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FACULTY OF HEALTH SCIENCIES

Study and evaluation of the efficacy of pancreatic proenzymes against the tumour microenvironment and chemoresistance in pancreatic cancer

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Para optar a la mención de "**Doctor Internacional**", la doctoranda realizó durante el periodo de formación una estancia de tres meses (15 Septiembre a 15 Diciembre de 2023) en el Laboratorio de la Prof. Dra. Elisa Giovannetti, Department of Medical Oncology, Lab. Medical Oncology - PI, Molecular Mechanisms of Drug Activity Group Location VUmc, Cancer Center Amsterdam, Vrije Universiteit Amsterdam.

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Certifica que **Dª María Belén Toledo Cutillas,** graduada en Biotecnología, ha realizado bajo su dirección y en el Departamento de Ciencias de la Salud, el trabajo titulado "Estudio y evaluación de la eficacia de proenzimas pancreáticas frente al microambiente tumoural y la quimiorresistencia" reuniendo las condiciones necesarias para optar al grado de Doctor.

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< Lo que hacemos en la vida tiene su eco en la eternidad >

Máximo Décimo Meridio, Gladiator

< What we do in life, echoes in eternity >

Maximus Decimus Meridius, Gladiator

“Para mamá y papá, que siempre habéis creído en mí mucho más
de lo que yo lo hacía.

Porque es más sencillo ser valiente y enfrentarme a mis miedos
cuando sé que estáis a mi lado.

Mi objetivo es que os sintáis tan orgullosos de ser mis padres
como yo me siento de ser vuestra hija.

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Gracias por hacer de nuestra casa un lugar ilimitado
de amor, respeto y apoyo.

Os quiero y os querré, en todas mis vidas.”

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GLOSSARY

GLOSSARY

2DG 2-Deoxyglucose
5-AZA 5-Azacytidine
5-FU: 5-Fluorouracil
α-CTLA-4 Alpha-Cytotoxic T-Lymphocyte-associated Protein 4
α-PD-L1 Alpha-Programmed death-ligand 1
αSMA Alpha Smooth Muscle Actin

A

ABC ATP-Binding Cassette
ACT Adoptive Cell Transfer
AI Artificial Intelligence
AKT Serine/Threonine Kinase
ALDH Aldehyde Dehydrogenase
ALK Anaplastic Lymphoma Kinase
AP-1 Activator protein 1
Arg2 Arginase 2
ASOs Antisense oligonucleotides
ATCC American Type Culture Collection
ATM Ataxia Telangiectasia Mutated
ATP Adenosine Triphosphate

B

Bcl-2 B-Cell Lymphoma 2
BER Base-Excision Repair
BM Bone Marrow
BMP2 Bone Morphogenetic Protein 2
BRAF B-Rar Proto-oncogene

C

CA Carbohydrate Antigen
CAFs Cancer-associated Fibroblasts
CAR Chimeric Antigen Receptor
CAR-T Chimeric Antigen Receptor-Engineered Autologous T
CAV-1 Caveolin-1
CCL C-C Motif Chemokine Ligand
CCR C-C Motif Chemokine Receptor
CDA Cytidine Deaminase
CDP Cytidine Diphosphate
CDTD Deoxycytidine Monophosphate Deaminase
CEFs CAF-educated Fibroblasts
Chi3L1 Chitinase-3-like protein 1
CM Conditioned Medium
COXs Cyclooxygenases
CRC Colorectal Carcinoma
CSCs Cancer Stem-Like Cells
CT Computed Tomography
Ct Threshold Cycle
ctDNA Circulating tumour DNA
CTGF Connective Tissue Growth Factor
CTLA4 Cytotoxic T Lymphocyte associated Protein-4
CTLs Cytotoxic T Lymphocyte
CXCL Chemokine (C-X-C motif) Ligand
CXCR C-X-C Motif Chemokine Receptor

D

DAPI 4'-6-diamidino-2-phenylindole
DCs Dendritic Cells
dCDA Deoxycytidine Deaminase
dCDP Deoxycytidine Diphosphate
dCK Deoxycytidine Kinase
DCR Disease Control Rate
dCTP Deoxycytidine Triphosphate
DDDs DNA-Damaging Drugs
DDRs DNA-Damage Responses
dFdC Gemcitabine
dFdCDP Gemcitabine Diphosphate
dFdCMP Gemcitabine Monophosphate
dFdCTP Gemcitabine Triphosphate
dFdU 2',2'-Difluorodeoxyuridine
dFdUMP 2',2'-Difluorodeoxyuridine Monophosphate
dFdUTP 2',2'-Difluorodeoxyuridine Triphosphate
DFDCDP Difluorodeoxycytidine Diphosphate
DFDCMP Difluorodeoxycytidine Monophosphate
DFDCTP Difluorodeoxycytidine Triphosphate
DMEM Dubelcco's Modified Eagle's Medium
DNA Deoxyribonucleic Acid
DPD Dihydropyrimidine Dehydrogenase
DSB Double Strand Breaks
dTMP Deoxythymidine Monophosphate
DTR Diphtheria Toxin Receptor

E

E2 17β-Estradiol
EBV Epstein-Barr Virus
ECM Extracellular Matrix
ECOG Eastern Cooperative Oncology Group
EFNB2 Ephrin-B2
EGF Epidermal Growth Factor
EGFR Epidermal Growth Factor Receptor
EMAP II Endothelial Monocyte Activating Polypeptide II
EMT Epithelial-to-mesenchymal Transition
EndMT Endothelial-to-mesenchymal Transition
EP 1-4 E-type prostanoid receptors 1-4
EPCs Endothelial Progenitor Cells
ER Endoplasmic Reticulum
ERK Extracellular Signal-regulated Kinase

