0.209	[218]
VEGF	Aflibercept + gem vs. gem	R III	587	6.5 vs. 7.8	Discontinued due to no improvement in primary end point, OS	0.159	[180]
Inhibition of tumour stroma							
Matrix metalloproteinase	Matrimastat + gem vs. gem	R III	239	5.4 vs. 5.4	No significant differences in all assessed parameters	0.95	[212]
SHH	Vismodegib + gem vs. gem	R Ib/II	106	6.9 vs. 6.1	No difference in PFS, OS or response rate was noted	0.84	[202]
PSCs	Candesartan + gem	SA II	35	9.1	Treatment was well tolerated but failed to show significant activity	–	[219]
Hedgehog (Smoothened)	IPI-926 + gem vs. gem	R Ib/II	122	–	Decrease in survival in IPI-926 arm caused closure of study	NR	[220]
Hyaluronic acid	PEGPH20 + gem	Ib	28	6.6	Well tolerated, may be beneficial, especially for patients with high HA levels (13 months OS)	–	[213]
	PEGPH20/Abraxane vs. Abraxane	R II	237		Ongoing		[214]
		R III	420		Ongoing		
Other targets							
JAK/STAT	Ruxolitinib + cape vs. cape	R II	127	4.5 vs. 4.2	Well tolerated, slight, but significant improvement in OS and PS	0.011	[183]
	2nd line therapy	R III	270		Phase III on larger population is ongoing		[184,221]
γ-secretase	RO4929097 2nd line	SA II	18	4.1	Study was discontinued as the primary endpoint-survival rate at 6 months—was not promising (27.8%)	–	[190]
Immunotherapy							
CTLA-4	Ipilimumab + GVAX vaccine vs. ipilimumab	R Ib/II	30	5.7 vs. 3.6	Despite the enhancement of the T cell repertoire ($p = 0.031$), no significant increase in OS or PFS was noted	0.51	[222]
Telomerase vaccination	GV1001 + gem + cape/gem + cape	R III	1062	8.4 vs. 6.9	No significant improvement in OS has been achieved	0.11	[223]

SA, single arm; R, randomized; OS, overall survival; PFS, progression-free survival; RR, response rate; cape, capecitabine; gem, gemcitabine.

7. Immunotherapy for Pancreatic Cancer

Another emerging option for treating advanced pancreatic cancer patients is immunotherapy. Induction of an anti-tumour immune response has been shown to be extremely effective in different advanced stage cancer types. However, immunotherapy trials in PDAC have shown conflicting results so far. An immunotherapy approach in pancreatic cancer therapy can be divided into a few categories: checkpoint inhibitors, vaccines, monoclonal antibodies, adoptive cell transfer, viruses, and use of cytokines. The first option, immune checkpoint inhibitors, by enhancement of stimulatory or blocking activity of immune system regulators, intensifies existing anti-cancer responses, enabling for better clearance of cancer cells. Programmed death receptor 1 (PD-1), a well as its ligand PD-L1, is one of the most important checkpoint pathways [224]. They are expressed on tumour-associated lymphocytes and are involved in suppression of immune responses observed during carcinogenesis, which is why they may be considered as one of the mechanisms of cancer immune resistance. Targeting this pathway should induce T cell activity and consequently cancer cell death. Therefore, antibodies targeting PD-1 receptor or PD-L1 are being investigated [225]. Phase I/II trials examining antibodies targeting another checkpoint inhibitor, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), (with e.g., ipilimumab, an FDA approved immunotherapy drug for melanoma) [226] are also ongoing. Nivolumab (anti-PD-1 antibody) alone (NCT02423954) or in combination with ipilimumab, anti-CTLA-4 Ab (NCT01928394); gemcitabine (NCT01473940), or other antibodies (NCT02526017, NCT02381314) are currently being tested. Studies of another anti PD-1 antibody, pembrolizumab (NCT02268825, NCT02305186) alone or in combination with gemcitabine and FAK inhibitor defactinib (NCT02546531), are also ongoing. Another combination of anti-PDL1 and anti-CTLA4 antibodies (durvalumab and tremelimumab respectively (NCT02558894, NCT02639026, NT02311361, NCT02527434) or durvalumab with mogamulizumab- anti-CCR4 Ab (NCT02301130) is being investigated in patients with advanced cancer, including pancreatic cancer. Unfortunately, preliminary data from the previously mentioned combinations indicate no significant improvement so far. To overcome the immunosuppressive activity of pancreatic cancer stroma, targeting CD40 has arisen as a novel strategy to increase anti-tumour activity. CD40 is a member of the tumour necrosis factor family expressed by immune cells, and its elevated expression and activity have been linked with different malignancies, including cancer [227]. Therefore, through immune system activation, targeting of CD40 can affect tumour growth. Several studies have been proposed, in which enhancing CD40 activity with its agonists may improve T-cell-dependent (macrophages activation and tumour stroma destruction) and independent immune responses and consequently induce cancer regression. A promising combination of gemcitabine and CD40 agonist antibody (CP-870,893) is being tested in clinical trials [228,229]. This combination enhances the accumulation of tumour-suppressive macrophages, increasing tumour regression. A Phase Ib/II trial of gemcitabine/nab-paclitaxel combined with indoximod (inhibitor of indoleamine-2,3-dioxygenase, tryptophan metabolite toxic to T cells) is ongoing, with preliminary data showing moderate and sustained activity [230]. Vaccine-based therapies are designed to enhance the immune system response against tumour-associated antigens. Unfortunately, no statistically significant clinical benefit over standard therapies has been achieved so far with vaccines, such as GV1001 [223] or PANVAC-V [231,232]. Nevertheless, a plethora of vaccine-based combinations clinical trials are currently ongoing (e.g., ipilimumab ±vaccine therapy [222], GVAX Pancreas vaccine (designed to secrete GM-CSF) ± nivolumab [233], GVAX, CRS-207 (vaccine targeting mesothelin protein) ± nivolumab (NCT02243371) or HyprAcute Pancreas (algenpantucel-L; NCT02405585) for both resectable and metastatic cancers. Various monoclonal antibodies are also currently used in cancer therapy. Cetuximab, an EGFR-targeting monoclonal antibody, despite promising phase I results, did not show any survival benefit in further studies [152]. Phase I and II clinical trials of, e.g., anti-HER3 antibody (MM-141, NCT02399137), Trop-2 antibody (IMMU-132, NCT01631552), or anti CA19-9 antibody (MVT-5873, NCT02672917), are currently being developed. Adoptive T cell therapy has emerged as another powerful tool in the enhancement of immune system responses. It is based on removal of the patient's T cells, followed by boosting their activity through genetic/chemical

re-engineering, and reintroduction into the patient. The modifications currently investigated include targeting anti-MAGE-A3 protein (NCT02111850), targeting NY-ESO-1 antigen (NCT01967823) or CAR T (chimeric antigen receptor T) cells reengineered to recognize mesothelin (NCT01583686). Virus therapies, such as ParvOryx (NCT02653313) or Reolysin, which replicates particularly in Ras-transformed cells (NCT02620423), are also currently assessed as anti-cancer tools, facilitating cancer cell self-destruction. Overall, different approaches to PDAC immunotherapy are presently being undertaken, with promising preclinical studies results. However, most of the studies are still in their early phases and much more effort needs to be made to fully assess their potential effectiveness and applicability in PDAC patients' treatment.

8. miRNAs in PDAC Therapy

Recently, the developing field of miRNA investigation has attracted interest as another possibility for expanding the repertoire of PDAC treatments. It has been demonstrated, in several independent studies, that these short (18–22 nucleotide) non-coding RNAs can regulate expression and activation of multiple signalling pathways responsible for cell development, growth, differentiation and apoptosis, suggesting their possible involvement in carcinogenesis [234]. In fact, miRNA expression profiling showed abnormal expression of a plethora of different miRNA in several cancers including PDAC. Increased levels of pro-oncogenic as well as reduced expression of tumour suppressive miRNAs have been found in cancerous, as well as pre-cancerous pancreatic samples [235], suggesting their importance in PDAC development. Because each single miRNA targets multiple genes, causing alteration in their expression, targeting miRNAs provides encouraging approach for PDAC treatment, in which by targeting of one molecule, activation of multiple pathways may be altered. However, the same concept raises similar amount of concern, since alteration of that significant number of genes might cause severe adverse effects.

Several different expression profiles in pancreatic tissues from different sources (fresh frozen tissue, paraffin-embedded or fine-needle biopsy) showed a significant number of aberrantly expressed miRNAs compared to healthy pancreatic tissues [236,237]. Among many, increased expression of miR-21, miR-221, miR-155 and decreased levels of miR-146a, miR-34 and miR-145 were regularly detected across all the studies [238]. Moreover, overexpression of miR-155 and miR-21 has been correlated with advanced cancer stage and poor prognosis, with the latter being involved in the transformation from normal tissue to PDAC [239–241]. miR-155 has been found to be significantly upregulated in pancreatic tissues as well as in PanIN-2 and PanIN-3 samples [242]. Moreover, its expression correlated with PDAC patients' survival and lymph node metastasis [243,244], suggesting the importance of miR-155 in PDAC carcinogenesis. By targeting the expression of molecules important in this process, such as suppressor of cytokine signalling 1 (SOCS1) or MLH1, miR-155 has been proposed as an important player in PDAC invasion and migration [244,245]. Its importance has been confirmed by in vitro studies, in which knockdown of miR-155 resulted in significant decrease in expression of EGFR and KRAS, proteins essential for PDAC development, as well as reduced cell proliferation and colony formation [246]. Similar correlation between cancer staging and miRNAs expression has been demonstrated for miR-221. Moreover, its expression has been also associated with metastasis and unresectable tumour status [247,248]. Inhibition of miR-221 significantly reduced PDAC cell proliferative capacity by targeting and blocking multiple genes, including *PTEN*, *P27* or *PUMA* [249]. Furthermore, increased miR-221 expression has been also detected in pancreatic stellate cells (PSCs) [250], suggesting its involvement not only in cancer cell proliferation but also in the tumour microenvironment. Similar observations have been reported for miR-146a; however, its expression has been found to be considerably decreased in PSCs [250]. It has been also shown that overexpression of miR-146a or its induction by isoflavone treatment, significantly decreased PDAC cell invasiveness by downregulation of, e.g., EGFR [251]. However, a separate study suggested that expression of miR-146a in PanINs was upregulated, suggesting its potential involvement in PDAC initiation [252]. All these findings make miR146a a controversial target for PDAC therapy. Three members of miR-34 family,

miR-34a, miR-34b and miR-34c have been found downregulated in PDAC and were correlated with lymph node metastasis and poor survival [241,253]. Their impact on cancer cell proliferation, invasion, epithelial-mesenchymal transition (EMT) and cell cycle regulation through targeting molecules such as Notch or Bcl2 has been confirmed [254]. Interestingly, miR-34a downregulation can be partially attributed to epigenetic regulation (hypermethylation), suggesting demethylating agents as a possible therapeutic drugs [255]. In fact, isoflavone treatment resulted in miR-34a upregulation and consequently, induction of apoptosis and suppression of tumour growth [256]. A number of studies have considered miR-21 as a suitable target for PDAC therapy. Its elevated expression was found in 79% of pancreatic cancer samples, whereas only 8% of benign tumours expressed this miRNA [257]. Its activation triggers the response of multiple oncogenic signalling pathways, inducing cell proliferation, differentiation and exerting an anti-apoptotic role [258]. Similar to others, its expression has been correlated with PanIN progression, dismal prognosis, increased proliferation and invasion. Conversely, downregulation of the expression of miR-21 reduced proliferation of multiple cancer cell types [259] and it was shown to be beneficial in the adjuvant settings, increasing drugs activity. Importantly, gemcitabine resistance has been associated with miR-21 expression and thus this could be considered as a prognostic marker for gemcitabine response [257,260–262]. It has been demonstrated that co-delivery of gemcitabine and miR-21 silencers had a synergistic anti-tumour effect and presents a promising strategy for novel anticancer therapy. Taking into consideration the pivotal role of multiple miRNAs in a variety of carcinogenic processes, different approaches for the regulation of their activity have been considered. Nanoparticle delivery of tumour suppressing miRNAs, such as miR-150 or miR-34a resulted in reduction of cell proliferation and invasion, as well as was able to suppress tumour growth [263,264]. Analogously, combination of miR-21 and miR-221 antisense nucleotides reduced growth of primary tumours and significantly inhibited metastasis [265]. Recently, co-delivery of miRNAs and chemotherapeutics emerged as another promising strategy. In particular, co-administration of miRNAs (or their inhibitors) involved in chemoresistance seems an attractive approach. Co-delivery of miR-205 and gemcitabine was able to reverse this resistance and reduce proliferation and invasion of highly resistant PDAC cell lines such as MiaPaCa-2 or Capan-1 [266]. Similarly, targeting of miR-21 using lentiviral vectors stimulated angiogenesis, enhanced gemcitabine delivery and provoked tumour regression [259–261]. All of these results make miR-21 a promising target, which needs to be further evaluated in more advanced clinical studies. Another strategy in targeting miRNAs for anti-cancer therapy is the use of natural agents [267], such as the aforementioned isoflavone, curcumin or 3,3′-diindolylmethane (DIM). It has been formerly demonstrated that isoflavones possess anti-cancer activity. Considering our current knowledge, miRNAs alteration may be one of the mechanisms responsible for this activity. As previously mentioned, treatment with the isoflavone genistein was able to suppress tumour growth through upregulation of miR-34a expression. Similarly, elevated expression of miR-146a and miR-200, as well as a decrease in miR-27a levels were detected after isoflavones treatment, which resulted in reduction of cell proliferation and invasion and increased sensitization of cells to gemcitabine treatment [251,268]. Anti-cancer activity of DIM is also exerted via regulation of different miRNAs, including miR-200, miR-221 or miR-146a. A decrease in cell proliferation and migration, reversal of EMT and sensitization to gemcitabine were induced after exposure of cancer cells to DIM [251,269]. Similar effects could be achieved with the curcumin analogue difluorinated-curcumin (CDF), which increased curcumin bioavailability. CDF treatment elevated expression of miR-101, miR-146a and miR-200 and decreased miR-211 levels. This activity results in inhibition of pancreatic cancer cell growth and migration, decreased colony formation, as well as downregulation of a plethora of pathways pivotal for PDAC progression, including EGFR, ERK or KRAS expression [270,271]. Other natural agents, such as Brucein D, resveratrol or rosemary extracts exerted similar effects on pancreatic cancer cells, through regulation of different miRNAs [272,273]. Overall, targeting miRNAs either by their re-expression or inhibition seems a novel and promising strategy in pancreatic cancer treatment. Furthermore, this approach has been shown to enhance cancer cell response to

chemotherapy, by reducing cancer chemoresistance. However, there is a need for in-depth preclinical and clinical studies to assess the efficiency and safety of this strategy. Alteration of single miRNA can result in the cascade of changes in activity of downstream effectors, contributing to elevated adverse effects. Moreover, considering the correlation between the expression of most of miRNAs, PDAC stage and patients' OS, miRNAs levels should be also further explored as novel, predictive biomarkers.

9. Second-Line Therapies

Limited options are available for patients whose disease has progressed after gemcitabine-based first line treatment. Oxaliplatin-based therapies are usually offered in these cases, but good performance status is a critical factor. The beneficial effect of oxaliplatin in addition to 5-FU and folinic acid, over individual therapies, has been observed in several trials (CONKO-01, CONKO-03); with an acceptable safety profile and almost doubling of the survival period [274,275]. However, contradictory results were obtained in the PANCREOX study, in which addition of oxaliplatin to mFOLFOX6 (infusional FU/LV) showed no benefit in patients who progressed on gemcitabine-based first line therapy [276]. A single-arm phase II study of docetaxel and oxaliplatin (DocOx) in gemcitabine-refractory patients has recently been conducted, and a median overall survival time of 10 months was noted [277]. Second line combinations of capecitabine and oxaliplatin (CapOx) have been also considered, with encouraging activity and safety profile of the combination [278]. FOLFOX treatment (Leucovorin, 5-FU and Oxaliplatin) also proved to be an efficient (mOS of 4.3 months) and considerably safe second-line treatment for metastatic patients with good PS [279]. Its activity was comparable with yet another agent tested for second-line PDAC treatment, FOLFIRI (Leucovorin, 5-FU and Irinotecan), which increased survival by approximately six months [280,281]. A combination of capecitabine and JAK-1 and JAK-2 inhibitor ruxolitinib, administered to patients who already received gemcitabine, is being investigated in a phase III JANUS study [184]. Studies analysing second-line therapies after FOLFIRINOX failure are also under investigation. Gemcitabine and nab-paclitaxel, as well as maintenance capecitabine, have shown promising results and further studies are planned [282–284]. An alternative option for a second line treatment was proposed in the NAPOLI-1 trial [285], in which nanolioposomal irinotecan combined with 5-FU and folinic acid significantly increased OS and PFS in a phase II study. Taken together, no optimal second-line therapy has been determined. Therefore, there is an increasing interest in defining most favourable strategy for treatment of advanced PDAC patients who failed to respond to conventional therapies.

10. Conclusions

Despite efforts made to develop more effective therapeutic strategies for PDAC, it still remains one of the most fatal malignancies, for which incidence constantly rises. Even though advances have been made in screening and treatment of other cancer types, PDAC therapy has not experienced significant improvement in the last decades. Gemcitabine and its doublets failed to provide considerable survival benefit. Multidrug therapies—Abraxane and FOLFIRINOX—have been recently developed moderately improving patients' outcomes; however, their efficacy still remains low and their usage is coupled with elevated adverse effects. Therefore, there is an urgent need for the development of novel and more effective treatments.

Thus far, tumour resection supported by adjuvant therapy has presented the only curative option for PDAC patients. However, less than 20% of patients have resectable tumours at the time of diagnosis, caused by local and distant metastasis. Therefore, efforts are being made to increase the percentage of patients able to undergo this procedure. Very early dissemination of pancreatic cancer provides ground for the applicability of neoadjuvant therapies, potentially increasing resection rates. However, despite theoretical advantage, no straightforward evidence of clinical applicability of neoadjuvant therapies is available. Preoperative treatment demonstrated considerable benefit in the increase in R0 resections, the main survival predictive factor. Unfortunately, the significant increase in R0 resection rates in patients subjected to neoadjuvant therapies did not fully translate

into patients' survival benefits. The lack of consensus on effectiveness in resectable patients, as well as contradictory results for BRPC, makes this strategy highly debatable. The controversy of the feasibility of neoadjuvant therapies is due to the inconsistency of the design of clinical trials and difficulty in data interpretation. Lack of standardization and perioperative quality control makes it difficult to properly assess the applicability of neoadjuvant treatments. Inconsistency in accurate tumour classification, varying between centres, single arm phase I/II trials, limited sample size and mostly retrospective data, analysing patients with different disease context, impairs proper data comparison, resulting in lack of consent on the use of neoadjuvant regimen for PDAC patients. In addition, currently available imaging tools do not accurately assess tumour burdens and make it difficult to distinguish treatment-induced fibrosis from extended tumour, disabling proper distinction between down-staged and untreated cancer and potentially depriving part of the population from successful R0 resections. Additionally, most of the studies involved the use of one or two therapeutics; however, recent evidence suggests that multidrug treatments (i.e., PEFG, PDXG or FOLFIRI) yielded significantly higher response rates, showing superiority in various retrospective studies. Therefore, prospective complex evaluation of multi-agent strategy should be more widely explored. Overall, it has been suggested that if properly designed, neoadjuvant treatment followed by surgery may increase five-year survival rates up to 40%. Therefore, the standardization of staging procedures and the initiation of higher number of prospective phase III trials might significantly add to patients' survival. Based on available data, several studies are focusing on providing an algorithm of action improving the decision-making and consequently providing better outcomes; however, so far only marginal benefit has been demonstrated [286]. An Alliance trial is currently being evaluated in order to assess the effectiveness of modified FOLFIRINOX as neoadjuvant agent and to establish reproducible standards for BRPC therapy. In general, despite the controversy and reluctance of some centres to apply neoadjuvant therapy to PDAC patients, this approach is supported by the National Comprehensive Cancer Network (NCCN) in the United States [84].