F

FA Fanconi Anemia
FACS Fluorescence-activated Cell Sorting
FAFs Fibrosis-associated Fibroblasts
FAK Focal Adhesion Kinase
FAPα Fibroblast Activation Protein-α
FASL Fas Ligand
FBS Fetal Bovine Serum
FDA Food and Drug Administration
FDUMP Fluorodeoxyuridine Monophosphate
FGF(R) Fibroblast Growth Factor (Receptor)
FLI1 Friend Leukemia Integration 1 Transcription Factor
FSP-1 Fibroblast-specific Protein 1

G

G1 1-(4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl)-Ethanone
GCs Gastrointestinal Cancers
GEM Gemcitabine, 2'-2'-difluorodeoxycytidine, dFdC
GFRs Growth Factor Receptors
GISTs Gastrointestinal Stromal Tumours
GLI Glioma-associated Oncogene
GLUT-1 Glucose Transporter 1
GM-CSF Granulocyte-Macrophage Colony-Stimulating Factor
GOF Gain of Functions
GPER G-Protein-coupled Estrogen Receptor
GR Gemcitabine Resistant
GSIs Gamma-Secretase Inhibitors
GSH Glutathione
GSK Glycogen Synthase Kinase-3
GSR Gemcitabine Sensitive Ratio

H

HA Hyaluronic acid
HCC Hepatocellular Carcinoma
HDACi Histone Deacetylase Inhibitor
HDR Homology-directed Repair
hENT Human Equilibrative Nucleoside Transporter
HGF Hepatocyte Growth Factor
HGSOC High-grade Serous Ovarian Cancer
HH Hedgehog
HIF1-α Hypoxia Inducible Factor 1-alpha
HK Hexokinase
HLA Human Leukocyte Antigen
HNSCC Head and Neck Squamous Cell Carcinoma
HPCs Hematopoietic Progenitor Cells
HPV Human Papillomavirus
HR Homologous recombination
HSF1 Heat Shock Factor 1
HUVECs Human Umbilical Vein-derived Endothelial Cells

I

IARC International Agency of Research on Cancer
IBC Invasive Breast Cancer
IC_{50} Half-Maximal Inhibitory Concentration
iCAFs Inflammatory-like Cancer-associated Fibroblasts
ICAM Intercellular Adhesion Molecule 1
IDH Isocitrate Dehydrogenase
IDO1 Indoleamine 2,3-Dioxygenase 1
IF Immunofluorescence
IGF Insulin-like Growth Factor
IGFR Insulin Growth Factor Receptor
IL Interleukin
IPMN Intraductal Papillary Mucinous Neoplasm

J

JAK/STAT Janus kinase-Signal Transducer and Activator of Transcription

K

KLF5 Kruppel-like Factor 5
KRAS Kirsten Rat Sarcoma Virus

L

LCC Lewis Lung Cancer
LDH-A Lactate Dehydrogenase
LPA Lipoprotein A

M

MAF Metastasis-associated Fibroblasts
MAPK Mitogen-activated Protein Kinase
MCN Mucinous Cystic Neoplasm
MCs Mast Cells
MDR Multidrug Resistance
MDSCs Myeloid-derived Suppressor Cells
MEK Mitogen-activated Protein Kinase
MET Methionine
MHC Major Histocompatibility Complex Class
MMPs Matrix metalloproteinases
MRI Magnetic Resonance Imaging
mRNA Messenger RNA
MSCs Mesenchymal Stem Cells
MSI Microsatellite Instability
mTOR Mammalian Target of Rapamycin
MTT 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
MVP Major Vault Protein
myCAFs Myofibroblast-like Cancer-associated Fibroblasts
MZF-1 Myeloid Zinc Finger-1

N

NAFs Normal Activated Fibroblasts
NDPK Nucleoside Diphosphate Kinase
NF-κβ Nuclear factor kappa-light-chain-enhancer of activated B cells
NER Nucleotide Excision Repair
NGF Nerve Growth Factor
NHEJ Non-Homologous End-Joining
NK Natural Killer
NM Normal Medium
nM Nanomolar
NMPK Nucleoside Monophosphate Kinase
NSCLC Non-Small Cell Lung Carcinoma
NTRK Neurotrophic Tyrosine Receptor Kinase

O

OATP Organic Anionic Transporter
OCT Organic Cation Transport
OPN Osteopontin
ORR Overall Response Rate
OS Overall Survival
OXPHOS Oxidative Phosphorylation

P

PanIN Pancreatic Intraepithelial Neoplasm
PARP Poly (ADP-ribose) Polymerase
PARPi Poly (ADP-ribose) Polymerase Inhibitor
PBS Phosphate-buffered Saline
PC Pancreatic Cancer
PD-1 Death Protein 1
PD-L Programmed Cell Death-ligand
PDAC Pancreatic Ductal Adenocarcinoma
PDGF Platelet-derived Growth Factor
PDGF-BB Platelet-derived Growth Factor-BB
PDK1 3-Phosphoinositide protein kinase-1
PDX Patient-derived Xenograft
PFA Paraformaldehyde
PFS Progression Free Survival
PGE2 Prostaglandin E2
PI3K Phosphoinositide-3-kinase
PIP3 Phosphatidylinositol (3,4,5)-trisphosphate
PLOD2 2-Oxoglutarate 5-Dioxygenase 2
POSTN Periostin
PRP Chymotrypsinogen and Trypsinogen 6:1
PSCs Pancreatic Stellate Cells
PTCH Protein Patched Homolog
PTEN Phosphatase and Tensin Homolog

Q

QoL Quality of Life

R

rDCs Regulatory DCs
RI Resistance Index
RNA Ribonucleic Acid
RNA-seq RNA sequencing
RNI Reactive Nitrogen intermediates
ROCK Rho-associated Protein Kinase
ROS Reactive Oxygen Species
RR Ribonucleotide Reductase
RT Room Temperature

S

SDF-1 Stromal Cell-Derived Factor 1
SDS-PAGE Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
Ser Serine
Shh Sonic Hedgehog
Smo Smoothened
SOCS1 Suppressor Of Cytokine Signaling 1
Sox2 SRY-Box Transcription Factor 2
SPARC Secreted Protein Acidic And Cysteine Rich
SRB Sulforhodamine B
SRF Serum Response Factor
STAT3 Signal Transducer and Activator of Transcription 3

T

TAMs Tumour-associated Macrophages
TANs Tumour-associated Neutrophils
TBST Tris-buffered Saline with Tween
TCF Ternary Complex Factor
TEP Tumour Educated Platelet
TGF-β Transforming Growth Factor beta
Tie1/2 Tyrosine Kinase with Immunoglobulin-like and EGF-like Domains 1/2
TIMP-1 Tissue Inhibitor of Metalloproteinase-1
TIn Tumourgenesis Index
TK1 Thymidine Kinase 1
TKI Tyrosine Kinase Inhibitor
TME Tumour Microenvironment
TN-C Tenascin-C
TNF tumour Necrosis Factor
Treg Regulatory T-cells
TS Thymidine Synthase

U

UV Ultraviolet

V

VCAM Vascular Cellular Adhesion Molecule
VDR Vitamin D Receptor
VEGF(R) Vascular Endothelial Growth Factor (Receptor)
VIM Vimentin
Vit Vitamine
VRAC Volume-Regulated Anion Channel

W

WFDC1 WAP Four-Disulfide Core Domain 1
WHO World Health Organization
WT Wild Type

Y

YAP1 Yes-associated Protein-1

Z

ZEB1 Zinc Finger E-box Binding Homeobox

SUMMARY
SAMENVATTING
RESUMEN

ENGLISH SUMMARY

Cancer ranks as the second leading cause of death in developed countries. It is well characterized by uncontrolled cell growth, invasion of neighboring tissues, and potential spread to other organs through metastasis. The uncontrolled growth is caused by genetic alterations that disrupt growth regulatory mechanisms, which can be spontaneous or induced by environmental factors, lifestyle, or hereditary factors. Pancreatic ductal adenocarcinoma (PDAC) is among one of the most aggressive and lethal type of cancer, representing approximately 90% of all pancreatic malignancies. Characterized by its late diagnosis and rapid progression, PDAC is often detected at an advanced stage, significantly limiting treatment options and resulting in a dismal five-year survival rate of less than 12%. The pathogenesis of PDAC is driven by a complex interplay of genetic mutations, including in key genes like KRAS, TP53, CDKN2A, and SMAD, alongside extensive epigenetic alterations. Additionally, the tumour microenvironment (TME), rich in desmoplastic stroma and cancer-associated fibroblasts (CAFs), contributes to the disease's resistance to conventional therapies. Despite advances in surgical techniques, chemotherapy, and radiotherapy, PDAC remains exceptionally challenging to treat, underscoring an urgent need for novel therapeutic strategies and early detection biomarkers.

Cancer involves three key processes: tumourigenesis (or oncogenesis), tumour progression and tumour dissemination (or metastasis). Tumourigenesis is the transition from a normal cell to a cancer cell; while tumour progression involves the tumour growth and interactions between cells that surround it, implying malignancy of the environment; and tumour dissemination implicates the movement of cancer cells from the primary site to distant organs. Notably, only 10% of cancer-related deaths are caused by the primary tumour. Therefore, the major therapeutic challenge lies in addressing metastatic or secondary tumours.

The absence of a universally effective treatment is largely due to the considerable heterogeneity of cancer. This heterogeneity is evident not only between different types of tumours and among patients with the same type of tumour (inter-tumour heterogeneity) but also within the diverse cell subpopulations that comprise a single tumour mass (intra-tumour heterogeneity). Additionally, the main treatment, which is based on chemotherapy, produces resistance that is favored by a malignant TME. Chemoresistant cells in PDAC represent a significant obstacle to effective treatment and a major contributor to the disease's poor prognosis. They exhibit an enhanced ability to survive despite aggressive chemotherapy regimens, such as Gemcitabine (GEM), due to various intrinsic and extrinsic mechanisms. Genetic mutations, epigenetic alterations, and the activation of survival pathways enable these cells to evade apoptosis. Moreover, the TME plays a pivotal role in fostering chemoresistance, providing a protective niche where cancer cells can thrive. This resilience leads to reduced efficacy of standard treatments, frequent recurrence, and metastasis, underscoring the urgent need for novel therapeutic strategies that target these chemoresistant populations and their interactions with the TME to improve patient outcomes in PDAC. Therefore, adjuvant therapies specifically targeting chemoresistant cells are essential to reduce tumour recurrence and prevent cancer spread. Within tumours, chemoresistant cells possess unique capabilities, such as self-renewal, quiescence, and epithelial-to-mesenchymal transition (EMT), which contribute to drug resistance and metastasis. The lack of effective treatments against this cell group makes tumours more aggressive and increases the rate of tumour recurrence.

The use of natural agents, particularly the pancreatic (pro)enzymes trypsinogen and chymotrypsinogen, has shown in previous studies its efficacy in both *in vitro* and *in vivo* studies against cancer stem cells (CSCs), indicating a high potential for clinical application. These (pro)enzymes could somehow affect the three key processes mentioned before (oncogenesis, tumour progression and metastasis), making their combination, known as the drug PRP, a promising therapeutic approach for treating PDAC.

In this thesis, we focused on the crucial role of the TME supporting

tumour progression, invasiveness, metastasis, and in promoting drug resistance. To this end, we first conducted an extensive review of studies focusing on the formation of the TME, its cellular composition, cellular interactions within the TME, and the formation of the pre-metastatic niche. We covered the molecular pathways that govern the evolution of the TME, focusing our interest on the malignification experienced by stromal cells due to the influence of tumour cells, and the crosstalk established between them.