The grim prognosis for PDAC patients and the disappointing therapeutic results are attributed to the highly proliferative and chemoresistant nature of PDAC. Therefore, targeting signalling pathways and mechanisms dysregulated during PDAC development has emerged as a new possibility and has opened the door for more personalized treatments. In the last years, the strategy of combining targeted agents with chemotherapy has been widely explored; however, although successfully introduced in multiple solid cancer types, targeted therapy failed to demonstrate any clinical benefit for pancreatic cancer patients. The only exception, erlotinib (Traceva), although only moderately improving OS, provided bases for further exploration of therapeutic possibilities. Huge heterogeneity and complexity of PDAC is regarded to be a major clinical obstacle in the development of successful therapies. Targeting individual molecules is not a sufficient approach, as it is counteracted by upregulation of members of adjacent pathways, contributing to therapy failure. Therefore, strategies combining chemotherapy with targeting multiple targets could considerably diminish this drawback. However, unpredictable adverse events of such a broad interference should not be neglected. Hitherto, most of the conducted studies were designed based on gemcitabine activity. Considering that gemcitabine is no longer the drug of reference, the focus of future studies should be placed on targeted therapies involving Abraxane or FOLFIRINOX, potentially improving achieved outcomes. In PDAC, high mutational variability is observed not only between patients, but also throughout individual samples. Therefore, another major flaw of current clinical trials is the lack of patients' selection and classification into prognostic subpopulations. In fact, less than 10% of conducted studies selected their patients on the basis of predictive molecular markers [287]. The individualised molecular pancreatic cancer therapy (IMPACT) trial is currently being evaluated in order to stratify patients and allow for more personalized treatments [288]. Additionally, a recent meta-analysis has shown that only a small subset of trials (40%) have been conducted after confirming drug efficacy in thorough pre-clinical studies. A small percentage of studies (30%) formulated their hypothesis based on in vivo studies, whereas the vast majority was based on in vitro, cell line studies [289]. Therefore, although most of the studies

demonstrated promising results during preclinical evaluation, the vast majority failed to proceed to more advanced clinical studies due to the lack of efficiency. Therefore, better models should be developed to more accurately recapitulate human disease and make pre-clinical studies more relevant.

On the other hand, novel, potent therapeutic targets should be explored. Considering the high variety of miRNAs aberrantly expressed in PDAC and their role in the control of cell proliferation, invasion and apoptosis, the strategy of altering their expression and activity in order to prevent cancer development and progression seems promising. Synthetic nanoparticle delivery of miRNAs, which are downregulated in cancer tissues, as well as inhibition of overexpressed miRNA, mainly with the use of natural agents has been explored. Both approaches showed promising in vitro and in vivo results; however, we are currently lacking knowledge about possible adverse events. Considering that each miRNA has multiple targets, their alteration might cause unpredictable modifications in many pathways, contributing to fatal consequences. Therefore, more advanced pre-clinical and clinical studies are needed to fully elucidate the potential of miRNAs modulation in PDAC therapy. Boosted research and clinical studies should be also focused on the role of pancreatic cancer stem-like cells, a subpopulation of slow-cycling highly metastatic cells showing increased chemoresistance. The ability to control this subpopulation of cancer cells, responsible for enhanced aggressiveness and invasion potential, could be of great clinical value. If successful, novel strategies targeting this subpopulation would make a breakthrough in PDAC therapy.

Altogether, pancreatic cancer is a complex disease that should be managed with an integrative approach. In order to fulfil the goal set by clinicians and scientists to double PDAC patients' survival by 2020, multidisciplinary strategy, determining best palliative techniques and tailoring specific therapeutic strategies aimed at specific subpopulations of patients is of crucial importance. Close collaboration between oncologists, radiologists and surgeons would allow for accurate patients' classification into proper modality. Disease stage, but also mutations, performance and nutrition status should also be considered.

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Abbreviations

ARBs	Angiotensin II receptor blockers
BRPC	Borderline resectable pancreatic cancer
CA19-9	Carbohydrate antigen 19-9
CAR T	Chimeric antigen receptor T
CXC	Chemokine
CDF	Difluorinated-curcumin
CDKN	Cyclin-dependent kinase inhibitor
CRP	C-reactive protein
CSF1R	Colony stimulating factor 1 receptor
CT	Computed tomography;
CTGF	Connective tissue growth factor
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
DIM	3,3′-diindolylmethane
DDL4	Delta like canonical notch ligand 4
ECE1	Endothelin converting enzyme 1
ECF	Extracellular matrix
EGF	Epidermal growth factor

FAK	Focal adhesion kinase
FDA	Food and Drug Administration
FLT3	Tyrosine-protein kinase
Gy	Gray
HA	Hyaluronic acid
hENT	Human equilibrative nucleoside transporter
IGF1R	Insulin-like growth factor 1 receptor
JAK	Janus kinase
LAPC	Locally advanced pancreatic cancer
LN	Lymph-node ratio
LV	Leucovorin
MAGE-A3	Melanoma-associated antigen 3
MAPK	Mitogen-activated protein kinase
MLH1	MutL homolog 1
neoCRT	Neoadjuvant chemoradiotherapy
OS	Overall survival
PanIN	Pancreatic intraepithelial neoplasia
PARP	Poly ADP ribose polymerase
PD	Pancreaticoduodenectomy
PDAC	Pancreatic ductal adenocarcinoma
PDGFR	Platelet-derived growth factor receptor
PD-L1	Programmed death-ligand 1
PFS	Progression free survival
PS	Performance status
PSCs	Pancreatic stellate cells
PV	Portal vein
RT	Radiotherapy
SMAD4	Mothers against decapentaplegic homolog 4
SMV	Superior mesenteric vein
SOCS1	Suppressor of cytokine signalling 1
PUMA	p53 upregulated modulator of apoptosis
TK	Tyrosine kinase
TNM	Tumour node metastasis
VEGFR	Vascular endothelial growth factor receptor

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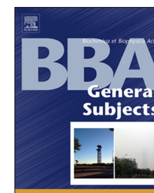
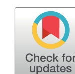


Mini Review

ABC transporters as cancer drivers: Potential functions in cancer development



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ABSTRACT

Background: ABC transporters have attracted considerable attention for their function as drug transporters in a broad range of tumours and are therefore considered as major players in cancer chemoresistance. However, less attention has been focused on their potential role as active players in cancer development and progression.
Scope of review: This review presents the evidence suggesting that ABC transporters might have a more active role in cancer other than the well known involvement in multidrug resistance and discusses the potential strategies to target each ABC transporter for a specific tumour setting.
Major conclusions: Emerging evidence suggests that ABC transporters are able to transport bioactive molecules capable of playing key roles in tumour development. Characterization of the effects of these transporters in specific cancer settings opens the possibility for the development of personalized treatments.
General significance: A more targeted approach of ABC transporters should be implemented that considers which specific transporter is playing a major role in a particular tumour setting in order to achieve a more successful outcome for ABC transporters inhibitors in cancer therapy.

1. Introduction

ATP-binding cassette (ABC) transporters belong to the most conserved protein superfamily, expressed from eukaryotes to vertebrates. Because of their ubiquitous expression, ABC transporters play crucial roles in the functioning of all the organisms.

ABC transporters utilize the energy derived from ATP hydrolysis in order to translocate specific substrates or regulate the activity of membrane channels. In the majority of ABC transporters, ATP hydrolysis is mediated by two nucleotide-binding domains (NBDs), which closely interact with two transmembrane domains (TMDs). Conformational changes occurring at the level of NBDs, upon ATP hydrolysis are further transmitted to TMDs, which bind a specific substrate and translocate it across the biological membranes [1].

The human ABC transporters superfamily lists 48 members distributed into seven subfamilies (ABCA-G). Usually localized in cellular plasma membrane, ABC transporters have been also reported to be expressed in the membranes of mitochondria, Golgi and endoplasmic reticulum [2]. Being responsible for the translocation of several substrates across these membranes, including steroids, phospholipids, glycolipids or xenobiotics, ABC transporters are engaged in diverse physiological processes such as membrane homeostasis, lipid trafficking, cell signalling, cell detoxification and drug resistance [3].

Despite the fact that a lot of emphasis has been placed on investigating the role of ABC transporters as protective pumps from exogenous compounds, xenobiotic excretion has been recently suggested not to be the primary function of these proteins [4,5]. Various other physiological roles have been assigned to ABC transporters; inter alia export of fatty acids, cholesterol, peptides and sterols, as well as defence against oxidative stress, detoxification and antigen presentation (Fig. 1) [6]. Notably, it has been shown that some members of this superfamily are able to translocate endogenous lipids to actively influence lipid homeostasis, lipid trafficking and signalling. These are crucial processes for cell functioning and, more importantly, they are involved in the development of multiple pathologies [7]. As the confirmation of the importance of ABC transporters in human physiology, the mutations or failure of nearly 50% of known ABC transporters are considered as the molecular basis of a plethora of human diseases (Table 1) [8].

In this review, we summarize all emerging evidence that suggests that ABC transporters play a more active role in cancer biology and progression. We will also suggest that revised strategies should be carried out to target these molecules in disparate cancer settings. This will make possible to obtain better results than those achieved so far

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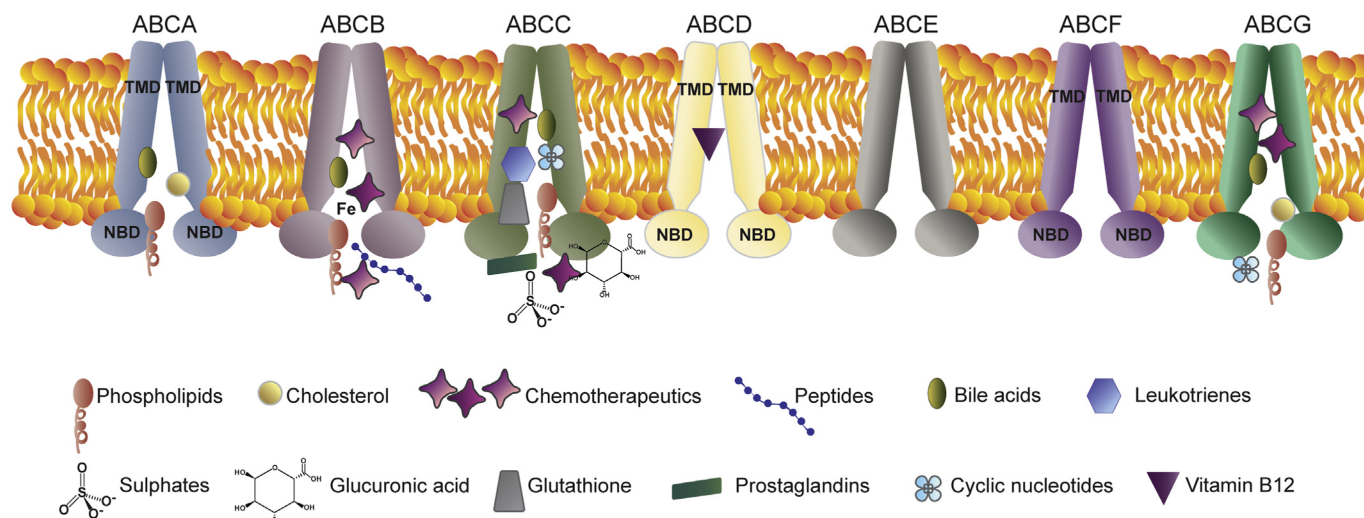

Fig. 1. ATP-binding cassette family of transporters utilize the energy derived from the hydrolysis of ATP to translocate a variety of lipophilic endogenous substrates outside the cells.
TMD, transmembrane domain; NBD, nucleotide-binding domain; Fe, iron.

focusing only on their involvement in drug efflux.

2. ABCA subfamily

Most of the ABC transporters in this subfamily are involved in lipids transport and homeostasis and in the regulation of membrane trafficking and function [9]. ABCA1 is involved in reverse cholesterol transport from the cells to circulating high-density lipoprotein (HDL), as well as phospholipids transport to the plasma membrane [10]. Similarly, an excessive cholesterol influx mediated by low-density lipoprotein (LDL), promotes overexpression of ABCA2, ABCA3 and ABCA7 proteins, suggesting that these transporters play a pivotal role in maintaining a healthy cholesterol homeostasis within the cells. Moreover, ABCA2 has been found to be highly expressed in neuronal cells where it regulates cholesterol homeostasis by modulating the expression of low-density lipoprotein receptor (LDLR) [11] and ABCA3 has been reported to efflux cholesterol in the alveolar cells [12]. In addition, the cluster of highly conserved ABCA5-related transporters including ABCA6, ABCA8, ABCA9 and ABCA10 is also involved in cholesterol and lipid efflux [13]. Interestingly, ABCA4 mediates transport of molecules essential for retinal photoreceptor cells. ABCA4 has been found expressed predominantly in photoreceptors, where it transports retinal and other vitamin A derivatives, suggesting a key role in the visual process [108].

2.1. ABCA subfamily and role in disease

In the ABCA subfamily, defective ABCA1 is linked to Tangier disease, characterised by lack of circulating high-density lipoprotein (HDL). In this recessive condition, a mutation of the *ABCA1* gene disrupts the outflow of free cholesterol, causing a toxic accumulation of cholesteryl esters (CE) within the cells [14]. ABCA7 is involved in the autoimmune disease affecting exocrine glands, known as Sjögren syndrome [15]. Furthermore, due to its role in the transport of Vitamin A and derivatives in photoreceptor cells, *ABCA4* mutations are linked to various forms of retinopathies, like retinitis pigmentosa and retinal degeneration [16]. Different members of the ABCA subfamily such as ABCA1, ABCA2, ABCA5 and ABCA7 seem to play a role in the pathology of neurodegenerative disorders and in particular Alzheimer's disease [17]. ABCA12 is a lipid transporter expressed by keratinocytes and different mutations of the *ABCA12* gene account for different types of congenital ichthyoses, including the most severe form, called harlequin ichthyosis [17].

Transporters of the ABCA subfamily have also been linked to tumour progression and poor prognosis. ABCA2 plays a role in drug efflux and thus it seems to be responsible for multidrug resistance in different cancer types such as lung cancer and estrogen-dependent cancers [18,19]. In addition, overexpression of ABCA2 together with ABCA3 correlates with poor prognosis in infant acute lymphoblastic leukaemia [20]. Similarly, amplification of the *ABCA13* gene is reported to confer poor prognosis in gastric adenocarcinoma where it increases the risk of developing lymphnode metastases [21]. Elevated *ABCA13* mRNA levels are also linked to reduced overall survival in patients with metastatic serous ovarian carcinoma [22]. This evidence indicates a possible role of ABCA13 in tumour metastasis and invasion [17].

3. ABCB subfamily

The ABCB subfamily is the most diversified, containing full and half transporters, with specificity for a wide range of substrates such as iron, peptides and drugs. The most characterised and the first described ABC transporter is ABCB1 (also known as P-glycoprotein or multidrug resistance protein 1, MRP1), a widely expressed protein with a broad spectrum of substrates and known to be responsible for the development of chemoresistance in cancer cells [2]. Other members of the ABCB subfamily, e.g. ABCB4 or ABCB11, exhibit higher substrate specificity, transporting phosphatidylcholine and bile salts. The endoplasmic reticulum membrane half-transporters ABCB2 and ABCB3 participate in MHC I-dependent antigen presentation [2]. ABCB4 is a transporter involved in lipid homeostasis. Predominantly expressed in the liver, ABCB4 mediates the transport of phosphatidylcholine from the canalicular membrane of hepatocytes to the biliary tree, reducing the toxicity of bile salts [23]. ABCB6-8 are yet to be fully characterised; nevertheless, together with ABCB10, they are speculated to be mitochondria-localized transporters involved in the transport of metals, especially iron, across mitochondrial membranes, contributing to tightly regulate iron metabolism and homeostasis. These mitochondrial transporters also translocate peptides, proteins and heme across mitochondrial membranes [2]. Furthermore, ABCB8 and ABCB10 seem to be involved in protection of cells from oxidative stress. ABCB8 has been reported to function as an ATP-dependent potassium channel (K_{ATP}) in rat cardiomyocytes, where it contributes to ablate oxidative stress damages leading to cell death [24,25]. ABCB10 is highly expressed in tissues exposed to elevated oxidative stress, like haematopoietic tissue,

Table 1

Summary of main ABC transporters families, physiological role and role in disease progression and development of multidrug resistance in different cancer types. The table indicates tissues where each transporter is mainly expressed and substrates exported in normal (neutral) conditions. The clinical significance indicates diseases associated with the mutated or defective ABC transporters. In addition, for each transporter overexpression in specific cancer types is listed. Overexpression of a particular ABC transporters in this table is linked to a poor prognosis.

ABC transporter	Tissue expression [90,91]	Natural substrates	Clinical significance [2,90–92]	MDR involvement [93]
ABCA				
ABCA1	Lung, colon, liver, brain, testicles	Phospholipids, phosphatidylcholine, phosphatidylserine, sphingomyelin, cholesterol [94]	Glioma, lung, testis, liver, colorectal, pancreatic, breast, renal cancer, Tangier disease	Cisplatin
ABCA2	Nervous system	Cholesterol [95]	Alzheimer's disease, melanoma, breast, breast, liver, colon cancer, leukaemia	Mitoxantrone, estramustine, methotrexate
ABCA4	Photoreceptors	Vitamin A, phosphatidylethanolamine [94]	Autosomal-recessive disease Stargardt macular dystrophy, fundus flavimaculatus, cone-rod dystrophy, retinitis pigmentosa, age-related macular degeneration, breast, ovarian cancer	None identified
ABCA7	Bone marrow Brain, kidney, colon, lung pancreas	Phosphatidylserine, β-amyloid peptides [94]	Melanoma, lung, cervical, stomach, endometrial, colorectal, pancreatic, breast cancer, Alzheimer's disease	None identified
ABCB				
ABCB1	Brain, blood-brain barrier, colon, liver, kidney, testis, placenta, small intestine, pancreas	Steroids, bile acids, lipids, bilirubin, platelet activating factor [96]	Ovarian, breast, colorectal, kidney, adrenocortical cancer, AML	Daunorubicin, epirobicin, doxorubicin, colchicines, paclitaxel, docetaxel, vincristine, vinblastine, imatinib
ABCB4	Liver	Phosphatidylcholine [97]	Liver, lung, pancreatic, renal cancer, melanoma, soft tissue sarcoma	Daunorubicin, digoxin, paclitaxel, vinblastine
ABCB5	Liver, testicles	Interleukin 1b [98]	Renal cancer, melanoma	5- fluorouracil, doxorubicin, ininotecan, topotecan, camptothecin, mitozantrone
ABCC				
ABCC1	Kidney, colon, pancreas, lymph nodes, liver, testis, brain, blood-brain barrier, breasts, spleen,	Lysophosphatidylinositol (LPI), leukotriene C4, prostaglandins, sphingosine-1-phosphate [99], glutathione, glutathione disulphide [100]	Breast, lung, ovarian or prostate cancer, neuroblastoma	Anthracyclines, vinca alkaloids, camptothecins, daunorubicin, imatinib, etoposide, vincristine, vinblastine, methotrexate
ABCC2	Brain, lymph nodes, liver, colon, kidney, lung, testis, breasts, pancreas	Bilirubin, leukotriene C4 [2], glutathione, glucuronate and sulfate conjugates [101]	Colorectal, liver, lung, gastric cancer, Dubin-Johnson syndrome	Doxorubicin, carboplatin, cisplatin, irinotecan, epirubicin, paclitaxel, vinblastine, topotecan, vincristine
ABCC3	Pancreas, liver, lymph nodes, lung, adrenal glands, colon, testis, spleen, small intestine	GSH [96], prostaglandins, leukotriene C4 (LT_4) [102]	Pancreatic, liver, lung, colorectal, stomach, renal, breast cancer	Etoposide, methotrexate, teniposide, vincristine
ABCC4	Brain, testis, colon, kidney adrenal glands, pancreas, liver, ovary, lung, spleen, breasts, skin, heart	Cyclic nucleotides, prostaglandins, trombocane A2, steroids, GSH conjugates, folate, urate [103]	Prostate, renal,liver, lung, breast, ovarian, stomach cancer, neuroblastoma	5-Fluorouracil, 6-mercaptopurine, Irinotecan, methotrexate, gemcitabine, topotecan, vinblastine
ABCC5	Lymph nodes, pancreas, kidney, testis, brain, colon, liver, heart, muscles	Cyclic nucleotides (cAMP and cGMP), folic acid, glutamate conjugates, *N*-acetylaspartylglutamate, hyaluronan [104]	Lung, urothelial, breast, cervical, renal, liver, pancreatic cancer, glioma	Gemcitabine, methotrexate, 6-mercaptopurine, doxorubicin, 5-fluorouracil
ABCG				
sABCG1	Pancreas, liver, colon, kidney, brain, lung, lymph nodes, testis	Phospholipids, cellular sterols [105]	Lung, renal, breast, endometrial, prostate, colorectal, cervical, pancreatic cancer, glioma	Doxorubicin
ABCG2	Intestine, testis, colon, placenta, liver, kidney, small intestine	Phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine [78], Sphingosine 1-phosphate GSH, androgens, haem, flavonoids, HIV-1 protease inhibitors [106]	Liver, testis, prostate, renal, non-small-cell lung cancer, glioma, Alzheimer's disease	Daunorubicin, doxorubicin, irinotecan, mitoxantrone, methotrexate, epirubicin, etoposide
ABCG4	Brain, endocrine, testis, colon, liver, kidney	Esmosterol and amyloid-β [107]	Glioma, melanoma, thyroid, head and neck, renal, testis, ovarian, endometrial, non-small-cell lung cancer	None identified

and in the heart where it plays a pivotal role in protecting cells from mitochondrial oxidative damage [26,27]. Transporters of the ABCB family also play a role in intracellular peptide transport (e.g. ABCB2 and ABCB3) and antigen presentation, DNA repair and chromosome recombination [28].