Second, we targeted CAFs testing the efficacy of the pancreatic (pro)enzymes treatment on this cell population. We developed an *in vitro* model of TME, based on 2D and 3D cell co-cultures and our findings demonstrated PRP multifaceted effects. In short, the treatment included: (i) inhibition of the acquisition of a pro-tumoural phenotype by healthy fibroblasts; (ii) enhanced expression of E-cadherin and decreased expression of EMT-associated genes; (iii) blockade of the crosstalk between CAFs and cancer cells, leading to a decrease in the proliferation and migration rate of the pancreatic cancer cells; (iv) modulation of CAFs-induced alteration on endothelial cells; (v) induction of Meflin expression, associated with a less pro-tumoural phenotype; (vi) inhibition of the TGF-β pathway, reducing the activation of Smad2/3 proteins involved in fibroblast malignification; (vii) selective induction of apoptosis in CAFs by PRP, as evidenced by increased expression of the BAX biomarker; (viii) impaired fibrotic tissue formation, as tested in an *in vivo* model of PDAC, by reducing the population of CAFs within the TME and reducing tumour growth and volume. Collectively, these results underscore the potential of PRP as a therapeutic candidate for disrupting the intricate interactions within the PDAC TME.

Third, we validated the (pro)enzymes pancreatic formulation on chemoresistant human pancreatic cancer cell lines (BxPC3, Suit2.007, and Patu-T). The idea of developing pancreatic cancer cell lines with a chemoresistant phenotype was to simulate a key issue in PDAC, such as the emergence of chemoresistance against current chemotherapeutic treatments. From there we were able to study different resistance mechanisms, investigate possible alternative therapies specifically directed at this subset of cells and evaluate the effectiveness of pro-enzymatic treatment against metastasis and tumour relapse.

Interestingly, (i) exposure of these chemoresistant pancreatic lines to PRP resulted in a significant decrease in their IC_{50} against GEM (especially in BxPC-3 and Suit2.007); (ii) in addition, their proliferation, clonogenicity, invasion and migration rates were also reduced; (iii) suppression of the expression of chemoresistant markers (metabolic and transporter) with this treatment was observed *via* RT-qPCR, immunofluorescence, and western blot; (iv) blockade of the EMT process in all three cell lines; (v) PRP also promoted the inhibition of the TGFβ pathway; (vi) as well as induced cell death through apoptosis in a broad tumour cell population.

The development of a coadjuvant treatment with PRP and GEM holds (based on PRP followed by later addition of GEM) significant promise for addressing the challenge of chemoresistance in pancreatic cancer. By targeting the TME, CSCs, and mechanisms of drug resistance, this combination therapy could provide a more effective and comprehensive approach to treating PDAC. Further clinical research and trials are necessary to validate the efficacy and safety of this combination, but the potential benefits make it a compelling avenue for improving outcomes in pancreatic cancer patients.

NEDERLANDSE SAMENVATTING

Kanker is de tweede belangrijkste doodsoorzaak in ontwikkelde landen. Het wordt voornamelijk gekenmerkt door ongecontroleerde celgroei, de invasie van nabijgelegen weefsels en de potentiële verspreiding naar andere organen via metastase. De ongecontroleerde groei wordt veroorzaakt door genetische veranderingen die de groeiregulerende mechanismen verstoren, welke spontaan kunnen zijn of veroorzaakt door omgevingsfactoren, levensstijl of erfelijke factoren. Pancreas ductaal adenocarcinoom (PDAC) is een van de meest agressieve en dodelijke vormen van kanker, en vertegenwoordigt ongeveer 90% van alle pancreatische maligniteiten. Gekenmerkt door laattijdige diagnose en snelle progressie, wordt PDAC vaak in een vergevorderd stadium ontdekt, wat de behandelingsopties aanzienlijk beperkt en resulteert in een vijfjaarsoverleving lager dan 12%. De pathogenese van PDAC wordt aangedreven door een complexe interactie van genetische mutaties, waaronder in sleutelgenen zoals KRAS, TP53, CDKN2A en SMAD, samen met uitgebreide epigenetische veranderingen. Bovendien draagt de tumormicro-omgeving (TME), rijk aan desmoplastisch stroma en met kanker geassocieerde fibroblasten (CAFs), bij aan de resistentie van de ziekte tegen conventionele therapieën. Ondanks vooruitgang in chirurgische technieken, chemotherapie en radiotherapie blijft PDAC uitzonderlijk moeilijk te behandelen, wat de dringende behoefte aan nieuwe therapeutische strategieën en vroege detectie biomarkers onderstreept.

Kanker omvat drie sleutelprocessen: tumorigenese (of oncogenese), tumorvoortgang en tumordisseminatie (of metastase). Tumorigenese is de overgang van een normale cel naar een kankercel; terwijl tumorvoortgang de tumorgroei en interacties tussen de omliggende cellen impliceert, wat de maligniteit van de omgeving inhoudt; en tumordisseminatie houdt de beweging van kankercellen van de primaire plaats naar verre organen in. Het is opmerkelijk dat slechts 10% van de aan kanker gerelateerde sterfgevallen door de primaire tumor wordt veroorzaakt. Daarom ligt de belangrijkste

therapeutische uitdaging in het aanpakken van metastatische of secundaire tumoren.

Het ontbreken van een universeel effectieve behandeling is grotendeels te wijten aan de aanzienlijke heterogeniteit van kanker. Deze heterogeniteit is evident niet alleen tussen verschillende typen tumoren en onder patiënten met hetzelfde type tumor (intertumorale heterogeniteit), maar ook binnen de diverse celsubpopulaties die één enkele tumormassa vormen (intratumorale heterogeniteit). Bovendien produceert de hoofdbehandeling, die gebaseerd is op chemotherapie, resistentie die wordt bevorderd door een kwaadaardige TME. Chemoresistente cellen in PDAC vormen een significante hindernis voor effectieve behandeling en dragen sterk bij aan de slechte prognose van de ziekte. Deze cellen tonen een verbeterd vermogen om te overleven ondanks agressieve chemotherapiebehandelingen, zoals Gemcitabine (GEM), door verschillende intrinsieke en extrinsieke mechanismen. Genetische mutaties, epigenetische veranderingen en de activering van overlevingspaden stellen deze cellen in staat apoptose te ontwijken. Bovendien speelt de TME een cruciale rol in het bevorderen van chemoresistentie, door een beschermende niche te bieden waarin kankercellen kunnen gedijen. Deze veerkracht leidt tot verminderde effectiviteit van standaardbehandelingen, frequente terugval en metastase, wat de dringende behoefte aan nieuwe therapeutische strategieën onderstreept die gericht zijn op deze chemoresistente populaties en hun interacties met de TME om de resultaten bij PDAC-patiënten te verbeteren. Daarom zijn adjuvante therapieën die specifiek gericht zijn op chemoresistente cellen essentieel om tumorrecidief te verminderen en de verspreiding van kanker te voorkomen. Binnen tumoren bezitten chemoresistente cellen unieke capaciteiten, zoals zelfvernieuwing, quiescentie en epitheliale-mesenchymale transitie (EMT), die bijdragen aan geneesmiddelresistentie en metastase. Het ontbreken van effectieve behandelingen tegen deze celgroep maakt tumoren agressiever en verhoogt de tumorrecidief percentage.

Het gebruik van natuurlijke middelen, vooral de pancreatische (pro)enzymen trypsinogeen en chymotrypsinogeen, heeft in eerdere studies zijn

effectiviteit aangetoond, zowel *in vitro* als *in vivo* tegen kankerstamcellen (CSCs), wat aangeeft dat er een hoog potentieel is voor klinische toepassingen. Deze (pro)enzymen zouden de drie eerder genoemde kernprocessen (oncogenese, tumorgroei en metastase) op een of andere manier kunnen beïnvloeden, waardoor hun combinatie, bekend als het geneesmiddel PRP, een veelbelovende therapeutische benadering wordt voor de behandeling van PDAC.

In deze scriptie hebben we ons gericht op de cruciale rol van de TME bij het ondersteunen van tumorvoortgang, invasiviteit, metastase en het bevorderen van geneesmiddelresistentie. Daartoe hebben we eerst een grondige review uitgevoerd van studies die zich concentreren op de vorming van de TME, de cellulaire samenstelling, cellulaire interacties binnen de TME en de vorming van de pre-metastatische niche. We hebben de moleculaire routes die de evolutie van de TME beheersen behandeld, waarbij we onze interesse richtten op de malignificatie die cellen uit het stroma ervaren door de invloed van tumorcellen en de communicatie die tussen hen wordt vastgesteld.

Ten tweede richtten we ons op CAFs door de effectiviteit van de behandeling met pancreatische (pro)enzymen op deze celpopulatie te testen. We ontwikkelden een *in vitro* model van TME, gebaseerd op 2D en 3D celco-culturen, en onze bevindingen demonstreerden de veelzijdige effecten van PRP. Kort gezegd omvatte de behandeling: (i) remming van de verwerving van een pro-tumoraal fenotype door gezonde fibroblasten; (ii) verhoogde expressie van E-cadherine en verminderde expressie van EMT-geassocieerde genen; (iii) blokkering van de communicatie tussen CAFs en kankercellen, wat leidde tot een afname van de proliferatie- en migratiesnelheid van de pancreatische kankercellen; (iv) modulatie van door CAFs geïnduceerde veranderinen op endotheelcellen; (v) inductie van de expressie van Meflin, geassocieerd met een minder pro-tumoraal fenotype; (vi) inhibitie van de TGF-β route, wat de activering van Smad2/3 eiwitten betrokken bij fibroblast malignificatie vermindert; (vii) selectieve inductie van apoptose in CAFs door PRP, zoals blijkt uit de toegenomen expressie van de BAX biomarker; (viii) verminderde vorming van fibrotisch weefsel, getest in een *in vivo* model van PDAC, door het

verminderen van de populatie van CAFs binnen de TME en het verkleinen van tumor groei en volume. Gezamenlijk benadrukken deze resultaten het potentieel van PRP als therapeutische kandidaat voor het verstoren van de complexe interacties binnen de PDAC TME.

Ten derde valideerden we de formulering van pancreatische (pro)enzymen op chemoresistente menselijke pancreaskankercellijnen (BxPC3, Suit2.007 en Patu-T). Het idee om pancreaskankercellijnen met een chemoresistent fenotype te ontwikkelen, was om een belangrijk probleem in PDAC te simuleren, zoals de opkomst van chemoresistentie tegen de huidige chemotherapeutische behandelingen. Vanuit daar konden we verschillende weerstandsmechanismen bestuderen, mogelijke alternatieve therapieën onderzoeken die specifiek op deze subset van cellen zijn gericht en de effectiviteit van pro-enzymatische behandeling tegen metastase en tumorrecidief evalueren. Interessant was dat (i) blootstelling van deze chemoresistente pancreatische lijnen aan PRP resulteerde in een significante afname in hun IC50 tegen GEM (vooral in BxPC-3 en Suit2.007); (ii) bovendien werden ook hun proliferatie-, clonogeniciteit-, invasie- en migratiesnelheden verminderd; (iii) onderdrukking van de expressie van chemoresistente markers (metabool en transporter) werd met deze behandeling waargenomen via RT-qPCR, immunofluorescentie en western blot; (iv) blokkering van de EMT in alle drie de cellijnen; (v) PRP bevorderde ook de inhibitie van de TGFβ route; (vi) evenals geïnduceerde celdood door apoptose in een brede populatie tumorcellen.