3.1. ABCB subfamily and role in disease and cancer progression

Mutations in *ABCB4* and *ABCB11* are responsible for progressive intrahepatic cholestasis (PFIC) [29]. Nevertheless, ABC transporters are attracting interest as key players in carcinogenesis and their activity often correlates with cancer progression and aggressiveness. As an example, ABCB1 is the best characterised multidrug resistance protein, being the first human ABC transporters to be cloned [2,30]. ABCB1 is known to transport a variety of hydrophobic drugs outside the cancer cells thus conferring chemoresistance to numerous tumour types, such as breast cancer, pancreatic cancer, lung cancer, hepatocellular carcinoma and neuroblastoma, leading to treatment failure and consequent tumour relapse [2,31]. Bebawy and colleagues highlighted a novel mechanism in which membrane microparticles (MPs), mediating intercell communication, can transfer ABCB1 from chemoresistant cells to sensitive ones. The latter are thus able to acquire drug resistance properties, and this non-genetic acquisition of multidrug resistance could explain metastatic spread and instruction of malignant cells in distant sites [31,32]. ABCB1 expression has been associated with tumour phenotype in colorectal cancer and soft tissue sarcomas, and its overexpression has been also linked with the progression of lymph node metastases. ABCB1 expression was also reported to be induced and elevated in chemoresistant breast and ovarian cancers [33,34]. Furthermore, ABCB1 is involved in the resistance to apoptosis, which is one of the hallmarks of cancer cells. In fact, inhibition of ABCB1 transporter results in cell cycle arrest and induction of apoptosis in leukaemia and colon cancer [35], whereas its overexpression leads to cells being less responsive to apoptotic stimuli [36]. Platelet activating factor (PAF) activity has also been associated with ABC transporters in the regulation of apoptosis. ABCB1 activity exporting PAF has been reported to enhance the anti-apoptotic signals by increasing the activity of proteins as BCL-2 or BCL-xl. Therefore, inhibition of PAF release may enable to make the cells more vulnerable to apoptosis [37]. Moreover, ABCB5 is responsible for interleukin 1b (IL1b) secretion, inducing the pro-inflammatory CXCR1 pathway [38].

4. ABCC subfamily

The ABCC subfamily is most known for containing the majority of drug transporters and multidrug resistance proteins (MRPs), as well as the cystic fibrosis transmembrane conductance regulator (CFTR/ABCC7), important regulator of chloride ion export [37]. ABCC transporters are also involved in lipid trafficking. As an example, ABCC1 exports lysolipids, such as sphingosine-1-phosphate (S1P) and lysophosphatidilinositol (LPI), both important signalling molecules and intracellular second messengers in tumour cell proliferation [39,40]. Classes of lipids like prostaglandins, together with steroid conjugates, folate and cyclic nucleotides are among the different signalling molecules exported by ABCC4 [41]. Together with leukotrienes, prostaglandins are responsible for the leak of vascular endothelium, contributing to cancer metastasis [42]. In addition, enzymes involved in prostaglandins synthesis, such as cyclooxygenase-2 (COX2), are highly expressed during cancer-related inflammation, and ABC- transported prostaglandins and leukotrienes influence inflammatory responses, as shown in mice lacking ABCC1 gene [43]. ABCC10 is known to act as a lipophilic anions transporter in physiological conditions, playing a role in detoxification processes.

Implication of various ABCC transporters in cell migration and invasion has also been reported. Most notably, migration of dendritic cells has been shown to be influenced by ABCC1 and ABCC4 activity in mice and human tissues respectively and their downregulation in vitro highly reduced dendritic cells migration [44]. Members of this family, such as ABCC5 and ABCC8, transport nucleotide and nucleoside analogs. Interestingly, ABCC8 has been recently shown to play a role in releasing an important mediator of chemotaxis, cAMP, synthetised and stored in microvesicular bodies and microvesicles in *Dictyostelium discoideum* [45].

4.1. ABCC subfamily and role in disease and cancer progression

Mutations in the gene of cystic fibrosis transmembrane regulator protein (*CFTR/ABCC7*), result in the development of cystic fibrosis defined by defective pancreatic secretions [2]. Mutated *ABCC2* causes the recessive liver dysfunction known as the Dubin-Johnson syndrome, linked to a defect in the excretion of bile acids [46].

Due to their role in multidrug resistance and drug efflux, members of the ABCC subfamily are also known as multidrug-resistance proteins (MRPs) and are found overexpressed in many cancer types where they play a key role in disease development and tumour progression. ABCC transporters contribute to cancer chemoresistance and treatment failure by exporting different classes of drugs, from amphipathic anions and non-ionic lipophilic compounds, including doxorubicin-related drugs (ABCC1/MRP1) to hydrophobic and amphipathic drugs conjugated with sulphates or glutathione and glucuronic acid [2,4].

Many of ABCC transporters, e.g. ABCC1, ABCC2, ABCC3, ABCC4, ABCC6, ABCC10 and ABCC11 are able to export Leukotriene C4 (LTC4) outside the cells [47]. Leukotrienes activate GPCRs, triggering signalling pathways, upregulated in several cancers that promote tumour cell proliferation and survival. Due to the proved contribution of LTC4 to pancreatic ductal adenocarcinoma (PDAC) progression, the leukotrienes-ABCC activated signalling pathways have been widely studied as potential drug targets. More specifically, ABCC1, ABCC2 and ABCC3 showed specificity towards leukotriene C4 translocation, whereas ABCC4 possesses the ability to transport prostaglandins and PGA_2 or thromboxane A_2 [2]. In particular, in addition to the direct inhibition of specific ABCC transporters, the inhibition of arachidonate 5-lipoxygenase (ALOX5), an enzyme upstream of LCT4, has been demonstrated to be effective in PDAC mouse models [48]. Therefore, the main inflammatory prostaglandin- and leukotrienes-mediated pathways, together with arachidonic acid and COX2, which are involved in their synthesis, are considered to play a fundamental role in cancer development. Arachidonic acid and COX2, are often found overexpressed in tumour samples, and, together with other phospholipids and molecules participating in prostaglandin and leukotrienes synthesis, they have attracted the interest of researches as potential pharmacological targets [49]. In PDAC, the prostaglandin-mediated tumour progression involves the activation of PI3K-Akt signalling pathway, increased expression of the vascular endothelium growth factor A (VEGFA) and consequent stimulation of angiogenesis in support of the inflammatory environment [50]. All these mechanisms contribute to promoting an inflammatory environment, supporting cancer progression.

Furthermore, ABCC10 seems to be involved in the development of chemoresistance in colorectal and breast cancer progression [13]. In neuroblastoma patients, overexpression of ABCC1 and ABCC4 is predictive of poor clinical outcome. ABCC1 is involved in the development of chemoresistance, as well as playing a role in promotion of cell proliferation and resistance to apoptosis [51], while ABCC4 transports signalling molecules relevant for cancer progression, like leukotrienes and prostaglandins [52,53]. Similarly, ABCC2 and ABCC3 are known to contribute to progression and poor prognosis of non-small-cell lung cancer and breast cancer [54,55]. Despite many studies suggesting the involvement of ABCC transporters in cell migration, no direct relationship between their expression and metastatic potential has been established yet. Nonetheless, an elevated expression of ABCC1 and ABCC4 has been observed in the cells dissected from metastatic tissues and metastatic lymph nodes compared to the cells derived from primary

tumours [56], suggesting a possible contribution of individual ABC transporters in the metastatic spread.

5. ABCD subfamily

The ABCD subfamily comprises four members which are half transporters and function as homodimers, with ABCD1-3 mainly localized in peroxisomes membranes and known to translocate very long chain fatty acids (VLCFA) into these organelles [57,58]. ABCD4 instead, has been reported to be residing in the endoplasmic reticulum (ER) and lysosomes where it plays an important role in the release of Vitamin B12 into the cytosol [59].

5.1. ABCD subfamily and role in disease

Diseases associated with mutations of members of the ABCD subfamily mainly involve peroxisomal dysfunctions. Different mutations of *ABCD1* are associated with X-linked adrenoleukodystrophy, which results in the toxic accumulation of VLCFA in tissues. Defects in ABCD3 have been recently identified to be associated with hepatosplenomegaly, while *ABCD4* mutations have been found in disrupted Vitamin B12 metabolism [57].

6. ABCE and ABCF subfamilies

To date, very little information is available about members of the ABCE and ABCF subfamilies, although ABCE1 seems to be a highly conserved protein in prokaryotes and eukaryotes. ABCE1 is formed by only two nucleotide binding domains (NBDs) and therefore, missing the transmembrane domain (TMD), it does not function as a transporter. Instead, it plays a fundamental role in cell division and initiation of protein translation [60,61]. Similarly, ABCF members do not function as transporters but seem to be involved in translational regulation [62].

7. ABCG subfamily

ABCG family members, especially ABCG1, are associated with the export of phospholipids and cholesterol, in particular from cholesterol-loaded macrophages to HDL acceptors [63]. ABCG2 is known as breast cancer resistance protein (BCRP) and plays a role in multidrug resistance, although its physiological role has been also described in human kidney as a urate exporter [64]. ABCG4 functions as a lipid exporter and localises mainly in the central nervous system, while ABCG5 and ABCG8 are mainly expressed in enterocytes, where they limit plant-derived cholesterol absorption, and in canalicular membrane of hepatocytes where they help exporting sterols through the bile ducts, back to the intestinal lumen [64].

7.1. ABCG subfamily and role in disease and cancer progression

Because of their important role in regulating cholesterol absorption in the gut and liver, mutations of the genes *ABCG5* and *ABCG8* in liver and gastro-intestinal (GI) tract cause toxic intracellular cholesterol loading in patients affected by sitosterolemia [65]. Similarly, because of its role as urate exporter, mutations of *ABCG2* have been linked to the accumulation of urate crystals in the blood and development of gout [66]. Nevertheless, ABCG2 is mostly known for its role in multidrug resistance, being first described as breast cancer resistance protein or BCRP [67]. ABCG2 is found overexpressed in numerous drug-resistant cancers including breast, ovarian, liver, lung and melanoma and it correlates with poor prognosis. In addition, ABCG2 is found particularly overexpressed in a subpopulation of slow-cycling cancer-stem like cells with self-renewal capacity and high chemoresistance [68].

8. ABC transporters beyond chemoresistance

Tumour chemoresistance represents a major challenge in the treatment of malignancies and several ABC transporters play a pivotal role in the development of multidrug resistance (MDR). MDR is characterised by upregulation of membrane-associated ABC transporters among which the most widely investigated are P-glycoprotein ABCB1 (MDR1), multidrug resistance protein ABCC1 (MRP1) and breast cancer resistance protein ABCG2 (BRCP) [4]. Overexpression of multidrug resistance transporters in cancer patients correlates with poor prognosis and lower survival rates mostly due to the failure to respond to chemotherapy. It has been hypothesised that the drug efflux mediated by ABC transporters in chemoresistance mechanisms is the result of their ability to export a diverse array of endogenous compounds and signalling molecules and, concomitantly, chemotherapy drugs [69]. Nevertheless, the mechanisms at the base of this process are still unknown and it is yet to be investigated whether cancer cells do overexpress MDR proteins in response to chemotherapy.

The role of ABC transporters in tumorigenesis depends on their involvement in the secretion of bioactive molecules and the transport of lipids that contribute to the activation of important signalling pathways leading to cancer progression. Lipid transport by various members of the ABC transporter family suggests an active role of these proteins in cancer progression, beyond drug resistance mechanisms. Work conducted by our group has investigated the role of ABCC1 in the transport and release of LPI in the extracellular milieu where, interacting with G-protein coupled receptor 55 (GPR55), it activates signalling pathways involved in cancer progression [70,71].

Our understanding of the role of ABC transporters in cancer is still very limited. However, we speculate that the ABC transporters play a key role in transporting lipids, prostaglandins, leukotrienes and other signalling molecules to promote cancer progression and, coincidentally, broad -spectrum transporters confer chemoresistance by exporting therapeutic drugs. Cancer cells overexpress ABC transporters and this often correlates with poor prognosis and increased tumour aggressiveness, but the mechanisms regulating ABC transporters overexpression are still mainly unknown. The majority of patients' databases are based on the level of mRNA expression and only few data are available at the protein level. This creates a discrepancy between mRNA and actual protein levels because overexpression of ABC transporters is often regulated post-transcriptionally by miRNAs [72]. At the gene level, it is important to outline that overexpression of ABC transporters in cancer cells, just as metabolic reprogramming, is driven by oncogenes. In neuroblastoma *MYCN* regulates the expression of ABCC1 and ABCC4 [73] while *P53*, together with *P63* and *P73*, seems to be involved in the regulation of ABCB1 expression [74].

9. Role of ABC transporters in cancer biology

Oncogene-driven metabolic reprogramming is characterised by enhancement of glycolysis at the expense of oxidative phosphorylation. This process guarantees a rapid, although less efficient, ATP production, with the main advantage of reducing the generation of potentially damaging reactive oxygen species (ROS), thus promoting rapid cancer cell proliferation. In cancer metabolic reprogramming, lipid metabolism plays an important role for tumour progression as lipids are used not only as signalling molecules activating tumorigenic pathways, but also as building blocks to sustain enhanced biogenesis and anabolic processes leading to tumour cell proliferation. Cancer cells have a distinctive plasma membrane lipid composition, which is different from normal cells, and here we argue that ABC transporters play an important role in maintaining this structure. Membrane lipid composition in malignancies is a unique signature not only distinguished from normal non-cancerous cells, but that also allows to discriminate between different tumour types, from benign compared to malignant cancers, and to identify the cancer stages, whether localized or

metastatic [75]. Other than being responsible of maintaining the lipid homeostasis, studies conducted using *Saccharomyces cerevisiae* have demonstrated that ABC transporters contribute to support plasma membrane asymmetry and stability [76,77]. Acting as lipid flippases, some ABC transporters regulate the level of membrane fluidity by increasing the transport of unsaturated fatty acids, thus decreasing membrane fluidity and permeability [78,79]. This important function has been primarily reported for ABCB1 and ABCB3, ABCC1 and ABCG2, because of their ability to flip phospholipids, mainly phosphatidylserine, phosphatidylcholine and phosphatidylethanolamine, across the lipid bilayer [7]. Similarly, other than just transporting cholesterol, ABCA1 and ABCG1 activity has been linked to the translocation of phospholipids across the plasma membrane [78]. The asymmetrically arranged lipids forming the lipid bilayer may be shifted across the membrane during differentiation or in pathological conditions, such as apoptosis, causing the loss of asymmetry [80].

Furthermore, cholesterol metabolism, is upregulated in cancer, which could explain the reason why ABC transporters are often overexpressed in cancer cells. Cholesterol is a fundamental component of the peculiar plasma membrane of cancer cells, but it is stored in large amounts also within the cells and it correlates with tumour aggressiveness in breast and prostate cancers [81,82]. The extrusion of cholesterol from cancer cells might have the same autocrine or paracrine role proposed for signalling molecules. At the same time, proliferating tumour cells release signalling molecules that influence the surrounding tissues and cells in order to create a tightly regulated microenvironment that supports and sustain cancer progression, mainly providing nutrients for an increased anabolic demand. Phospholipids, together with sphingolipids, are synthesized form fatty acids, which levels in turn are controlled by ATP availability, a simultaneous determinant of ABC transporter activity.

10. ABC transporters and tumour microenvironment

Tumour progression is far from being a merely enhanced proliferative capacity of malignant cells. In the past two decades it has become evident that cancer development and propagation is a complex and heterogeneous process, involving extensive metabolic reprogramming and remodelling in order to create the tumour microenvironment [83]. Tumour microenvironment not only provides support to tumour proliferation, but also acts as a physical and biochemical barrier for chemotherapy. Cancer-induced remodelling of the microenvironment and consequent tumour migration to distant sites for metastatic progression is mediated by an extensive network of autocrine and paracrine cell-to-cell communication. Extracellular vesicles (EVs) are secreted in abundance by cancer cells and play a pivotal role in this communication network [84,85]. EVs are classified according to their biogenesis and include microvesicles (MVs) derived from blebbing of the plasma membrane and exosomes, derived from late endosomes. EVs are shed by all cells in the body, including cancer cells, and they are cargoes transporting an array of signals that promote tumour progression, migration and establishment of distant metastatic niches [85]. It has been shown that ABC transporters are present in the membranes of exosomes and MVs. MVs blebbing from chemoresistant human acute lymphoblastic leukaemia cells can transfer ABCB1 to recipient sensitive cells that acquire multidrug resistance [32]. Moreover, multidrug resistance proteins seem to be involved in transporting and packing chemotherapy drugs into vesicles that are then exported outside the cells [86]. It has been hypothesised that in cancer cells EVs, MVs and exosomes, transport signals that are then released and promote migration and invasion. This release of chemotactic signals from EVs has been shown to involve ABC transporters [85]. In particular, a mechanism of release has been demonstrated with a recent study by Kriebel and colleagues investigating the role of cAMP released from shed microvesicles in *Dictyostelium discoideum*. Authors showed that MVs synthetise and secrete the cAMP, promoting chemotaxis, via the ABC transporter ABCC8 [45]. Similar mechanisms might regulate the release of signals from tumour EVs to promote extravasation and metastatic spread to distant sites as well as reprogramming of cells in the tumour microenvironment. In solid malignancies characterised by a dense desmoplastic stroma, like pancreatic cancer adenocarcinoma (PDAC) and breast cancer, the tumour microenvironment contributes to the development of chemoresistance which, in turns, enhances the chance of tumour relapse. Desmoplastic stroma is composed by a heterogeneous array of cell types among which tumour associated macrophages (TAMs) are known to overexpress MRP1 (ABCC1) and MRP3 (ABCC3), thus further contributing to both tumour development and chemoresistance [87]. Cancer-mediated reprogramming of the tumour microenvironment though EVs and transfer of MDR also includes remodelling of the immune system in order to escape the organism immune response and enhance cancer survival and progression [88]. Resident macrophages are the first-line immune response to malignant cancer cells, although tumours activate mechanisms to elude this surveillance. Jaiswal and colleagues have elegantly demonstrated how EVs shed by multidrug resistant breast cancer cells can bind inflammatory macrophages and impair their migration and engulfing activity. Instead, impaired macrophages are phagocytised by tumour cells scavenging for nutrients. In addition, pro-inflammatory cytokines released by impaired macrophages can act as attractant stimuli for extravasation of cancer cells and to further recruit TAMs in the establishment of a metastatic niche [88]. These findings suggest a role of ABC transporters in mediating the paracrine signals involved in tumour microenvironment remodelling by transferring MDR to chemo-sensitive neighbouring cells, as well as immune elusive response by reprogramming macrophages activity.

11. ABC transporters and cancer cell energy balance

A particular aspect of ABC transporters functioning in cancer cells needs to be carefully considered. These transmembrane proteins need ATP to function and the more ABC transporters are expressed in a cell, the more ATP is required. Actively proliferating cancer cells are characterised by a rapid metabolic rate and have been reported to rely mainly on glycolysis for energy production, thus, in cancer cells, ATP is a limited and precious resource. Utilisation of this resource by an increased number of ABC transporters in cancer cells must confer a selective advantage, promoting tumour progression despite scarcity of ATP. We hypothesize that one of the strategies to maintain this balance is related to cancer stem-like cells (CSCs), a population of slow cycling cells characterised by self-renewal capacity and elevated tumorigenic potential, that contribute to tumour relapse due to an enhanced chemoresistance [89]. Cancer stem-like cells are quiescent, compared to the fast-growing bulk of the tumour, they rely more on oxidative phosphorylation rather that glycolysis and, as discussed in our recent review, CSCs overexpress ABC transporters [68]. Oxidative phosphorylation provides the cells with more ATP (32 molecules per molecule of glucose) compared to glycolysis (two molecules) which could sustain the elevated expression and activity of ABC transporters. It is therefore evident that more investigation on energy balance in cancer cells is required to explain the importance of maintaining a costly set of ATP-dependent multidrug transporters when energy availability is a crucial element necessary to sustain the high demand of metabolic power of cancer cells.