De ontwikkeling van een adjuvante behandeling met PRP en GEM (gebaseerd op PRP gevolgd door later toevoeging van GEM) biedt veelbelovend potentieel om de uitdaging van chemoresistentie bij alvleesklierkanker aan te pakken. Door te richten op de TME, CSCs en mechanismen van geneesmiddelresistentie, zou deze combinatietherapie een effectievere en meer alomvattende benadering kunnen bieden voor de behandeling van PDAC. Verdere klinische onderzoeken en trials zijn noodzakelijk om de effectiviteit en veiligheid van deze combinatie te bevestigen, maar de potentiële voordelen maken het een aantrekkelijke weg om de uitkomsten bij patiënten met pancreaskanker te verbeteren.

RESUMEN ESPAÑOL

El cáncer se clasifica como la segunda causa principal de muerte en los países desarrollados. Se caracteriza principalmente por el crecimiento celular descontrolado, la invasión de tejidos vecinos y la posible diseminación a otros órganos a través de la metástasis. El crecimiento descontrolado es causado por alteraciones genéticas que interrumpen los mecanismos de regulación del crecimiento, los cuales pueden ser espontáneos o inducidos por factores ambientales, el estilo de vida o factores hereditarios. El adenocarcinoma ductal pancreático (PDAC) se encuentra entre los tipos de cáncer más agresivos y letales, representando aproximadamente el 90% de todas las malignidades pancreáticas. Caracterizado por su diagnóstico tardío y rápida progresión, el PDAC suele detectarse en una etapa avanzada, limitando significativamente las opciones de tratamiento y resultando en una tasa de supervivencia a cinco años inferior al 12%. La patogénesis del PDAC está impulsada por una compleja interacción de mutaciones genéticas, incluidas en genes clave como KRAS, TP53, CDKN2A y SMAD, junto con extensas alteraciones epigenéticas. Además, el microambiente tumoral (TME), rico en estroma desmoplásico y fibroblastos asociados al cáncer (CAFs), contribuye a la resistencia de la enfermedad a las terapias convencionales. A pesar de los avances en técnicas quirúrgicas, quimioterapia y radioterapia, el PDAC sigue siendo excepcionalmente difícil de tratar, subrayando la urgente necesidad de nuevas estrategias terapéuticas y biomarcadores de detección temprana.

El cáncer involucra tres procesos clave: la tumorigenesis (o oncogénesis), la progresión tumoral y la diseminación tumoral (o metástasis). La tumorigenesis es la transición de una célula normal a una célula cancerosa; mientras que la progresión tumoral implica el crecimiento del tumor y las interacciones entre las células que lo rodean, lo que implica la malignidad del entorno; y la diseminación tumoral se refiere al movimiento de las células cancerosas desde el sitio primario a órganos distantes. Cabe destacar que solo el 10% de las muertes relacionadas con el cáncer son causadas por el tumor

primario. Por lo tanto, el principal desafío terapéutico radica en abordar los tumores metastásicos o secundarios.

La ausencia de un tratamiento universalmente efectivo se debe en gran medida a la considerable heterogeneidad del cáncer. Esta heterogeneidad es evidente no solo entre diferentes tipos de tumores y entre pacientes con el mismo tipo de tumor (heterogeneidad intertumoral), sino también dentro de las diversas subpoblaciones celulares que componen una única masa tumoral (heterogeneidad intratumoral). Además, el tratamiento principal, que se basa en la quimioterapia, produce resistencia que es favorecida por un microambiente tumoral (TME) maligno. Las células quimiorresistentes en el PDAC representan un obstáculo significativo para un tratamiento efectivo y son un gran contribuyente al mal pronóstico de la enfermedad. Estas células exhiben una capacidad mejorada para sobrevivir a pesar de regímenes de quimioterapia agresivos, como la Gemcitabina (GEM), debido a varios mecanismos intrínsecos y extrínsecos. Las mutaciones genéticas, las alteraciones epigenéticas y la activación de vías de supervivencia permiten que estas células evadan la apoptosis. Además, el TME juega un papel crucial en fomentar la quimiorresistencia, proporcionando un nicho protector donde las células cancerosas pueden prosperar. Esta resiliencia conduce a una eficacia reducida de los tratamientos estándar, a frecuentes recurrencias y a metástasis, lo que subraya la urgente necesidad de nuevas estrategias terapéuticas que apunten a estas poblaciones quimiorresistentes y sus interacciones con el TME para mejorar los resultados en los pacientes con PDAC. Por lo tanto, las terapias adyuvantes que se dirigen específicamente a las células quimiorresistentes son esenciales para reducir la recurrencia tumoral y prevenir la propagación del cáncer. Dentro de los tumores, las células quimiorresistentes poseen capacidades únicas, como la autorrenovación, la quiescencia y la transición epitelio-mesenquimal (EMT), que contribuyen a la resistencia a los fármacos y a la metástasis. La falta de tratamientos efectivos contra este grupo celular hace que los tumores sean más agresivos y aumenta la tasa de recurrencia tumoral.

El uso de agentes naturales, particularmente las (pro)enzimas pancreáticas tripsinógeno y quimotripsinógeno, ha demostrado en estudios previos su eficacia tanto *in vitro* como *in vivo* contra las células madre cancerosas (CSCs), lo que indica un alto potencial para su aplicación clínica. Estas (pro)enzimas podrían de alguna manera afectar los tres procesos clave mencionados anteriormente (oncogénesis, progresión tumoral y metástasis), lo que convierte su combinación, conocida como el fármaco PRP, en un enfoque terapéutico prometedor para el tratamiento del PDAC.