12. Concluding remarks

In conclusion, in this review we have discussed the role of ABC transporters in cancer progression and highlighted how their involvement in multidrug resistant mechanisms strongly depends upon their physiological function in cancer cell biology. Despite the expanded knowledge on the molecular characterization of ABC transporters and their involvement in chemoresistance, the specific substrates and the

roles of the majority of these proteins are still elusive. In addition, it is worth to note that individual ABC transporters might have different functions in diverse cellular context and diseases [7]. Consequently, we hypothesised that the role and the substrate of a specific ABC transporter might differ in a certain cancer setting compared to its normal physiological function. Some ABC transporters members, are important exporters of lipids, including fundamental signalling molecules promoting cancer progression, cancer associated inflammation and tumour-stoma crosstalk [88]. We propose that overexpression of ABC transporters in cancer cells has a function beyond chemoresistance, which needs to be addressed and revisited. ABC transporters are energetically expensive to maintain for cancer cells that are fast proliferating and mainly relying on glycolysis for the production of ATP, thus we propose that the reason why tumour cells are overexpressing these transmembrane proteins should be further investigated. Moreover, it is paramount to understand the role of ABC transporters in the cancer-associate stroma in solid tumours and the tumour-microenvironment interaction mediated by signalling lipids and other signals excreted by ABC transporters, as well as the role of cancer stem-like cells that overexpress multidrug resistance proteins and play a role in tumour relapse and metastatic spread. In addition, the genetic and epigenetic mechanisms regulating ABC transporters expression are still unknown, especially in the reciprocal interplay between cancer cells, stroma and immune system. Finally, in order to implement a personalized treatment targeting a specific ABC transporter, we need reliable and clinically validated assays to detect the expression of ABC transporter at the protein level. Subsequently, we need more specific and less promiscuous inhibitors that efficiently target a specific transporter and possibly resulting in less toxic effects.

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REVIEW

ATP-binding cassette transporters in progression and clinical outcome of pancreatic cancer: What is the way forward?

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most aggressive diseases and is characterized by high chemoresistance, leading to the lack of effective therapeutic approaches and grim prognosis. Despite increasing understanding of the mechanisms of chemoresistance in cancer and the role of ATP-binding cassette (ABC) transporters in this resistance, the therapeutic potential of their pharmacological inhibition has not been successfully exploited yet. In spite of the discovery of potent pharmacological modulators of ABC transporters, the results obtained in clinical trials have been so far disappointing, with high toxicity levels impairing their successful administration to the patients. Critically, although ABC transporters have been mostly studied for their involvement in development of multidrug resistance (MDR), in recent years the contribution of ABC transporters to cancer initiation and progression has emerged as an important area of research, the understanding of which could significantly influence the development of more specific and efficient therapies. In this review, we explore the role of ABC transporters in the development and progression of malignancies, with focus on PDAC. Their established involvement in development of MDR will be also presented. Moreover, an emerging role for ABC transporters as prognostic tools for patients' survival will be discussed, demonstrating the therapeutic potential of ABC transporters in cancer therapy.

Key words: Pancreatic ductal adenocarcinoma; Multi-drug resistance; ATP-binding cassette transporters; Targeted therapies; Pancreatic ductal adenocarcinoma prognosis; Predictive markers

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Core tip: Pancreatic cancer is one of the deadliest cancers due to its highly aggressive biology and resistance to broad range of therapeutics. Expression of ATP-binding cassette (ABC) transporters by cancer cells is one of the main mechanisms responsible for the lowered drug accumulation. However, the attempts made in multidrug resistance reversal by the inhibition of their activity have not provided satisfactory results in clinical trials. Nevertheless, current knowledge on the role played by ABC transporters in carcinogenesis beyond chemoresistance, could create the opportunity for the development of novel, direct targeted therapeutic strategies. Additionally, the association between ABC transporters expression and pancreatic ductal adenocarcinoma patients' prognosis and response to applied therapies confirms their pharmacological potential.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is one of the most fatal diseases in western world. Although not one of the leading causes of death, PDAC is certainly to be considered amid the most unfavourable cancers, ranking at 4th place in terms of death rate, with a 7%-8% chance of 5-year survival in United States[1]. Despite the progress made in understanding the biology and in the treatment of different cancer types, the mortality of PDAC patients still nearly equals its incidence and has not changed remarkably for the last few decades. The dismal prognosis of PDAC is the result of multiple factors including an aggressive nature, chemo- and radio-resistance and the lack of effective treatments and diagnostic tools. Therefore, when diagnosed, the vast majority of PDAC patients present with metastatic disease, not susceptible for surgery[2]. Only one fifth of the patients have the tumour resected and, unfortunately, most of them eventually relapse. Post-operative chemo- and radiotherapy are usually applied in order to delay tumour recurrence; nevertheless, high resistance and the heterogeneous nature of pancreatic tumours impede its treatment[3].

Pancreatic cancer pathology is a multistep process. It arises as an accumulation of abnormalities, both genetic and physiological, progressing through 3 stages of precursor lesions called pancreatic intraepithelial neoplasias (PanINs) before transforming into a fully differentiated tumour[4]. The substantial number of genetic modifications and consequent dysregulation of the wide range of essential signalling pathways accompanying these processes make PDAC highly heterogeneous[5]. Also, the variability of mutations between patients as well as within the same tumour contributes to its high resistance to applied therapy. High heterogeneity of PDAC is expressed also phenotypically. Genetically diverse subclones, possessing different metabolic and functional characteristics, exist within a tumour. Recent evidence shows that one of the populations acquires characteristics similar to stem cells, which enables it to survive during stressful conditions and is partly responsible for cancer relapse after treatment[6]. Furthermore, PDAC cell plasticity, which plays a role in epithelial to mesenchymal transition (EMT), facilitates metastatic spread and adds to the dismal prognosis[7]. Moreover, one of the main characteristics of PDAC, responsible for therapies' failure, is the formation of dense desmoplastic reaction, influencing cancer progression and impeding drug delivery to the tumour[8]. The interplay between tumour cells and stromal components (pancreatic stellate cells (PSCs), immune cells, cytokines or extracellular matrix proteins) influences cell metabolism, drug delivery and distribution. In addition, the existence of a rich tumour microenvironment (TME), influencing cancer cell functions and favouring chemoresistance, has been recently claimed to be an essential factor in cancer stem cell initiation and promotion[9].

PDAC RESISTANCE TO THERAPIES

On account of PDAC aggressive nature and its resistance to therapies, no successful treatment has been introduced so far[10]. In fact, until recently the gold standard in PDAC treatment was gemcitabine. Applied as a first line therapy drug since 1997, gemcitabine modestly improved patients' perspectives, increasing overall survival (OS) for 6 mo compared to previously used fluorouracil (5-FU)[11]. Since that time, attempts have been made to increase the efficacy of PDAC treatment and prolong patient survival; however, only modest or statistically insignificant improvements have been achieved so far. In the last years, two new drug regimens, ABRAXANE and FOLFIRINOX have been introduced[12,13]. However, their application did not increase OS to a meaningful degree when compared to gemcitabine, at the same time escalating the frequency of adverse events. Nevertheless, both treatments have obtained FDA approval and currently ABRAXANE combined with gemcitabine is acknowledged as a standard first-line therapy for pancreatic cancer. Considering the high number of genes altered during PDAC progression, targeted therapies emerged as a potential therapeutic tool. Many small inhibitors have been developed as single agents or applied in combination with gemcitabine or ABRAXANE to enhance their efficacy[14-18]. However, the vast majority of them failed to improve patients' survival in the clinical settings. Therefore, it remains pivotal to gain better knowledge on the mechanisms of PDAC chemoresistance and to

find novel therapeutic strategies in order to develop more effective treatment regimens.

Among other factors, the failure of PDAC treatment has been attributed to local recurrence and liver metastasis and importantly, to its high chemoresistance, both intrinsic and acquired. The phenomenon called multi-drug resistance (MDR), which is characterized by resistance to a broad spectrum of structurally diversified compounds, has been confirmed as one of the main reasons for the inefficiency of PDAC therapies, leading to tragic health and economic consequences.

There are multiple factors contributing to the development of MDR in pancreatic cancer, such as decreased drug uptake, accelerated drug metabolism and DNA repair, blocking of apoptotic pathways, metabolic changes and the presence of highly resistant stem-like cells. Also, high heterogeneity of the tumour, dense stroma and hypoxia impairing drug delivery and constitutive activation of several signalling pathways, including K-Ras, PI3K/Akt, Notch or NF-κB, with the latter being additionally enhanced during chemo- and radiotherapy, all confer the modest response of PDAC to applied therapies[19-23]. Moreover EMT, frequently observed in PDAC tumours, has been implicated in conferring its resistance. Also, acquired mutations in targeted genes and reactivation of parallel pathways add to the therapy failing. However, in most cases the interplay between several of these processes is essential for chemoresistance development[24]. Additionally, high expression of transmembrane proteins belonging to the ATP-binding cassette (ABC) transporter family in tumour specimens is one of the major factors contributing to increased drug efflux and has been connected with MDR, adding to the poor response of PDAC to treatments[25-28]. Apart from drug extrusion, as integral membrane constituents, ABC transporters normally regulate the distribution of a wide variety of molecules, influencing different pathways and biological processes, which suggests their more direct impact on cell physiology and possibly, carcinogenesis. Therefore, the understanding of the role of ABC transporters both in healthy physiology and in cancer is crucial for the development of specific, potent and safe inhibitors that might be used in PDAC therapy.

ABC TRANSPORTERS AS MULTI-DRUG RESISTANCE MECHANISM

One of the main obstacles in cancer therapy is the resistance, both constitutive and acquired to administered drugs. As aforementioned, one of the processes responsible for drug resistance is the decreased intracellular accumulation of the drugs caused by their efflux from the cells induced by the expression of membrane drug transporters belonging to the ABC family.

The family of ABC transporters is a highly conserved family of proteins, expressed in all organisms, which implies their relevance in many biological functions. To date, 48 human genes and one pseudogene encoding the members of ABC family have been described and grouped into 7 subfamilies (ABCA-G), based on their sequence and structural similarity[29,30]. ABC transporters are integral transmembrane proteins which, by utilizing energy obtained from ATP hydrolysis, which drives the progressive conformational changes in their domains, shuffle molecules across the plasma and intracellular membranes against their gradient[31,32] (Figure 1). The structure of ABC transporters is highly conserved and consists of two hydrophobic transmembrane domains (TMDs), which form a pore in the membrane creating substrate-binding environment linked to two hydrophilic nucleotide-binding domains (NBDs) localized in the cytosol[33,34]. ABC transporters are reported to export a wide variety of structurally diverse endogenous ligands including amino acids, peptides, vitamins, sugars, hormones, ions, lipids and xenobiotics[26,32,35-37]. For example, ABCB1 has been reported to be able to transport more than 200 structurally diversified molecules[38-41]. Additionally, ABC transporters are known to excrete toxins form kidneys, gastrointestinal tract and liver, demonstrating a protective role in those tissues[42]. Few ABC transporters, *e.g.,* ABCC7- cystic fibrosis transmembrane conductance regulator (CFTR) or ABCC8- the sulphonyl urea receptor (SUR1), are not directly involved in transport of molecules across the membrane but use the ATP hydrolysis to regulate the activity of Cl^- and K^+ channels respectively[43]. In healthy physiology, ABC transporters are expressed in a wide variety of tissues, mainly associated with biological barriers (Table 1). As an example, ABCC1 is expressed in kidneys, intestine, ovaries, adrenal glands, colon, stomach, testes, lungs and blood-brain barrier and ABCB1 is mostly expressed in gastrointestinal tract, pancreas, kidneys, brain and adrenal glands, where they are involved in diverse physiological functions and in excreting toxins from the cells[40,44,45]. However, their enhanced levels have been found in different cancer types, suggesting the relevance of ABC transporters in cancer and its chemoresistance. So far, 15 of the transporters have been attributed the role of drug pumps, contributing to MDR *in vitro*[46]. Especially, P glycoprotein (P-gp)/ABCB1, breast cancer resistance protein (BCRP)/ABCG2, multidrug resistance protein 1 (MRP1)/ABCC1 and other members of ABCC subfamily (*e.g.,* ABCC2, ABCC3) have been reported to be responsible for PDAC chemoresistance[47].

Up to date, most research has been focused on P-gp, a member of the ABCB subfamily of transporters[48,49]. It exports a wide variety of molecules of "amphipathic nature" including anthracyclines, HIV-protease inhibitors, calcium channel blockers, steroid hormones, antibiotics, lipids, taxanes and alkaloids[50,51]. P-gp overexpression has been observed in several cancers including ovarian, colon, kidney or adrenocortical cancer, correlating with poor prognosis[52,53]. Additionally,

Table 1 Selected ATP-binding cassette transporters, their normal physiological expression and overexpression in cancer tissues[117,171]

ABC transporter	Tissue expression	Cancer overexpression	Correlation with PDAC survival (5-yr survival)
ABCA			
ABCA1	Lung, colon, liver, brain, testicles	Glioma, lung, testis, liver, colorectal, breast, renal cancer,	H: 21% L: 29%
ABCA7	Bone marrow, brain, kidney, colon, lung pancreas	Melanoma, Lung, cervical, stomach, endometrial, colorectal, pancreatic cancer	H: 38% L: 0%
ABCB			
ABCB1	Brain, blood-brain barrier, colon, liver, kidney, testis, placenta, small intestine, pancreas	Ovarian, breast, colon, kidney, adrenocortical cancer, AML	H: 34% L: 20%
ABCB4	Liver	Liver, lung, renal cancer, melanoma	H: 49% L: 22%
ABCC			
ABCC1	Kidney, colon, pancreas, lymph nodes, liver, testis, brain, blood-brain barrier, breasts, spleen,	Breast, lung, ovarian or prostate cancer, neuroblastoma	H: 13% L: 43%
ABCC2	Brain, lymph nodes, liver, colon, kidney, lung, testis, breasts, pancreas	Colorectal, liver, lung, gastric cancer	H: 29% L: 27%
ABCC3	Pancreas, liver, lymph nodes, lung, adrenal glands, colon, testis, spleen, small intestine	Pancreatic, liver, lung, colorectal, stomach, renal, breast cancer	H: 13% L: 41%
ABCC4	Brain, testis, colon, kidney adrenal glands, pancreas, liver, ovary, lung, spleen, breasts, skin, heart	Prostate, renal, lung, breast, ovarian, stomach cancer	H: 32% L: 23%
ABCC5	Lymph nodes, pancreas, kidney, testis, brain, colon, liver, heart, muscles	Lung, urothelial, breast, cervical, renal cancer, glioma	H: 34% L: 0%
ABCG			
ABCG1	Pancreas, liver, colon, kidney, brain, lung, lymph nodes, testis	Lung, renal, breast, endometrial, prostate, colorectal, cervical, pancreatic cancer, glioma	H: 34% L: 0%
ABCG2	Intestine, testis, colon, placenta, liver, kidney, small intestine	Liver, testis, prostate, renal cancer, glioma	H: 32% L: 23%
ABCG4	Brain, endocrine, testis, colon, liver, kidney	Glioma, melanoma, thyroid, head and neck, renal, testis, ovarian, endometrial cancer	H:43% L: 23%

The correlation between the overexpression of the transporters in PDAC and observed 5-year survival is also demonstrated[117]. H: High expression of the transporter; L: Low expression of the transporter. Statistically significant association is highlighted in bold. AML: Acute myeloid leukaemia; PDAC: Pancreatic ductal adenocarcinoma; ABC: ATP-binding cassette.

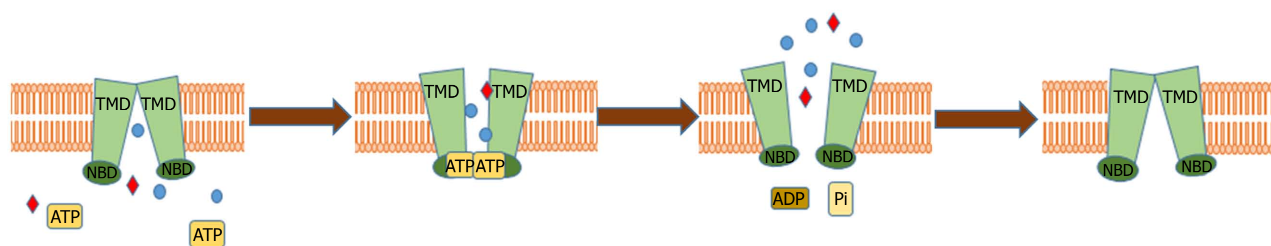

Figure 1 The schematic presentation of the mechanism of ATP-mediated ATP-binding cassette transporter substrate translocation. TMD: Transmembrane domain; NBD: Nucleotide-binding domain.

treatment-induced increase in ABCB1 expression has been noted in acute myeloid leukaemia (AML)[54], breast and high-grade bladder cancer[55]. ABCB1 is known to be responsible for developing drug resistance to neutral and cationic hydrophobic compounds, *e.g.,* to anthracyclines (daunorubicin, doxorubicin), colchicines, taxanes (paclitaxel, docetaxel), vinca alkaloids (*e.g.,* vincristine, vinblastine) and tyrosine kinase inhibitors (imatinib)[56-58].

The main role in xenobiotic transport and drug resistance in many cancers has been attributed to the ABCC subfamily of transmembrane transporters[59], with 9 out of 12 members being involved in MDR[47,59]. The most studied of MDR proteins, ABCC1 (MPR1) has been demonstrated to be expressed in several cancers, including breast, lung, ovarian and prostate cancer, showing the correlation between the expression of ABCC1 and poor patients' outcome[60]. It has been suggested that ABCC1 expression may confer resistance to methotrexate, vinca alkaloids, anthracyclines and camptothecins[47,61,62], influencing drug resistance in plethora of cancers. Additionally, cyclic nucleotides and their analogues (*e.g.,* gemcitabine) may be transported by ABCC4 and ABCC5[62-64], potentially contributing to their ineffectiveness in PDAC therapy.

Resistance to doxorubicin, mitoxantrone, anthracyclines and topotecan (quinolone topoisomerase inhibitor)[65] has been attributed to ABCG2 transporter[66,67], which

functions mainly in the ovaries, brain, liver, prostate, placenta and small intestine[68]. Additionally, increased ABCG2 expression has been reported in pluripotent stem cells, suggesting its role in the maintenance and protection of stem cells[69].

Regardless of the remarkable increase in the knowledge on the ABC transporters structure and MDR induction achieved in the past few decades, the actual function and significance of these proteins is highly underexplored. It is known that in healthy physiology, ABC transporters are involved in drug absorption, distribution and elimination, determining bioavailability of administered drugs. Both apical and basolateral membranes of gastrointestinal tract and biological barriers, in which ABC transporter expression has been demonstrated, need to be penetrated by the drug to reach its target. Therefore, ABC transporters expression may influence pharmacokinetic characteristics of administered chemotherapeutics. Additionally, various other physiological roles have been assigned to ABC transporters such as export of fatty acids, cholesterol, peptides, sterols and xenobiotics. Many ABC transporters are involved in secretion of bioactive molecules and in the transport of signalling lipids, which contribution to cancer progression has been well established. As an example, ABCA1 is involved in reverse cholesterol transport as well as phospholipids transport to plasma membrane[70,71]. Interestingly, recent studies demonstrated ABCC1 as an active player in progression of ovarian and prostate cancer[72,73], by extrusion of lipids (lysophosphatidylinositol, sphingosine 1-phosphate) that have been previously attributed a crucial role in carcinogenesis[73,74]. The changes in cancer cell proliferation, migration, invasion and resistance to apoptosis mediated by the activity of ABC transporters have been also widely documented[75]. Considering that information, attention has been brought to the pivotal role played by ABC transporters in carcinogenesis beyond chemoresistance and to the correlation between their expression with cancer progression and aggressiveness. Nevertheless, this area is still overlooked and more studies need to be focused on this aspect of ABC transporters' activity in order to fully elucidate their role in cancer.

ABC TRANSPORTERS- DRIVERS OF PDAC PROGRESSION?

There have been very limited studies on the role and expression of ABC transporters in pancreatic cancer; however, strong correlation between few of their members and PDAC has been recently suggested. On the basis of mRNA analysis, the expression of ABCC1, ABCC3, ABCC4, ABCC5 and ABCG2 in both pancreatic cancer samples and in healthy pancreas has been demonstrated[76,77] and was correlated with cell resistance to commonly applied chemotherapeutics[78]. At the same time, ABCC6, ABCC8 and ABCC9 could not be detected in any of the studied pancreatic cancer cell lines[79]. Furthermore, more in depth analysis showed that although ABCG2, ABCC1 and ABCC4 levels did not differ significantly between tumour and healthy tissues, ABCC3 and ABCC5 were found to be remarkably overexpressed in PDAC specimens. Moreover, although expression of none of them could be coupled with cancer stage, the differentiation status and tumour grading were related with increased ABCC3 levels and correlated with poor survival, whereas no such correlation could be found for ABCC5.

ABCC3 transporter is involved in transporting of bile salts and organic ions[80,81]. It has been also implicated in mediation of drug resistance, *e.g.,* to vincristine, methotrexate or etoposide; compounds used in clinical studies for PDAC treatments, which demonstrated only marginal effects[82]. Moreover, its expression levels have been correlated with survival of patients after resection, suggesting possible predictive aspect of ABCC3 expression in PDAC.

ABCC5 is involved in transport of nucleotide analogues; therefore, it is tempting to speculate its involvement in excessive efflux of nucleotide analogues-based drugs, such as 5-FU or gemcitabine. In fact, although still controversial, it has been shown that ABCC5 is responsible for gemcitabine resistance in pancreatic cancer[64,79,83]. Analysis of PDAC specimens demonstrated overexpression of ABCC5 transporter in samples resistant to gemcitabine, suggesting its involvement in the decreased efficiency of the drug. Furthermore, exposure of different PDAC cell lines to gemcitabine, as well as 5-FU/gemcitabine combination significantly increased the expression of ABCC5 demonstrating drug induced mechanism of PDAC cell resistance to the treatment[79,84]. Therefore, although not directly associated with PDAC progression, the importance of ABCC5 in PDAC chemoresistance, both inherent and acquired, makes it a valuable drug target for the enhancement of the efficacy of applied therapies.

While the role of ABC transporters in mediating chemoresistance is well established, little is known about their direct, drug-efflux independent contribution to pancreatic cancer progression. Nevertheless, intensive studies in recent years suggest that beyond their role in drug resistance, the biological functions of ABC transporters are more complex. It has been proposed that tumour-promoting functions of ABC transporters are based on their ability to export active signalling molecules and hormones, which by autocrine or paracrine regulation activate cancer cells as well as tumour environment. Increasing interest in this area has demonstrated the significant impact of these proteins on invasion, migration and differentiation of malignant cells[75]. Also, changes in metabolism as well as redox status, characteristics pivotal in PDAC tumorigenesis, may be induced by ABC transporters-released molecules.

One of the major events in PDAC development

is the metabolic switch, which occurs in response to decreased nutrient and oxygen supply[85-87]. Increased glucose dependence and use or aerobic glycolysis for energy production, known as Warburg effect, allows quickly proliferating PDAC cells to survive under harsh conditions and is considered as one of the hallmarks of cancer[88]. Additionally, glutamine dependence and increased protein breakdown add to cancer cell high proliferative abilities. However, a small population of cells with stem-like characteristics, which reside the areas of the tumour lacking oxygen and glucose supply, are known to rely on mitochondrial oxidative phosphorylation rather than glycolysis, which results in increased ATP production. This phenomenon may add to increased activity of ABC transporters observed in cancer cells. Therefore, low oxygen and nutrient supply may contribute to PDAC resistance by increase of the ABC transporters levels and their ATP-dependent substrate transport, suggesting a possible mechanism of hypoxia-induced chemoresistance, tumour maintenance and cancer progression.

Apart from glucose and glutamine addiction, increased lipid metabolism and demand has been recently demonstrated for PDAC[89,90]. Bioactive phospholipids are directly involved in the induction of cancer cell proliferation and thereby, cancer progression[91]. Increase in the levels of saturated lipids helps cancer cells to acquire additional resistance to oxidative stress by consolidating the membranes. Both, de novo lipid synthesis and their increased uptake have been reported in PDAC[92,93]. Moreover, enzymes involved in lipolysis and lipogenesis are overexpressed in PDAC and are usually correlated with poor prognosis[90]. It has been demonstrated by our work that, in prostate and ovarian cancer, ABCC1-transported lysophosphatidylinositol activates GPR55 receptor forming an autocrine loop, which activation triggers signalling cascade inducing cell proliferation[72]. Phospholipids transport has been also reported for another member of ABC transporter family, ABCG1. Therefore, it is tempting to suspect the existence of a similar mechanism, involving ABC transporter-mediated phospholipid activation of cancer cells in PDAC. An essential factor in PDAC cell survival is also cholesterol availability. As a component of lipid rafts, it influences membrane composition and integrity and interacts with membrane-bound proteins, facilitating activation of phosphorylation cascades[90]. The essential role played by cholesterol in PDAC tumorigenesis limits the growth and division of PDAC cells, depending on its availability[94]. A recent study by Mohelnikova-Duchonova *et al*[95] showed an upregulation in transcript levels of several ABC transporters in PDAC compared to non-neoplastic tissues. Particularly, upregulation of 2 members of ABCA family, ABCA1 and ABCA7 involved in cholesterol export, together with expression of ABCG1 transporting phosphatidylserine, phosphatidylcholine and sphingomyelin, suggests their involvement in cellular cholesterol imbalance in the disease[95]. Another of the characteristics of PDAC is the highly inflammatory environment, which actively promotes cancer cell proliferation and survival, angiogenesis and assists the metastatic spread[96]. Chronic inflammation, that aids the tumorigenesis and at the same time is one of the main factors contributing to its initiation, is mediated by prostaglandin-mediated pathways. Therefore, the main inflammatory molecules- prostaglandins and leukotrienes are considered as significant players in PDAC development. The prostaglandin-mediated PDAC progression may involve activation of PI3K-Akt signalling pathway, a major player in PDAC progression, increase in VEGFA expression and stimulation of angiogenesis and support of the inflammatory environment[97]. It is now known that several ABC transporters, mainly belonging to the ABCC subfamily (ABCC1, ABCC2, ABCC4) are involved in prostaglandins efflux outside of the cells, enabling the activation of the G protein-coupled receptors, triggering cancer progression[75,98]. Therefore, the manipulation of ABC transporter activity blocking prostaglandin signalling represents an additional potential therapeutic tool. Additionally, due to the proved contribution of leukotriene C4 (LTC4) to PDAC progression[99], its induced pathways have been widely studied as potential drug targets. Regarding the involvement of ABC transporters in leukotriene release, their inhibition presents an additional possibility for LCT4-signalling blockade, influencing cancer development.

Elevated levels of reactive oxygen species (ROS), inducing oxidative stress are also implicated in PDAC initiation and progression[100]. One of the molecules responsible for the maintenance of redox status in homeostasis is glutathione (GSH)[101], which transport is activated in response to oxidative stress. It is also involved in several signalling processes regulating cell proliferation, apoptosis or immune response. Several members of ABCC family (ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC7) and ABCG2 mediate glutathione transport, suggesting their involvement in cellular response to the oxidative stress. Also, ABCB10 has been implicated in cellular protection from oxidative stress[102]. Moreover, oxidative stress induces the activation of NF-κB and Nrf2 signalling, which in turn enhances expression of ABCB1, ABCG2 and ABCC2, additionally contributing to cancer cell resistance[103,104]. Therefore, manipulation of the activity of ABC transporters in cancer cells might potentially increase their antioxidant capacity, which has been shown to provide additional anti-tumorigenic protection[105].

Additionally, tumour environment and its engagement in cancer progression and metastatic spread has emerged as key player in carcinogenesis. Considering the significant role of stroma in PDAC progression and in the development of tumour chemoresistance, targeting its components presents a tempting approach in the development of novel therapies. However, the attempts to deplete stroma have not provided satisfactory results so far. The most promising combination of gemcitabine

and Hedgehog inhibitor IPI-926-03 tested by Olive *et al*[106] has failed due to high toxicity and lack of effectiveness in clinical trials[107]. Currently, molecules targeting hyaluronic acid, combined with chemotherapy, are being tested in phase II and III clinical trials[108]. Nevertheless, investigation of new approaches to target stroma in order to increase chemotherapy efficiency, as well as restraining tumour expansion remains essential. Recently, expression of several of ABC transporters in PDAC stroma has been reported. One of the main stromal components- macrophages- have been demonstrated to express several of the drug transporters, inter alia ABCC1 and ABCC3, contributing to both chemoresistance and tumour progression[109]. Therefore, considering the involvement of ABC transporters in chemoresistance and an emerging role in tumorigenesis, therapies targeting ABC transporters might prove to be useful in depleting or reprograming cancer stroma and reversing cancer resistance to applied drugs. Additionally, expression of few of ABC transporters in non-neoplastic tissues has been recently reported to influence PDAC progression and to be predictive of patients' overall response.

Finally, the most aggressive tumours are composed of non-differentiated cells possessing highly proliferative abilities[75], called cancer stem cells (CSCs). In particular, the existence and high importance of CSCs in cancer resistance to chemotherapy and its involvement in disease recurrence has been suggested for PDAC. Interestingly, high expression of ABC transporters has been reported in less differentiated tumour zones, conferring them a more aggressive phenotype[110-112], also in PDAC[76]. Therefore recently, the interest in CSCs as drivers of resistance and aggressive nature has emerged in PDAC[113,114]. A noticeable characteristic of cancer stem cells is the high expression of members of the ABC transporters family compared to more differentiated cells[115]. Also, it is speculated that their expression profile may be considered as the indicator of stem cell formation and carcinogenic potential of the tissue[116]. Considering the association of cell differentiation levels with its proliferative potential, the overexpression of ABC transporters in cancer stem cells highly supports their contribution to the more aggressive nature of the PDAC. Overexpression of ABC transporters in cancer stem cells may assist in their survival by efflux of xenobiotics, exhibiting protective roles, sustaining their proper performance and maintaining self-renewal characteristics. Additionally, their enhanced expression and activity in cancer cells and especially in CSCs, suggests an additional role in maintaining cancer cells aggressive biology and makes them an attractive therapeutic target.

ABC TRANSPORTERS EXPRESSION PROFILES AS PROGNOSTIC MARKERS IN PDAC

Although the investigation on the role of ABC transporters in PDAC is still in its outset, the initial analysis suggests their probable contribution to PDAC development and points at potential beneficial clinical consequences. Database analysis showed that the high importance and the potential of ABC transporters as pharmacological targets in PDAC is reflected in the association of the expression of its individual members with the prognosis of patients' survival[117]. Notable correlation between observed 5-year survival and expression of a majority of ABC transporters has been observed (Table 1); however, this discovered association is not uniform. Significant reduction in survival probability has been attributed to high expression of *e.g.*, ABCA1, ABCA12, ABCB1, ABCC1, ABCC3 or ABCC7. Expression of few other ABC transporters showed similar trend, nonetheless, their relationship with the OS was not remarkably pronounced. On the other hand, higher expression of a substantial number of ABC transporter genes has been correlated with increased chance of PDAC patients' survival. Among others, the expression of ABCA2, ABCA7, ABCB6 ABCB8, ABCC5 or AGCG1 in PDAC tissues most markedly correlated with prolonged 5-year survival, suggesting their-mediated release of molecules of anti-tumorigenic characteristics and favourable prognostic potential.

Considering the elevated expression of multiple ABC transporters in a vast majority of cancers and their redundancy in substrate specificity and activity, determination of their expression profiles and their clustering in prognostic groups, rather than analysis of individual members, also raised a lot of interest in the last years. The existence of ABC transporters expression signatures in PDAC and their correlation with clinic-pathological characteristics of the tumours has been studied by Mohelnikova-Duchonova *et al*[95], and dysregulation of expression of several members of ABC family has been observed. Upregulation of ABCB4, ABCB11, ABCC1, ABCC3, ABCC5, ABCC10 and ABCG2 has been noted in PDAC, compared to non-neoplastic tissues. Surprisingly however, expression of few ABC transporters in non-neoplastic tissues also could be correlated with tumour progression and survival. Moreover, higher levels of T3 and T4 stages were associated with ABCA1 and ABCB3 upregulation and ABCG1 and ABCG2 downregulation. In contrast, smaller size tumours were connected with the cluster, in which ABCA8, ABCB5, ABCA9, ABCA10 and ABCC9 were upregulated, while downregulation of ABCA12, ABCA13, ABCC3, ABCC7 and ABCC13 has been noted. Similarly, ABCB9 and ABCC4 upregulation correlated with N1 status, while ABCA3, ABCD1 overexpression and ABCA6 and ABCC10 downregulation corresponded with increased angioinvasion.

This and previous studies demonstrated the correlation of ABC transporter expression in tumour specimens with clinic-pathological features in different cancer types[118]. Nevertheless, the high importance of tumour microenvironment and its proposed involvement in PDAC progression, suggests that ABC transporter

Table 2 Selected ATP-binding cassette transporters responsible for the development of multi-drug resistance, their experimental inhibitors and drug specificity

ABC transporter	Inhibitor	MDR	Ref.
ABCB1	I generation:Cyclosporine A, Verapamil II generation: Valspodar, zosuquidar III generation: Tariquidar, OC144-093	Daunorubicin, epirobicin, doxorubicin, colchicines, paclitaxel, docetaxel, vincristine, vinblastine, imatinib	[46,145,146,171]
ABCC1	MK571, probenecid, ibrutinib, 3ATA	Anthracyclines, vinca alkaloids, camptothecins, daunorubicin, imatinib, etoposide, vincristine, vinblastine, methotrexate	[46,145,146,172]
ABCC2	Metothrexate, cyclosporine A, fluorescein, MK571	Doxorubicin, cisplatin, irinotecan, epirubicin, vinblastine	[46,145,146,171,173]
ABCC3	Indomethacin, sufinpyrazone, probenecid, benzmromarone	Etoposide, methotrexate, teniposide	[46,145,146,171,173]
ABCC5	Curcumin, trequensin, sildenafil	Gemcitabine, methotrexate, 6-mercaptopurine	[46,145,146,171,173]
ABCG2	Fumitremorgin C, Ko143, GF120918	Daunorubicin, doxorubicin, irinotecan, mitoxantrone, methotrexate, epirubicin, etoposide	[46,145,146,171]

MDR: Multi-drug resistance; ABC: ATP-binding cassette.

expression in non-neoplastic tissues might have important clinical implications. Following the analysis of 27 non-neoplastic pancreatic tissues and pairing them with 32 PDAC samples, 4 different clusters could be distinguished based on the gene expression profiles in cancer *vs* normal specimens. PN1 and PN2 clusters were characterized by upregulation of the majority of ABC transporters genes and correlated with significantly shorter patients' overall survival (OS) than patients grouped into PN3 and PN4 clusters, in which significant downregulation of genes or heterogeneous gene expression has been observed[119]. Especially, ABCA2, ABCA4, ABCA5, ABCC2 and ABCD4 signatures showed significant difference in patients' survival when comparison between upregulated and downregulated genes was carried out. Additionally, tumour-node-metastasis, age, gender, disease stage, margin status, therapy and survival have been analysed; however, no significant correlation between those features and ABC profiles could be established. Although the study presented few limitations, such as small group size or the distance between collected tumours and control tissue, created expression clusters could be successfully implemented into clinical practice. Moreover, reduction of the analysed genes to the limited group showing most distinct expression, did not have any impact on the statistical significance of observed clinic-pathological correlations, creating more practical and convenient clinical prognostic tools.

ABC TRANSPORTERS IN CANCER THERAPY

Looking at the key role played by ABC transporters in cancer chemoresistance and the emerging knowledge on their crucial contribution to tumorigenesis, the development of targeted therapies, aiming to block or modulate their activity has become a crucial area in cancer research. Inhibition of transporter activity, arrest of the transcription factors regulating their expression or blockade of the transporter-induced signalling pathways represent the options for impeding ABC transporters activity[120]. So far, 3 generations of ABC transporters modulators, directed mainly against ABCB1, have been developed[120,121] (Table 2). The first generation inhibitors, such as verapamil, quinine or cyclosporine A, compounds previously established for other conditions, in spite of promising *in vitro* activity[122,123], showed significant toxicity, unacceptable for further usage[123,124]. Lack of potency and specificity, combined with pharmacokinetic complications restrained their further investigation[125]. Structural modifications of existing inhibitors, aiming to enhance their efficacy and specificity, at the same time decreasing observed adverse effects, also did not provide satisfactory results. Valspodar (cyclosporine A derivative), a second generation ABCB1 inhibitor, demonstrated enhanced efficiency accompanied by decreased toxicity[126]. However, it showed unsatisfactory results in the majority of clinical trials, in which its co-administration with chemotherapeutics, *e.g.*, carboplatin, paclitaxel or doxorubicin did not exhibit any benefits, and in some cases deteriorated patients' outcome[127,128]. Likewise, application of dofequidar or biricodar citrate (VX-710)[129] did not result to be favourable, as their use has been restricted by the potential interactions with anti-cancer therapeutics (vincristine or paclitaxel)[130]. All these limitations led to the development of a third generation of inhibitors which potency, due to the rational QSAR design, has been described as 200-fold higher than the previously developed anti-ABCB1 molecules, greatly enhancing drug accumulation[131]. Additionally, only minimal drug-drug interactions have been reported. Clinical trials have been commenced for zosuquidar (LY335979)[132], elacridar (F12091)[133], mitotane (NSC-38721)[134], annamycin[135] or tarquidar (XR9576)[136]. Nevertheless disappointingly, most of the clinical trials testing their applicability have been

discontinued due to lack of significant positive response and off-site effects.

There are several reasons for the lack of success of the ABC transporters inhibition. Increased toxicity caused by off-target action in healthy tissues, as well as their high doses were the main reasons for the discontinuation of the trials for first and second generation inhibitors[42]. Increasing evidence of substrate similarities between ABC transporters and CYP450, enzyme involved in drug metabolism, suggests interactions of tested compounds with the enzyme, which influences pharmacokinetic properties of co-administrated chemotherapeutics, changing their activity, lowering the efficacy and, as a consequence, increasing the toxicity[137]. Therefore single-agent application of ABC transporters inhibitors should be considered in future research. Another reason for high toxicity of these modulators has been attributed to decreased clearance of anticancer agents and natural xenobiotics caused by unspecific blockade of the transporters. As an example, ABCB1 inhibition, apart from cancer cells may also result in its blockade in canalicular membrane in healthy liver or kidney, reducing the clearance of chemotherapeutics[42,138]. The involvement of some of the ABC transporters (mostly ABCB and ABCC subfamilies) in the immune system is another obstacle, as disruption of its proper functioning may result in undesirable deterioration in anti-cancer immune responses[139]. The ineffectiveness of targeted therapies may also lay in the functional redundancy of several ABC transporters, highly impairing full efficiency of the blockade of individual protein. Another limitation in the presented approach has been the fact that the vast majority of studies have been focused on ABCB1. Nevertheless, with increasing evidence of the role of other ABC transporters in cancer, the inhibitors of ABCC1 (*e.g.,* probenecid, sulindac, biricodar, BAY-u9773 or MK571)[129,140-142], ABCG2 (Ko143, fumitremorgin C, genistein, biochanin A)[143,144] or ABCC3 (indomethacin or sulfinpyrazone)[145,146] have been considered (Table 2). However, some of them similarly to ABCB1 blockers, exhibited unfavourable toxicity levels when combined with chemotherapy. Additionally, several non-selective ABCB1 inhibitors have been tested for their activity towards other ABC transporters[146]. Nonetheless, as the interest in ABC transporters increased only recently, the efficacy of the abovementioned therapeutic approach still needs to be evaluated. Also, the majority of the studies conducted so far have been focused on the reversal of chemoresistance rather than influencing cancer progression. However, current knowledge on the additional, or maybe principal role of ABC transporters in tumorigenesis might shed more light on the basis of current inhibitors toxicity as well as could allow for exploration of novel more specific molecules, aiming at slowing down cancer progression, rather than reversing MDR.

Considering the marginal effectiveness of ABC transporters inhibitors achieved so far, alternative concepts for ABC transporters targeting are being tested (Figure 2). RNA interference, use of monoclonal antibodies, antisense oligonucleotides or the use of transcription regulators is currently under consideration[147-150]. miRNA use has been also claimed as a possible way for ABC transporter regulation and reversal of chemoresistance[151,152]. As crucial players in carcinogenesis, also confirmed in PDAC, miRNA regulation has been proposed as an interesting therapeutic tool[153]. To date, several miRNAs have been reported to inhibit the expression of different ABC transporters, having chemotherapeutic effects[154-156]. Moreover, it has been demonstrated that tyrosine kinase inhibitors may block ABC transporters by binding to their transmembrane domain at substrate-binding sites[157]. Imatinib, nilotinib, sunitinib or lapatinib, drugs tested for the PDAC therapy independently of their ABC-inhibiting properties, have been demonstrated to block ABCB1, ABCC2 or ABCC10[158-161]. However, this approach also needs further evaluation.