En esta tesis, nos centramos en el papel crucial del microambiente tumoral (TME) en el apoyo a la progresión del tumor, la invasividad, la metástasis y la promoción de la resistencia a los fármacos. Para ello, primero realizamos una revisión extensa de estudios que se centraron en la formación del TME, su composición celular, las interacciones celulares dentro del TME y la formación del nicho premetastásico. Abordamos las vías moleculares que rigen la evolución del TME, centrándonos en la malignificación que experimentan las células estromales debido a la influencia de las células tumorales y el "crosstalk" establecido entre ellas.

En segundo lugar, nos enfocamos en los CAFs probando la eficacia del tratamiento con (pro)enzimas pancreáticas en esta población celular. Desarrollamos un modelo *in vitro* del microambiente tumoral (TME), basado en co-cultivos celulares en 2D y 3D, y nuestros hallazgos demostraron los efectos multifacéticos del PRP. En resumen, el tratamiento incluyó: (i) la inhibición de la adquisición de un fenotipo pro-tumoral por parte de fibroblastos sanos; (ii) una mayor expresión de E-cadherina y una disminución en la expresión de genes asociados con EMT; (iii) el bloqueo del "crosstalk" entre los CAFs y las células cancerosas, lo que llevó a una disminución en la tasa de proliferación y migración de las células de cáncer pancreático; (iv) la modulación de la alteración inducida por los CAFs en las células endoteliales; (v) la inducción de la expresión de Meflin, asociada con un fenotipo menos pro-tumoral; (vi) la inhibición de la vía TGF-β, reduciendo la activación de las proteínas Smad2/3 involucradas en la malignificación de los fibroblastos; (vii) la inducción selectiva de apoptosis en los CAFs por el PRP, como lo evidenció el aumento en la

expresión del biomarcador BAX; (viii) la formación alterada de tejido fibrótico, como se probó en un modelo *in vivo* de PDAC, mediante la reducción de la población de CAFs dentro del TME y la disminución del crecimiento y volumen del tumor. En conjunto, estos resultados subrayan el potencial del PRP como candidato terapéutico para interrumpir las interacciones intrincadas dentro del TME del PDAC.

En tercer lugar, validamos la formulación de (pro)enzimas pancreáticas en líneas celulares de cáncer pancreático humano quimiorresistentes (BxPC3, Suit2.007 y Patu-T). La idea de desarrollar líneas celulares de cáncer pancreático con un fenotipo quimiorresistente fue simular un problema clave en el PDAC, como es la aparición de quimiorresistencia contra los tratamientos quimioterapéuticos actuales. A partir de ahí, pudimos estudiar diferentes mecanismos de resistencia, investigar posibles terapias alternativas dirigidas específicamente a este subconjunto de células y evaluar la efectividad del tratamiento pro-enzimático contra la metástasis y la recaída tumoral.

Curiosamente, (i) la exposición de estas líneas pancreáticas quimiorresistentes al PRP resultó en una disminución significativa de su IC_{50} contra GEM (especialmente en BxPC-3 y Suit2.007); (ii) además, se redujeron sus tasas de proliferación, clonogenicidad, invasión y migración; (iii) se observó la supresión de la expresión de marcadores quimiorresistentes (metabólicos y de transporte) con este tratamiento a través de RT-qPCR, inmunofluorescencia y western blot; (iv) se bloqueó la EMT en las tres líneas celulares; (v) el PRP también promovió la inhibición de la vía TGFβ; (vi) así como indujo la muerte celular por apoptosis en una amplia población celular tumoral.

El desarrollo de un tratamiento coadyuvante con PRP y GEM (basado en PRP seguido de la adición posterior de GEM) presenta una promesa significativa para abordar el desafío de la quimiorresistencia en el cáncer de páncreas. Al dirigirse al TME, las CSCs y los mecanismos de resistencia a los fármacos, esta terapia combinada podría ofrecer un enfoque más efectivo y completo para tratar el PDAC. Se requiere más investigación clínica y ensayos para validar la eficacia y seguridad de esta combinación, pero los beneficios potenciales hacen que sea

una vía atractiva para mejorar los resultados en pacientes con cáncer de páncreas.

INTRODUCTION

INTRODUCTION

1. CANCER – AN OVERVIEW

According to the World Health Organization (WHO), cancer ranks alongside cardiovascular diseases as one of the leading causes of death globally. The latest WHO data from 2023 is particularly alarming, with approximately 10 million annual deaths attributed to cancer [1]. This doctoral thesis specifically focuses on the study of pancreatic cancer, a type of malignancy whose incidence and mortality rates have doubled or even tripled in recent years [2]. In the United States, between 2014 and 2019, pancreatic cancer was the fourth leading cause of cancer-related deaths, despite its relatively low but increasing incidence (ranking tenth in this regard) [3], highlighting its high lethality. Projections have indicated that pancreatic ductal adenocarcinoma (PDAC) could rise to the second leading cause of cancer-related deaths by 2030 due to its growing incidence and lethality [4].

The processes involved in cancer include tumourigenesis or oncogenesis, which is the transition from a normal cell to a cancer cell; tumour progression, which consists on tumour growth; and tumour dissemination or metastasis, which is the progression of the tumour from its primary site to distant organs through blood vessels or the lymphatic system [5]. The uncontrolled growth is caused by genetic alterations such as errors in cell division, DNA damage, and hereditary factors [6]. These alterations disrupt normal regulatory mechanisms, leading to the accumulation of abnormal cells with resistance to apoptosis. The hallmarks of cancer have been deeply described, genome instability, inflammation, reprogramming of energy metabolism, immune system evasion, non-mutational epigenetic reprogramming, phenotypic plasticity, senescent cells, and polymorphic microbiomes [7].