Currently, the use of nanoparticles for the delivery of therapeutics to the target cells has emerged as a growing area of interest[162,163]. Their small size, together with increased surface area, enhances the stability and solubility of the administered drugs, improving their bioavailability[164]. Additionally, controlled, prolonged release and protection form degradation present further advantages of that therapeutic approach. Co-delivery of the inhibitors of ABC transporters and chemotherapeutics with the use of nanoparticles is also applied to minimize observed side effects occurring as a result of drug-drug interactions. Nevertheless, the emerging field of the manipulation of ABC transporter activity for therapeutic purposes is still in its outset and more studies are needed to fully assess their pharmacological potential.

DISCUSSION

In the last years, ABC transporters have attracted remarkable attention of researchers from different scientific areas. The role of ABC transporters in different physiological and pathological conditions, including cancer, has been widely reported, increasing the interest in the development of their specific inhibitors. Especially, the well-known involvement of ABC transporters in the development of multi-drug resistance (MDR) led to the investigation of the potential of its reversal by blocking ABC transporter activity. Clinical relevance of several ABC transporters in multi-drug resistance reversal has been primarily attributed to P-gp, ABCG2, ABCB4 and 4 members of ABCC subfamily- ABCC1, ABCC2, ABCC3 and ABCC4[165]. Therefore, the main focus so far has been placed on these proteins in terms of their pharmacological potential. However, in spite of the initial enthusiasm regarding ABCB1 inhibitors, their efficacy in clinical settings has failed to provide any improvements, leading to the early closure of the trials[166,167]. Considerably high toxicity caused by lack of specificity and changes in pharmacokinetic of co-applied chemotherapeutics, decreasing their efficacy were

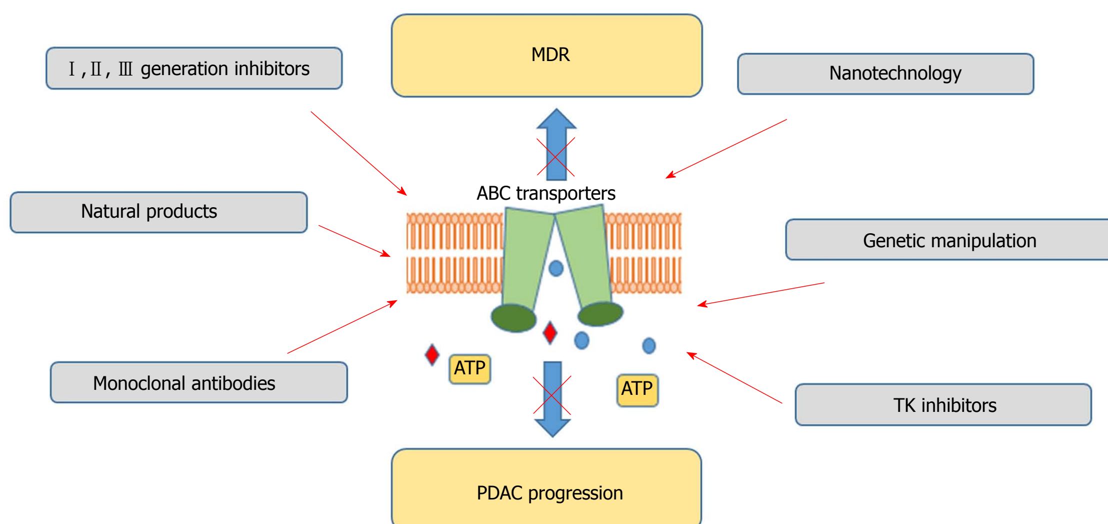

Figure 2 Pharmacological approaches towards inhibition of ATP-binding cassette transporters. MDR: Multi-drug resistance; PDAC: Pancreatic ductal adenocarcinoma; ABC: ATP-binding cassette; TK: Tyrosine kinase.

some of the reasons for the disappointing results[168]. The successful implementation of developed inhibitors was strongly impeded by the complexity of ABC transporters functioning. The correlation between cancer chemoresistance and ABC transporters expression is two-sided and forms a specific loop, which may increase cancer resistance to applied therapies. On one hand, their expression contributes to enhanced drug efflux from the cells, diminishing their efficacy, on the other hand, many studies have reported increased expression of the transporters, induced by drugs application, complementing formed loop. Therefore, in spite of the enhancement of drug accumulation and reversal of induced chemoresistance demonstrated *in vitro*, little success has been reported during clinical trials. Also, increased toxicity and insufficient potency observed during clinical trials restrained the majority of tested compounds from the clinical use. Additionally, the majority of carried clinical trials were performed on patients previously treated with several anticancer therapeutics. Therefore, the assessment of the protein levels might have been misevaluated due to drug-induced enhancement of expression of ABC transporters. Moreover, several of the studies were designed without proper patient stratification for ABC transporters expression. As an example, little success rate in ovarian cancer patients, might be explained by low expression rate of P-gp in this tumour type[127]. Although reversal of the drug resistance was the principal goal of ABC-targeted therapies, considering the increasing awareness of the pivotal role of ABC transporters beyond chemoresistance, their specific inhibition might not only aid to increase the activity of other therapeutics, but directly balk tumour development and progression[56], encouraging their further exploration. Therefore, the repertoire of ABC transporters against which inhibitors are being developed should be expanded for those playing an active role, not only in MDR, but in the expulsion of bioactive molecules. Looking at the wide variety of substrates transported by ABC transporters, together with their increased expression in cancer cells and especially cancer stem cells, the role of these proteins in the transport of signalling molecules, which activity promotes cancer progression, has become an area of interest. High impact of bioactive lipids, including phospholipids, sphingolipids or cholesterol on PDAC tumorigenesis and an emerging role of ABC transporters in their release presents a novel opportunity for targeting the disease. It has been previously demonstrated that one of the hallmarks of PDAC is lipid-dependence and that the decrease of the lipids levels may reduce cancer progression. Accordingly, aiming to block specific ABC transporters responsible for their extrusion, mainly members of ABCA and ABCC subfamilies, and depriving cancer cells of the necessary fuel may highly contribute to slowing down PDAC development. In fact, it has been demonstrated in several cancers that targeting of ABC transporters involved in lipid transport (*e.g.*, ABCC1 in prostate or ovarian cancer or ABCC4 in neuroblastoma) showed significant improvement in *in vitro* and *in vivo* models[75], slowing down cancer progression. Therefore, single-agent therapies based on ABC transporter inhibition should be considered to target cancer progression. Moreover, patients' treatment with ABC transporters single inhibitors would eliminate the risk of drug-drug interactions, reducing the risk of adverse events.

Importantly, the expression of ABC transporters may not only be explored in terms of their pro-tumorigenic activity but may also serve as prediction of therapy efficiency and patients' outcome. Database analysis demonstrated strong influence of the expression of the transporters *e.g.,* ABCC3 or ABCC1 on reported 5-year survival. However, positive association of other transporters (*e.g.,* ABCC5 or ABCA7) with the increased survival demonstrates the complexity of the role of ABC

transporters in PDAC tumorigenesis. It also shows the necessity for enhanced research in this area to fully understand and explore the therapeutic potential of these transmembrane proteins in PDAC therapy. The enhancement of chemotherapy efficacy, *e.g.,* by ABCC5 blocking, has been demonstrated for the gemcitabine-based therapies. However, considering the favourable association of this transporter with PDAC patients' survival, it is tempting to speculate that its inhibition might interfere with some of the protective functions that ABCC5 might exhibit and, as a consequence, deteriorate patients' outcome. Also, despite being overexpressed in a majority of cancer types, the role of ABC transporters is not uniform. Negative or positive correlation of the protein expression and survival observed in PDAC patients, is not invariably reflected in other cancer types. As an example ABCA7, expressed at a similar level in pancreatic and lung cancer, although positively correlated with 5-year survival in the PDAC (38% high expression *vs* 0% low expression), has no statistically significant effect in the latter case (48% *vs* 43%)[117]. Therefore, studying the context accompanying ABC transporters expression and functioning is of high importance in order to stratify their individual members in context of their pharmacological potential in diverse cancers. Additionally, the focus of research should not be placed only on the potential of the inhibition of ABC transporters that have undermining roles in carcinogenesis. Hence, the investigation of the characteristics of the ABC transporters that favour the survival of PDAC patients should be also explored to study the mechanisms and molecules responsible for their protective function.

Finally, ABC transporters profiling in cancer has proven to provide a potent tool in estimation of patients' response to applied therapies. As an example, analysis of 21 breast cancer specimens before and after neoadjuvant treatment showed different expression of several ABC transporters[169]. Similarly, 6 ABC transporters genes in AML samples allowed for their organization in two expression groups, correlated with resistance and patients' prognosis[170]. Correspondingly, generation of ABC transporter expression profiles in PDAC has allowed for creation of clusters, characterized by differentiated expression of their individual members. Correlation of each cluster with a variety of disease parameters (*e.g.,* number of metastases or drug response) and more importantly, with patients' survival suggested the gene profiling for ABC transporters expression as a clinically relevant prognostic tool.

CONCLUSION

Although a lot of advancement has been achieved in the identification of new druggable targets involved in PDAC progression and chemoresistance, no significant improvement in transferring that knowledge into clinical practice has been accomplished, leaving PDAC patients with grim prognosis. As critical players in PDAC chemoresistance and disease development, ABC transporters seem a promising target for the development of novel targeted therapies. However, despite their remarkable pharmacological potential demonstrated *in vitro*, acquired knowledge has not been successfully implemented in the clinic yet. Nevertheless, the knowledge learnt from previous mistakes and the potential reasons for the failed implementation of the inhibitors should be considered in the development of new studies and treatments. In the light of recent data, the potential of few ABC transporters beyond MDR reversal should be further explored to fully scrutinize the applicability of ABC transporter inhibition for clinical practice. More emphasis on the ABC transporters involvement in PDAC progression should be placed in prospective studies, leading to the determination of the proteins with the most pharmacological potential followed by design of single-agent treatment. The knowledge on the involvement of ABC transporters in cancer metabolic swift, their role in tumour-microenvironment cross-talk should be additionally expanded. Animal models of pancreatic cancer should be implemented in the development of new potential inhibitors to investigate their impact on abovementioned processes. In conclusion, proper study design and patients stratification regarding ABC transporters expression leading to tailored therapies should be elucidated in order to add to the efficiency of administered drugs.

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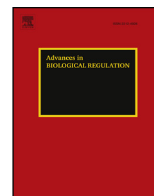

ABCC3 is a novel target for the treatment of pancreatic cancer

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ABSTRACT

Pancreatic Ductal Adenocarcinoma (PDAC) is a very aggressive disease, lacking effective therapeutic approaches and leaving PDAC patients with a poor prognosis. The life expectancy of PDAC patients has not experienced a significant change in the last few decades with a five-year survival rate of only 8%. To address this unmet need, novel pharmacological targets must be identified for clinical intervention. ATP Binding Cassette (ABC) transporters are frequently overexpressed in different cancer types and represent one of the major mechanisms responsible for chemoresistance. However, a more direct role for ABC transporters in tumorigenesis has not been widely investigated. Here, we show that ABCC3 (ABC Subfamily C Member 3; previously known as MRP3) is overexpressed in PDAC cell lines and also in clinical samples. We demonstrate that ABCC3 expression is regulated by mutant p53 via miR-34 and that the transporter drives PDAC progression via transport of the bioactive lipid lysophosphatidylinositol (LPI). Disruption of ABCC3 function either by genetic knockdown reduces pancreatic cancer cell growth *in vitro* and *in vivo*. Mechanistically, we demonstrate that knockdown of ABCC3 reduce cell proliferation by inhibition of STAT3 and HIF1α signalling pathways, previously been shown to be key regulators of PDAC progression. Collectively, our results identify ABCC3 as a novel and promising target in PDAC therapy.

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th leading cause of cancer-related deaths in the Western world (Garrido-Laguna and Hidalgo, 2015). Lack of distinctive symptoms leading to late diagnosis, early metastatic spread and huge genetic and phenotypical heterogeneity of PDAC contribute to its aggressive nature and high chemoresistance, making most therapies ineffective (Adamska et al., 2017, 2018; Falasca et al., 2016). Surgical resection represents a therapeutic option only for 15–20% of PDAC

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patients presenting with local or locally advanced disease (Michalski et al., 2007), the majority of whom unfortunately relapse. Radiation therapy and chemotherapy remain the only options for advanced and metastatic patients. However, this approach only marginally extends the overall survival (Vaccaro et al., 2015). Up until recently, gemcitabine was the main available, FDA-approved chemotherapeutic; however, it prolonged patient survival by only a few weeks (Burris et al., 1997). Currently, Abraxane (albumin-bound paclitaxel) and FOLFIRINOX are additionally applied as a standard-of-care therapy, providing modest improvement in survival rates but accompanied by a higher incidence of adverse effects (Conroy et al., 2011; Von Hoff et al., 2013). The high mutational heterogeneity and the plasticity of PDAC limit the options for the development of targeted therapies (Adamska et al., 2017; Borja-Cacho et al., 2008; Heinemann et al., 2000). There is a need, therefore, to identify novel pharmacological targets and develop more effective and safe therapeutic options for PDAC patients. ABC transporters have previously been linked with poor outcome in cancer and this has generally been attributed to chemoresistance (Gottesman et al., 2002; Hagmann et al., 2011). ABC transporters, particularly ABCB1 (P-glycoprotein), ABCG2 (BCRP) and ABCC1 (MRP1) are capable of effluxing a wide variety of substrates, including drugs, across the plasma membrane, lowering their intracellular concentration. The majority of studies have therefore focused on the role of ABC transporters in drug resistance and on its reversal. However, the ability of ABC transporters to also efflux bioactive molecules that play essential roles in cancer progression, suggests a more direct, active contribution of ABC transporters to carcinogenesis (Adamska and Falasca, 2018; Domenichini et al., 2018; Fletcher et al., 2010; Pineiro et al., 2011). In particular, signalling lipids such as phospholipids, which role in several malignancies including pancreatic cancer has been well documented (Suh and Cocco, 2016), were proposed as ABC transporters ligands. However, this area has been overlooked and the therapeutic potential of ABC transporter inhibition in counteracting PDAC progression has not yet been fully explored. Recently, we described the existence of an autocrine loop in which LPI (lysophosphatidylinositol)-activated GPR55 stimulates proliferation of PDAC cell lines that harbour p53 mutations (Ferro et al., 2018; Ruban et al., 2014). We showed that the blockade of LPI receptor- GPR55 significantly reduced disease progression in mouse models of PDAC. Considering their involvement in phospholipid efflux from cells (Tarling et al., 2013), we proposed that ABC transporters may mediate LPI transport in PDAC (Ruban et al., 2014).

In this study we investigated the role and the potential of targeting ABC transporters in PDAC therapy. We showe that a member of the ABCC family, ABCC3, is highly expressed in PDAC tumours and that its expression is dependent on mutation of TP53. We also show that ABCC3 is required for LPI-mediated PDAC progression via STAT3 and HIF1α signalling pathways, which have previously been shown to be involved in PDAC onset and progression (Corcoran et al., 2011; Hoffmann et al., 2008).

2. Materials and methods

2.1. Cell lines and plasmids

Cell lines were purchased from ATCC (VA, USA) and cultured as per manufacturer's instructions in *Mycoplasma*-free conditions: AsPC1 (CRL-1682™), HPAFII (CRL-1997™), CFPAC-1 (CRL-1918™), BxPC-3 (CRL-1687™), Capan-1 (HTB-79™), Capan-2 (HTB-80™), hTERT-HPNE (CRL-4023™). Mouse primary cell lines (PZR1, PZPR1, PZPflR) were kindly provided by Owen Sansom (Beatson Institute, Glasgow, UK). Cells were authenticated and regularly tested for *Mycoplasma*. Wild-type ABCC3 cDNA encoded by recombinant pcDNA3.1 plasmid (pcDNA3-ABCC3) was a kind gift from Prof. Susan Cole (Oleschuk et al., 2003).

2.2. RNA interference

For transient ABCC3 knockdown, four ABCC3-targeting siRNA sequences (siABCC3-1 (Hs_ABCC3_6), siABCC3-2 (Hs_ABCC3_15) QIAGEN; siABCC3-3 (J-007312-05), siABCC3-4 (J-007312-06) (Dharmacon®) and control siRNA (siSCR) were used at a working concentration of 75 nM. Cells were collected at 24 h (CFPAC-1) or 48 h (AsPC1, HPAFII) after transfection. Western blotting was used to verify knockdown efficiency.

The siABCC3-3 sequence was used to generate pSuper retro–based vectors that express short hairpin RNA (shRNA). Control vector pSuper 4Mut contains a four-point mutated sequence unable to target the human ABCC3. Retroviral stocks were generated as previously described (Sala et al., 2012) and infected CFPAC-1 cells were selected with 1 μg/ml of puromycin. Knockdown efficiency was determined by Western blotting. For proliferation analysis, stably transfected CFPAC-1 cells (shABCC3 and 4Mut) were seeded at a density of 10,000 cells/well in a 12-well plate in the presence of 1 μg/ml puromycin and incubated for 6 days. Cells were counted daily in duplicate with trypan blue exclusion.

2.3. Cell viability and colony formation assays

PDAC cell lines seeded at a density of 5×10^4 cells/well in 12-well or 2×10^4 cells/well in 24-well cell culture plates were treated in duplicate, DMSO was used as a negative control. After 72 h cells were counted with trypan blue exclusion.

To validate the effects of therapies on the ability of cancer cells to form colonies in anchorage-independent condition, soft agar colony formation assay was performed (Domenichini et al., 2019). Colonies were grown for 4 weeks and then fixed in 10% Acetone/Methanol, stained with a 0.05% crystal violet solution and counted.

2.4. RT-qPCR

Total RNA was extracted using GeneJET TNA Purification (Thermo Scientific, #K0732) according to the manufacturer's

instructions, followed by cDNA synthesis (Thermo Scientific, # EP0742). RT-qPCR was performed according to the manufacturer's instructions (Fermentas, #K0222) using an ABI 7500 RT-QPCR system. As a control, QARS cDNA was also amplified. Changes in gene expression, relative to control, was calculated using relative $\Delta\Delta C_T$ analysis.

2.5. Protein analysis

Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer and sonicated. Proteins were separated by SDS-PAGE and detected by Western blotting according to standard procedures using the following antibodies at 1:1000 dilution: ABCC3 (Invitrogen, #PA5-23653), HIF1α (Novus Biologicals, #NB100-479), pSTAT3 Tyr705 (CST, #9131), GAPDH (CST, #5174), β-actin (CST, #4970), α-actinin (CST, #3134), α/β tubulin (CST, #2148). Anti-rabbit secondary antibody (CST, #7074) was used at 1:20000 dilution. Immunoblots were quantified using ImageJ and Image Lab 5.2.1.

2.6. Caspase 3/7 activity

Following ABCC3 silencing, PDAC cells were incubated with Caspase 3/7 reagent (1:1000 dilution) (Essen Bioscience) accordingly to manufacturer's instruction and monitored for up to 72 h using IncuCyte Life Cell Analysis Imaging System (Sartorius).

2.7. LPI stimulation and release analysis

PDAC cells were serum-starved overnight before LPI stimulation. Cells were incubated with 1 µM LPI (MerckMillipore, cat# 440153) for 8 min (acute stimulation) or 0.5 µM LPI for 72 h (long-term stimulation). Cell viability and protein analysis were performed as described above.

HPAFII cells were transfected with siRNAs targeting ABCC3 and cPLA2. Twenty-four hours post-transfection, cells were labelled with $[^3H]$myo-Inositol for 48 h. Cells were then incubated with or without EGF (20 ng/ml) for 1 h. Lipids were extracted from cell supernatants by phase separation and radioactivity was assessed by scintillation counting.

2.8. Radiolabelled LPI preparation

HEK293T cells were fed tritiated myo-inositol to convert into ^{3}H-LPI. The ^{3}H-LPI released by the cells was separated by thin layer chromatography and recovered.

2.9. 3H-LPI transport assay

Membrane vesicles were prepared as described previously (Byrne et al., 2002) from HEK293T (untransfected cells or cells transiently-transfected with pcDNA3-ABCC3). Vesicles containing 60 µg of membrane protein were incubated at 37 °C for 15 min in 150 µl total volume containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 10 mM ATP, 10 mM $MgCl_2$, 100 µg/ml creatine kinase (Roche, UK), 10 µM creatine phosphate (Roche, UK) and 2 µM LPI (cold-LPI (Sigma-Aldrich) spiked with 0.5 nCi ^{3}H-LPI prepared as described above) in the presence or absence of 100 µM vanadate (Sigma-Aldrich). The reaction was stopped by adding ice-cold buffer (50 mM Tris-HCl, 250 mM sucrose, pH 7.5). The vesicles were recovered by rapid filtration through cellulose nitrate discs (0.2 µm pore size, 25 mm diameter Whatman; Fisher, UK) using a 1225 Sampling Manifold (Millipore) and washed four times with of ice-cold transport buffer. The ^{3}H-LPI accumulated in the vesicles was measured in a 1049 DSA scintillation counter (Wallac).

2.10. Mouse xenograft model

All animal experiments were performed accordingly to standards of national and institutional guidelines. Xenograft work was approved by the Italian Ministry of Health (N.484/2016-PR). All animals were kept at 21 °C in ventilated cages, with 12 h light/12 h dark cycle. Cages were changed twice weekly. 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* and the animals' health status was monitored daily. Stably silenced for ABCC3 expression CFPAC-1 cells were produced as described in the materials and methods (RNA interference). Athymic CD-1 nude mice (n = 20) were injected subcutaneously with 3×10^6 of CFPAC-1 sh4Mut or CFPAC-1 shABCC3 cells. Tumours of the different xenografts were monitored every week using a calliper and volumes were calculated using the formula: tumour volume = (length * width^2)/2.

2.11. Immunohistochemistry

Immunohistochemical analysis with the anti-ABCC3 antibody was performed on tissue microarrays (TMA) constructed by removing 2-mm diameter cores of histologically confirmed ductal pancreatic cancer areas from 60 invasive primary tumours. After antigen retrieval (microwave treatment at 750 W for 35 min in Tris-EDTA buffer, pH 9.0), TMA sections were incubated overnight (+4C°) with the anti-ABCC3 (sc-5776, S.Cruz, CA) goat polyclonal antibody at 1:100 dilution. The LSAB kit (K0690, Dako, Glostrup, Denmark) was used for signal amplification. In control sections, the specific primary antibody was replaced with non-immune goat serum. Tissues were counterstained with Haematoxylin. The normal pancreatic tissue presented in Fig. 1C was obtained from the

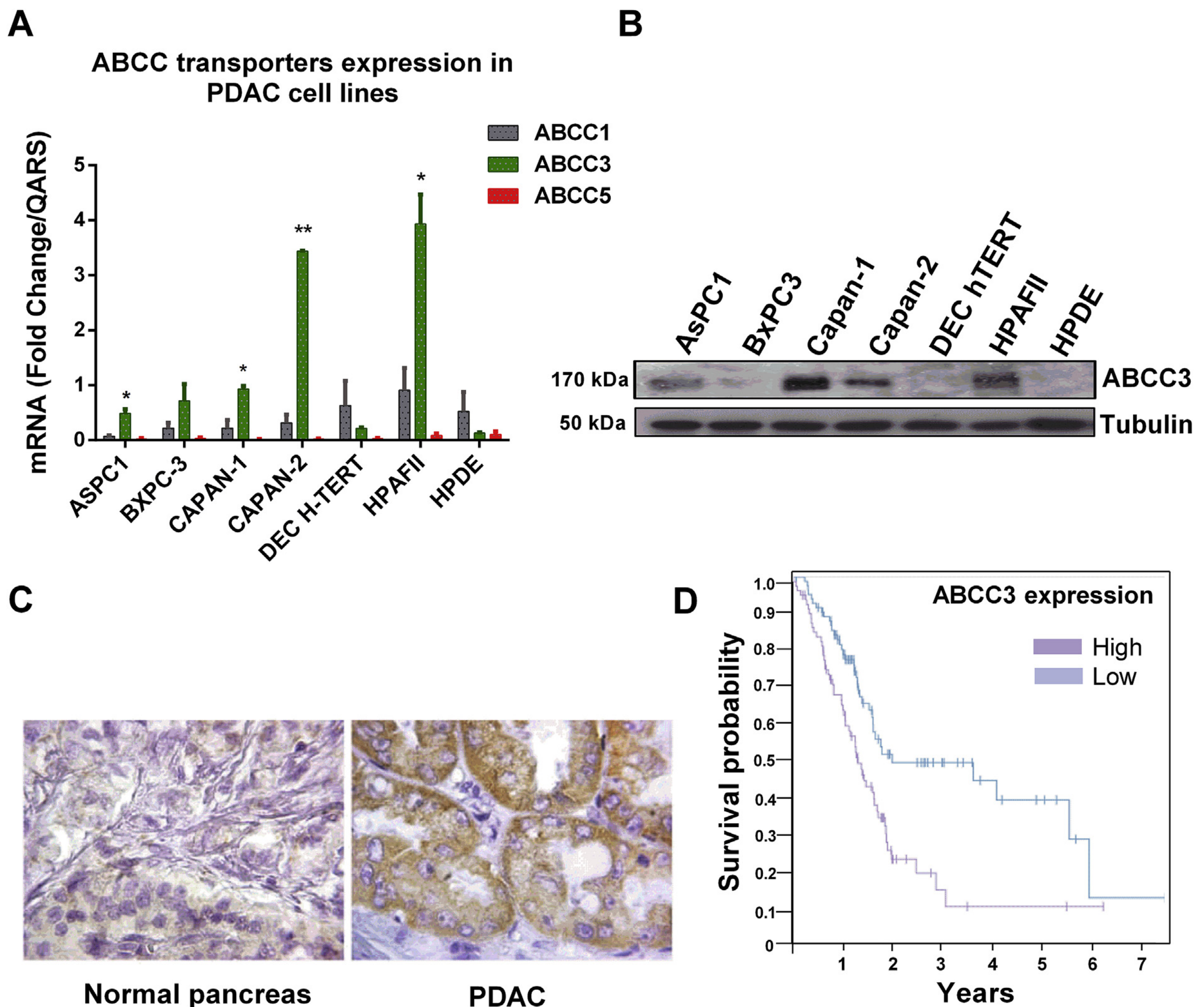

Fig. 1. ABCC3 is overexpressed in PDAC and regulates its progression and survival.
Expression analysis of ABCC1, ABCC3 and ABCC5 in PDAC cell lines compared to non-neoplastic pancreatic cell lines (HPDE, DEC-hTERT) at (A) mRNA and (B) protein level *p < 0.05, **p < 0.01; (C) Immunohistochemistry (IHC) analysis of ABCC3 expression in human pancreatic tissues confirming overexpression of ABCC3 in tumour specimens (right) compared to healthy pancreas (left) (Magnification: 63x); (D) Correlation of levels of ABCC3 expression (high or low) and survival rates of the patients (http://www.proteinatlas.org/ENSG00000108846ABCC3/pathology/tissue/pancreatic+cancer).

peritumoral tissue. The expression of ABCC3 was quantitatively assessed according to the percentage of positive tumour cells.

2.12. Statistics

The sample size for each experiment was assessed based on previous work. Results are represented as a mean ± SEM and the statistical analysis was performed for at least three independent experiments using GraphPad PRISM® V6.0 software (GraphPad Software, CA, USA). Unpaired, one-sided *t*-test (Western blot and IHC quantification), one-way ANOVA (cell growth) and two-way ANOVA (tumour growth) were used assuming independent samples and normal distributions. A 95% confidence interval was used for statistics and $P < 0.05$ was considered significant.

3. Results

3.1. ABCC3 is overexpressed in pancreatic cancer and correlates with poor prognosis

Enhanced expression of ABCC transporters in PDAC was suggested by previous studies (Konig et al., 2005). More detailed screening for ABCC1, ABCC3 and ABCC5 revealed significant upregulation of ABCC3 in PDAC cell lines both at the mRNA and protein

levels (Fig. 1A and B), suggesting that ABCC3 may be an important, but as yet unexplored player in PDAC progression. Consistent with this, our recent proteomic analysis of AsPC1 PDAC cell line showed increased expression of ABCC3 compared to undetectable levels in normal pancreatic cell lines (HPDE and hTERT-HPNE) (Emmanouilidi et al., 2019). Immunohistochemical (IHC) analysis of human tissues from normal pancreas (peritumoral tissue) and PDAC samples (Fig. 1C) confirmed high levels of ABCC3 protein in cancer specimens. Importantly, analysis of publicly available datasets (http://www.proteinatlas.org/ENSG00000108846-ABCC3/pathology/tissue/pancreatic+cancer) showed that ABCC3 is an unfavourable prognostic marker in pancreatic cancer patients in whom high expression significantly correlates with lower survival rates (Fig. 1D).

3.2. ABCC3 is an important player in PDAC growth

To investigate the role of ABCC3 in PDAC growth and progression, we stably silenced ABCC3 in CFPAC-1 pancreatic cancer cells with the use of short hairpin RNA (shRNA) (Fig. 2A) and measured their growth rate *in vitro* and *in vivo* following xenograft implantation in mice. Decreased ABCC3 expression resulted in significant reduction of CFPAC-1 growth *in vitro* (Fig. 2B). More importantly, implantation of cells with ABCC3 stable knockdown in immunocompromised mice remarkably reduced tumour growth in the mouse xenograft model (Fig. 2C), suggesting that ABCC3 expression is important for PDAC progression. We confirmed the impact of ABCC3 expression on PDAC anchorage-dependent and independent growth by transient knockdown of ABCC3 in three pancreatic cancer cell lines: AsPC1, CFPAC-1 and HPAFII. Two different short interfering RNAs (siRNAs) were used to allow direct comparison of the effects of ABCC3 knockdown in different cell lines. Decreased ABCC3 expression, confirmed by Western blotting (as shown in Fig. 4A), correlated with a significant reduction of PDAC anchorage-dependent (Fig. 2D) and anchorage-independent growth (Fig. 2E) in all three PDAC cell lines with at least two different siRNAs. These data highlight the important role of ABCC3 in PDAC growth.

3.3. ABCC3-mediated LPI release regulates progression of PDAC through STAT3 activation and induction of apoptosis

In order to investigate the role of ABCC3 in promoting PDAC progression at the molecular level, we transiently-expressed the transporter in naïve HEK293T cells and generated inside-out vesicles to test the hypothesis that ABCC3 was indeed the efflux transporter for the GPR55 ligand LPI. A seven-fold increase in accumulation of LPI was measured for the ABCC3-containing inside-out vesicles compared to naïve vesicles (Fig. 3A). Accumulation was dependent on added extravesicular ATP and inhibited by vanadate (Vi), as would be expected for primary-active transport by ABCC3. siRNA gene silencing experiments confirmed that EGF-dependent LPI release was both ABCC3- and PLA2-mediated, suggesting that the phospholipase is responsible for LPI production and the transporter for its release (Fig. 3B and C). Furthermore, we confirmed that the addition of exogenous LPI in serum-free conditions stimulates PDAC cell line proliferation (Fig. 3D), which we have previously shown to be dependent on GPR55 (Ferro et al., 2018). These results confirmed the involvement of LPI in the mechanisms regulating the proliferation and growth of PDAC cells. It also suggests that the inhibition of LPI synthesis or release from the cells could substantially reduce PDAC cell proliferation and decrease disease progression.

To gain further insight into the mechanisms of ABCC3-mediated regulation of PDAC cell proliferation, signalling pathways regulated by the transporter were investigated. Expression levels of relevant signalling molecules with a proven role in PDAC tumorigenesis were studied following transient knockdown of ABCC3. Both pSTAT3 Y705 and HIF1α were shown to play a key role in both PDAC carcinogenesis and cancer stroma signalling (Corcoran et al., 2011; Hoffmann et al., 2008; Wormann et al., 2016). Western blot analysis of all three PDAC cell lines revealed suppression of phosphorylated STAT3 (pSTAT3 Y705) levels, together with a reduction in HIF1α protein levels following ABCC3 siRNA knockdown (Fig. 4A). Conversely, stimulation of PDAC cell lines with 1 μM LPI significantly enhanced the phosphorylation of STAT3 at tyrosine 705 (Fig. 4B), strongly suggesting that LPI is the mediator of ABCC3-dependent signalling.

In addition, we hypothesized that ABCC3-mediated regulation of tumour cell proliferation and growth might be partly due to the involvement of ABCC3 in apoptosis. Increased activity of caspase 3/7 was observed in cells after knockdown of ABCC3, as indicated by caspase 3/7 probe activity (Fig. 4C). These observations demonstrate the role of ABCC3 in PDAC progression through regulation of cell proliferation and apoptosis.

3.4. p53 regulates ABCC3 expression through miR-34C

Defining the genetic determinants of ABCC3 upregulation could identify a cohort of patients that might benefit from ABCC3 targeted therapy. We therefore investigated a possible mechanistic link between ABCC3 regulation and mutation of key genes in PDAC. Mutations in *TP53* are present in 50–70% of PDAC cases and are known to play an important role in pancreatic cancer progression (Garrido-Laguna and Hidalgo, 2015). A negative correlation between ABCC3 and WT p53 expression was observed by Western blot in a panel of mouse cell lines. High expression of ABCC3 was detected in cell lines with mutated ($p53^{R172H/+}$, PZPR1) or deleted ($p53^{fl/+}$, PZPflR) *TP53* gene (Fig. 5A). Conversely, lower levels of ABCC3 were observed in a cell line bearing wild-type p53 (p53 WT, PZR1) (Fig. 5A). IHC analysis of ABCC3 expression in human PDAC tissues validated these findings, showing a statistically significant increase in ABCC3 expression in specimens with p53 immunostaining, an immunohistochemical surrogate marker of *TP53* mutation (Table 1). Analysis of an available PDAC dataset (TCGA provisional, from cBioPortal: http://www.cbioportal.org/) also confirmed increased ABCC3 mRNA expression in specimens with *TP53* mutations (Fig. 5B). Database analysis also suggested that miR-34C, whose expression is p53-dependent (Corney et al., 2007; He et al., 2007) and is typically downregulated in PDAC, might regulate ABCC3 expression (Fig. 5C). For instance, Capan-2 cells characterized by low expression of miR-34C (Ji et al., 2009), show

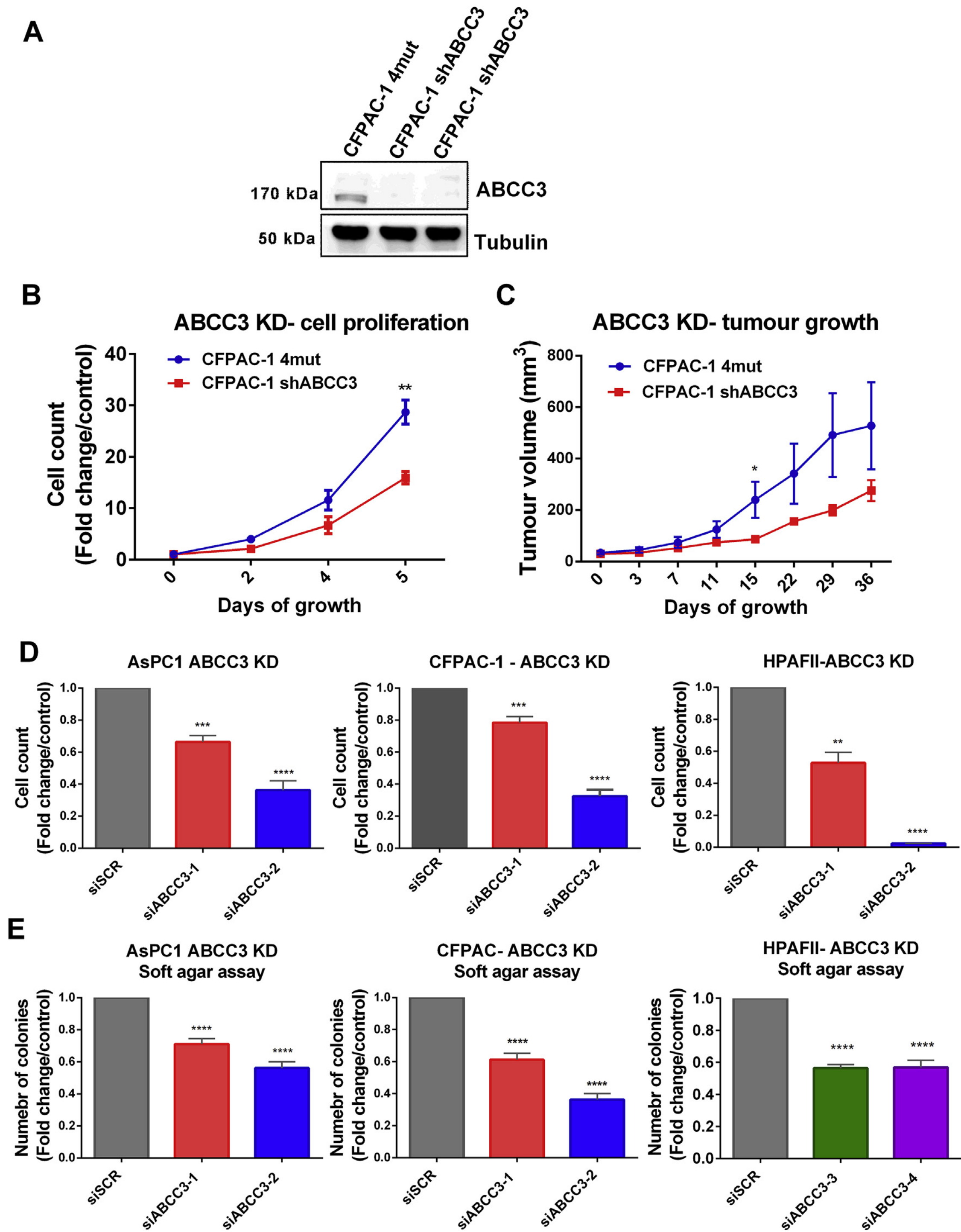

Fig. 2. ABCC3 as an important player in PDAC progression.
(A) Stable silencing of ABCC3 expression with short hairpin RNA (shRNA) confirmed by Western blotting of two independent samples. The reduction in the *in vitro* (B) cell growth and *in vivo* (C) tumour growth induced by ABCC3 knockdown in PDAC cells *p < 0.05, **p < 0.01. The data is the mean ± SEM of 3 independent experiments. Blue line indicates control group, red line indicates ABCC3-silenced group; The effect of knockdown of ABCC3 expression with specific siRNAs targeting ABCC3 (siABCC3-1, si-ABCC3-2, siABCC3-3, siABCC3-4) or non-targeting control siRNA (siSCR) on anchorage dependent cell proliferation (D) and anchorage-independent soft agar colony formation (E); The experiments was performed in 5 (AsPC1), 4 (HPAFII) and 7 (CFPAC-1) independent repetitions measured as mean ± SEM, **p < 0.01, ***p < 0.001, ****p < 0.0001.

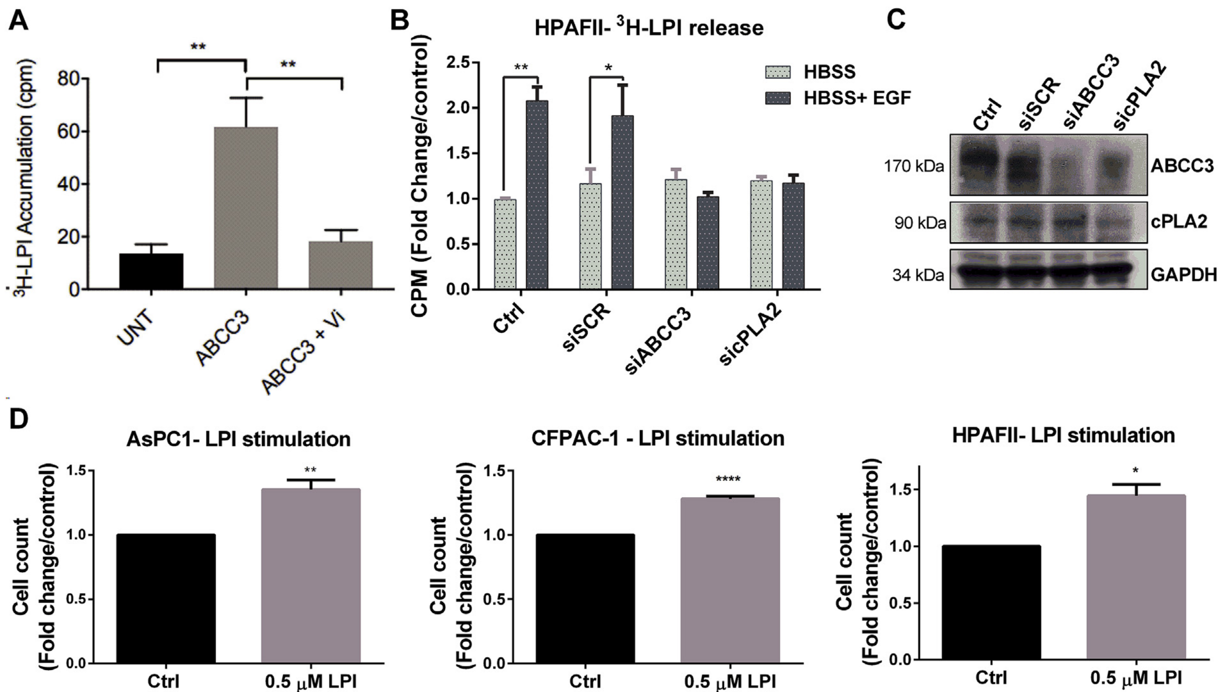

Fig. 3. ABCC3- mediated LPI release regulates progression of PDAC.
(A) Comparison of ^{3}H LPI accumulation in HEK293T inside-out vesicles prepared from untransfected cells and cells transiently expressing ABCC3 in the presence and absence of the ATPase inhibitor vanadate (Vi) $**p < 0.01$ (B) The effect of genetic knockdown of ABCC3 and $cPLA_2$ expression in HPAFII PDAC cell line on the [^{3}H]myo-Inositol efflux induced by EGF. Results are expressed as fold change of radioactivity detected in supernatants from untreated cells $*p < 0.05$, $**p < 0.01$; (C) Representative Western blot image showing the effects of the knockdown of ABCC3 and cPLA2 on the expression of the two proteins; (D) The effect of long-term stimulation with exogenous LPI (0.5 μM) of serum-starved PDAC cells (AsPC1, HPAFII and CFPAC-1) on the proliferation of cells measured after 72 h. The results are presented as mean ± SEM of 4 (CFPAC-1) and 3 (AsPC1, HPAFII) independent experiments, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$.

elevated expression of ABCC3 (Fig. 1B). Also, microRNA target prediction using miRWalk 2.0 (Dweep and Gretz, 2015) revealed that ABCC3 was a predicted target of miR-34C. Indeed, transient overexpression of miR-34C in PDAC cell lines significantly decreased ABCC3 expression (Fig. 5D), thus demonstrating a direct mechanism for p53-dependent regulation of ABCC3 expression. Notably, miR-34C is a tumour suppressor miRNA, and low expression of miR-34C correlates with a reduced survival rate in patients (Donahue et al., 2012; Vogt et al., 2011).

4. Discussion

PDAC is an aggressive malignancy whose prognosis has not changed in recent decades compared to other cancer types. Late diagnosis of the disease and high chemoresistance of pancreatic tumours greatly restrict available therapeutic options (Adamska et al., 2017), leaving PDAC patients with a grim prognosis. Considering the marginal effects obtained with the use of standard chemotherapy, novel therapeutic approaches are necessary. Few recent studies showed that non-standard therapeutic strategies may represent a chance for the improvement of patients' perspectives. As an example, a study by Abrams et al. demonstrated that the natural product berberine and its chemically modified analogues are potent inhibitors of pancreatic cancer cell proliferation (Abrams et al., 2019). Similarly, metformin, a drug commonly prescribed for type II diabetes, was shown to enhance the effectiveness of co-administered therapeutics, increasing sensitivity of pancreatic cancer cells (Candido et al., 2018). Nevertheless, the identification of new therapeutic targets in PDAC that can be explored pharmacologically is still pivotal. The contribution of ABC transporters to the failure of chemotherapy has been well documented in several cancer types. Due to their ability to transport a wide variety of substrates, including xenobiotics and drugs, ABC transporters associated with cancer have been mostly studied so far for their involvement in chemoresistance (Gottesman et al., 2002; Hagmann et al., 2011) and the potential of their inhibition to reverse the resistance and increase the efficacy of applied therapies has been explored (Choi, 2005; Falasca and Linton, 2012). Nevertheless, the involvement of ABC transporters in transporting several bioactive molecules involved in cancer progression, including prostaglandins, leukotrienes or phospholipids and their expression in immune cells and cancer stem cells, raises the question of a more direct role for ABC transporters in carcinogenesis, beyond their contribution to chemoresistance (Adamska and Falasca, 2018; Begicevic and Falasca, 2017; Domenichini et al., 2018). There have been limited studies on the role and expression of ABC transporters in pancreatic cancer, most of which focused on ABC transporter-induced resistance (Hagmann et al., 2010; Mohelnikova-Duchonova et al., 2013). Only one study, after analysing several ABC transporters, indicated the overexpression of two transporters,

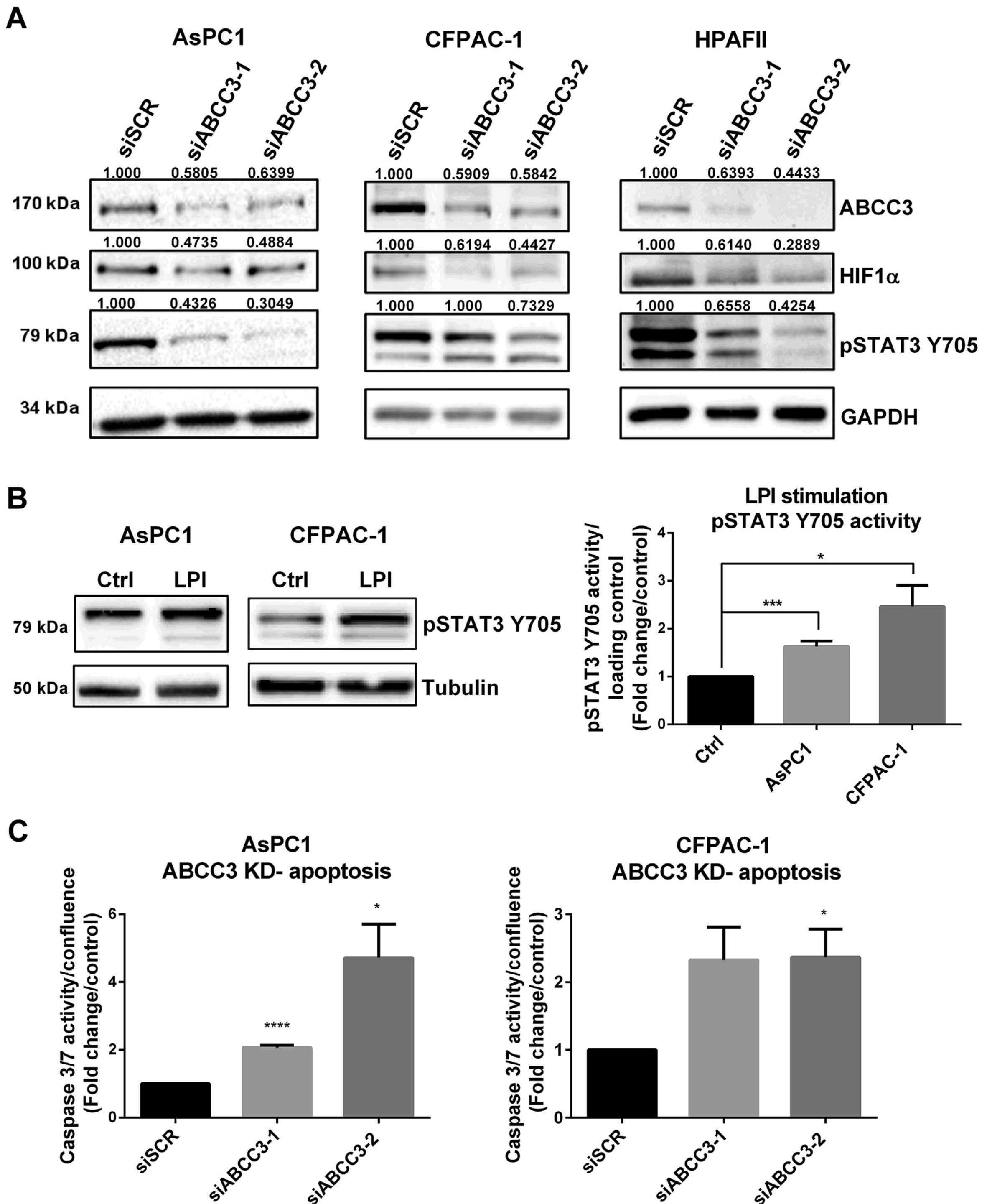

Fig. 4. ABCC3- mediated LPI release regulates progression of PDAC through STAT3.
(A) Representative Western blot images showing the effects of transient knockdown of ABCC3 with 2 specific siRNAs (siABCC3-1, siABCC3-2) on the expression of pSTAT3 Y705 and HIF1α, quantitative analysis, normalised to control, is presented as a mean of 3 independent experiments; (B) Representative Western blot images of the stimulation of AsPC1 and CFPAC-1 cells with 1 μM LPI (8 min) on the phosphorylation of STAT3 at tyrosine 705, quantitative analysis, normalised to control, is presented as a mean ± SEM of 3 independent experiments, $*p < 0.05$, $***p < 0.001$ (C) The effects of knockdown of ABCC3 with 2 specific siRNAs (siABCC3-1, siABCC3-2) and treatment with 10 μM S3 on the Caspase 3/7 activity (72 h post-treatment) measured with Caspase 3/7 probe Each experiment was performed in triplicate and the results are presented as the mean ± SEM, $*p < 0.05$, $****p < 0.0001$. Quantitative analysis of Western blots was performed with the use of ImageJ and Image Lab software.

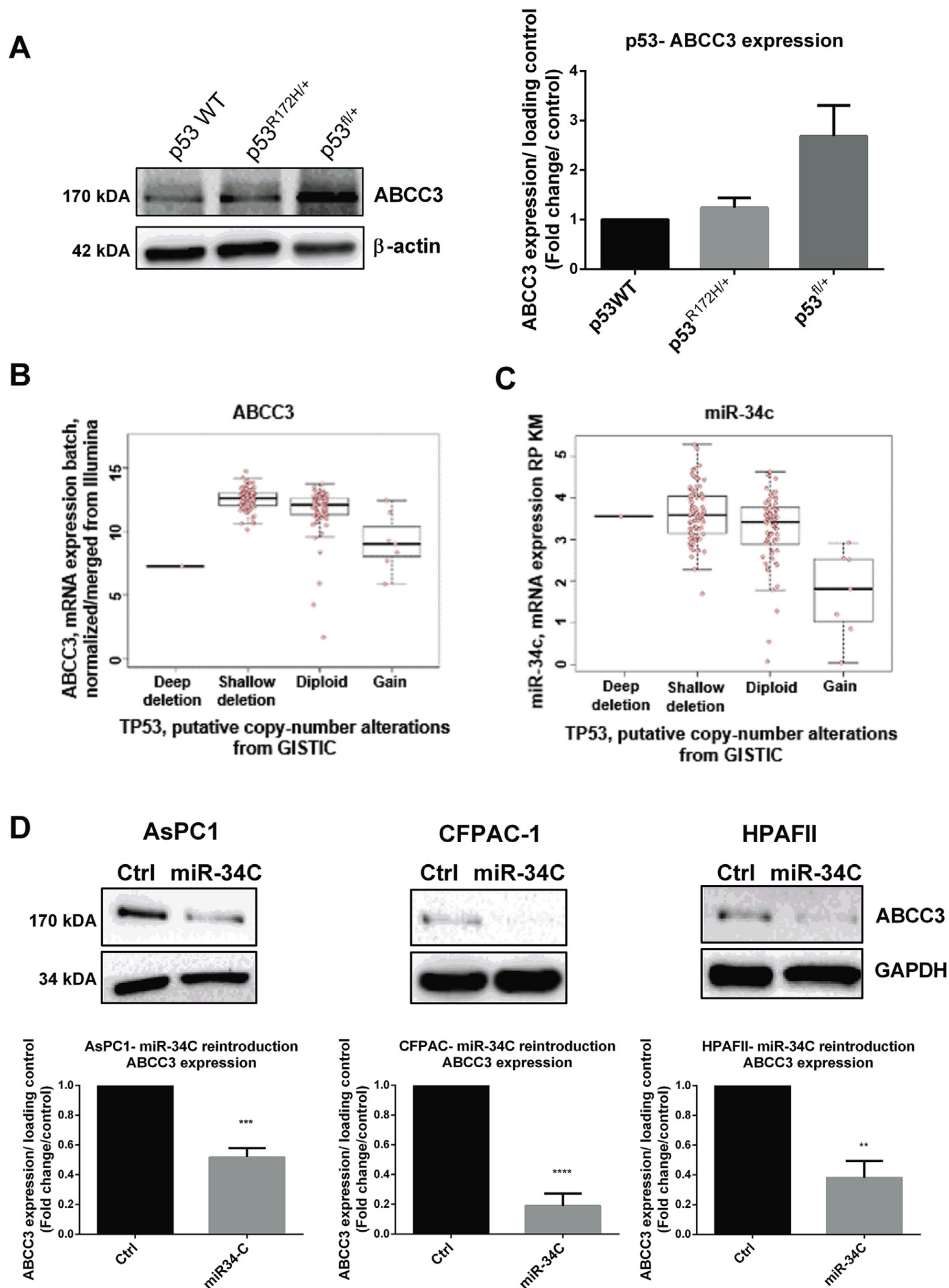

Fig. 5. ABCC3 expression is p53-dependent.
(A) Representative western blot analysis of ABCC3 expression in mice cell lines with different p53 status (p53 WT- PZR1, $p53^{R172H/+}$-PZPR1, $p53^{fl/+}$-PZPflR); Database analysis of TCGA pancreatic adenocarcinoma showing negative correlation between p53 status and (B) ABCC3 and (C) miR-34C expression; (D) Representative western blot images and quantitative analysis of ABCC3 expression following reintroduction of miR-34C into 3 different PDAC cell lines. All results are presented as a mean ± SEM of 3 independent experiments *p < 0.5, **p < 0.01, ***p < 0.001, ****p < 0.0001.

ABCC3 and ABCC5, in PDAC ducts, which for ABCC3 correlated positively with the tumour grading (Konig et al., 2005). However, no study has so far reported the involvement of ABC transporters in PDAC development or identified their potential as pharmacological targets in PDAC.

Here, we have demonstrated that the ABC transporter ABCC3 is a novel and key player in PDAC biology, playing an active role in its progression. We showed that ABCC3 is highly expressed in PDAC specimens and the available bioinformatic data concurs that its

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Table 1
Expression of membrane ABCC3 in PDAC (n = 60) according to p53 IHC status.

	ABCC3 mean ± SE[a]	P[b]
p53 wild-type (n = 33)	4.9 ± 2.7	0.042
p53 mutated (n = 27)	17.2 ± 5.6	

[a] Mean percent of positive tumour cells ± Standard Error (SE).
[b] Independent-sample *t*-test.

expression correlates with poor prognosis for patients. We also showed that ABCC3 regulates PDAC cell proliferation *in vitro* and *in vivo* through the release of lysophosphatidylinositol (LPI), whose importance in PDAC progression we recently reported (Falasca and Ferro, 2016; Ferro et al., 2018). Having identified the essential role of LPI in PDAC progression and the transporter responsible for its secretion, we now have the opportunity to target the transporter and reduce the level of LPI in the tumour environment and interfere with cancer progression.

Initial indications that ABCC3 is a viable therapeutic target in PDAC was evident from genetic knockdown experiments which reduced PDAC anchorage-dependent and independent cell growth. Moreover, we showed that ABCC3 regulates STAT3 and HIF1α signalling pathways, key regulators of PDAC development and progression. It has been reported that constitutive activation of STAT3 signalling negatively affects the survival of PDAC patients (Wormann et al., 2016). It is known that STAT3 signalling is triggered by IL6 activation of gp130 (Corcoran et al., 2011; Lesina et al., 2011). However, a recent study suggested the existence of gp130-independent STAT3 activation in PDAC (Wormann et al., 2016), which is consistent with our findings of ABCC3-mediated STAT3 induction. These results suggest that ABCC3-regulated function of STAT3 and HIF1α may represent the potential mechanism of ABCC3- mediated PDAC progression.

Apart from the high chemoresistance of PDAC tumours, the unsuccessful outcome of the majority of clinical trials in PDAC can also be attributed to the lack of proper stratification of patients into cohorts and to the failure to target therapies based on the mutational landscape. We show herein that the expression of ABCC3 is dependent on the genetic status of *TP53*, one of the main genes dysregulated in PDAC. Wild type p53 levels negatively correlate with ABCC3 mRNA and protein levels and this relationship appears to be mediated by miR-34C whose expression is dependent on p53 activity and is therefore usually downregulated in pancreatic cancer (He et al., 2007; Ji et al., 2009) (Fig. 6). It has been previously documented that in PDAC, constitutive activation of both HIF1α and STAT3 pathways is dependent on the *TP53* mutation or deletion (Wormann et al., 2016), which is consistent with our findings. It has also been shown that one of the mechanisms regulating the expression of the miR34 family involves pSTAT3, whose increased expression in *TP53* mutated samples blocks the activity of miR34a (Slabáková et al., 2017). Similarly, in colorectal cancer, HIF1α activity in hypoxic conditions also represses miR34a expression and affects STAT3 signalling (Li et al., 2017). It is tempting to speculate whether the activity of miR34-C might also be affected by STAT3 and HIF1α signalling in pancreatic cancer. Therefore, STAT3 and HIF1α downregulation through ABCC3 blockade might eliminate their inhibitory effect on miR34-C activity, which in turn would lower ABCC3 expression. This feed-forward loop might provide the mechanism by which pharmacological targeting of ABCC3 could reprogram pancreatic cancer cells and potentially slow down the disease progression and increase patient survival.

Our data, therefore, propose a mechanism by which pancreatic cancer cells might regulate the expression and activity of pro-tumorigenic proteins like ABCC3. It also shows the importance of genetic screening before the selection of patients for clinical trials and the therapy selection. Our data also provides an explanation for the recently demonstrated correlation between the presence of WT-p53 and the chemosensitivity of PDAC cells (Abrams et al., 2018). It has been additionally demonstrated that the beneficial effect of drugs such as Nutlin-3a and MDM-2 inhibitor, depends on the TP53 status (Candido et al., 2019). Similarly, the presence of WT53, which we have shown would decrease the levels of the ABCC3 protein, could indicate a lower response to potential ABCC3-targeting

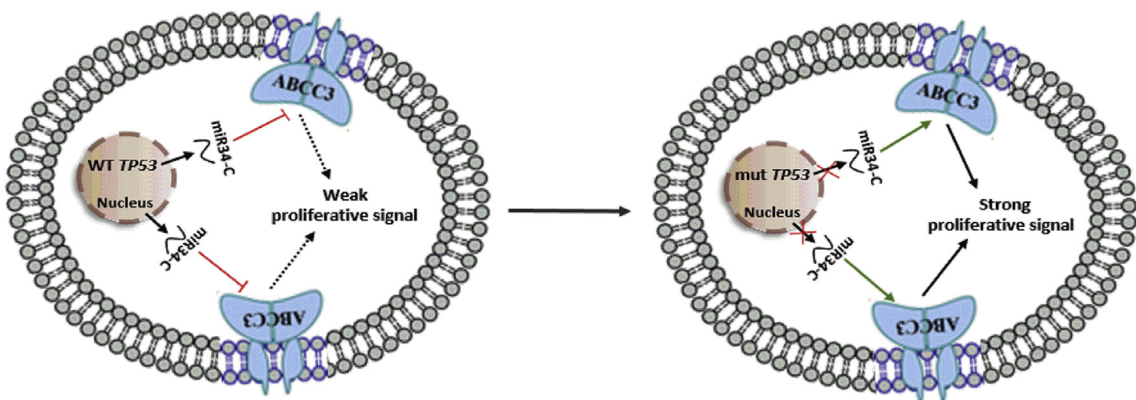

Fig. 6. ABCC3- mediated regulation of PDAC progression depends on p53 status.
The model of regulation of ABCC3 expression and activity in PDAC specimens characterized by *TP53* mutations.

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therapies.

In conclusion, our data demonstrate for the first time the key role played by ABCC3 in PDAC progression. The involvement of ABCC3 in PDAC cell proliferation *in vitro* and tumour growth *in vivo* xenograft model was demonstrated. The correlation of ABCC3 expression with p53 status, as well as LPI-mediated regulation of key signalling pathways in PDAC biology, reinforce the importance of ABCC3 in PDAC. Collectively, our data identify ABCC3 as a promising therapeutic target in pancreatic cancer, which potential should be explored clinically. It also suggests a basis for the selection of a cohort of patients that might benefit from ABCC3-targeted therapies.

Conflicts of interest

The authors declare that they have no conflicts of interest with the publication of this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbior.2019.04.004.

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