To improve the clinical management of cancer in general (and pancreatic cancer in particular) and achieve personalized treatment, it is crucial to understand the biology and evolution of the disease, which varies from patient to patient.

Although various models of tumour progression have been proposed, they often rely on oversimplifications that hinder the development of effective treatments. Therefore, there is an urgent need for a deeper understanding of the molecular foundations of cancer, enabling the establishment of a more accurate and comprehensive model of tumour progression.

1.1 Epidemiology

According to the latest data provided by GLOBOCAN, there were a total of 20.0 million new cases of cancer worldwide in 2022, and 9.7 million deaths due to this disease. Globally, the top five most common types of cancer are lung (12.4%), breast (11.6%), colorectum (9,6%), prostate (7.3%) and stomach (4.9%). However, the top five types of cancer with the highest mortality rates are lung (18.7%), colorectum (9,3%), liver (7.8%), stomach (7,7%) breast cancer (6,9%) and pancreas (4.8%) (Figure 1a).

In men, lung cancer is the most frequent cancer (both cases and deaths), followed by prostate and colorectal cancer for new cases and liver and colorectal cancer for deaths (Figure 1b); whereas breast cancer is the most commonly diagnosed cancer in women and the leading cause of cancer death, followed by lung and colorectal cancer for both (cancer cases and deaths) (Figure 1c).

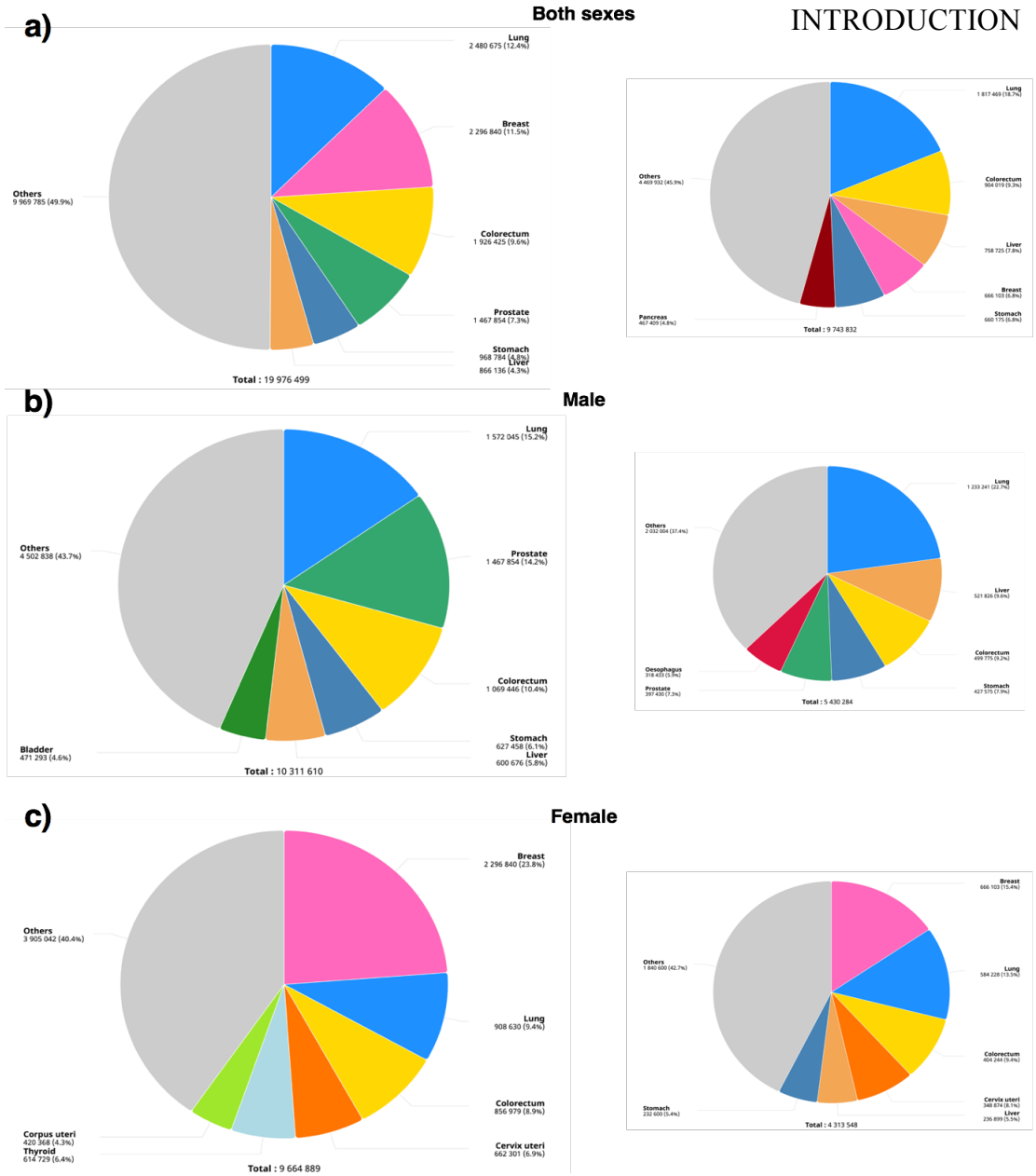


Figure 1. Pie charts present the distribution of cases and deaths for the top five cancers in 2022 for (A) both sexes, (B) males, and (C) females. The area of the pie chart reflects the proportion of the total number of cases or deaths. Source: GLOBOCAN 2022.

According to estimates, by 2030 the incidence of cancer will increase from 20.0 million to 24.6 million cases in both men and women. Furthermore, mortality rates are expected to increase as well, rising from 9.7 million to 12.9 million deaths (Figure 2).

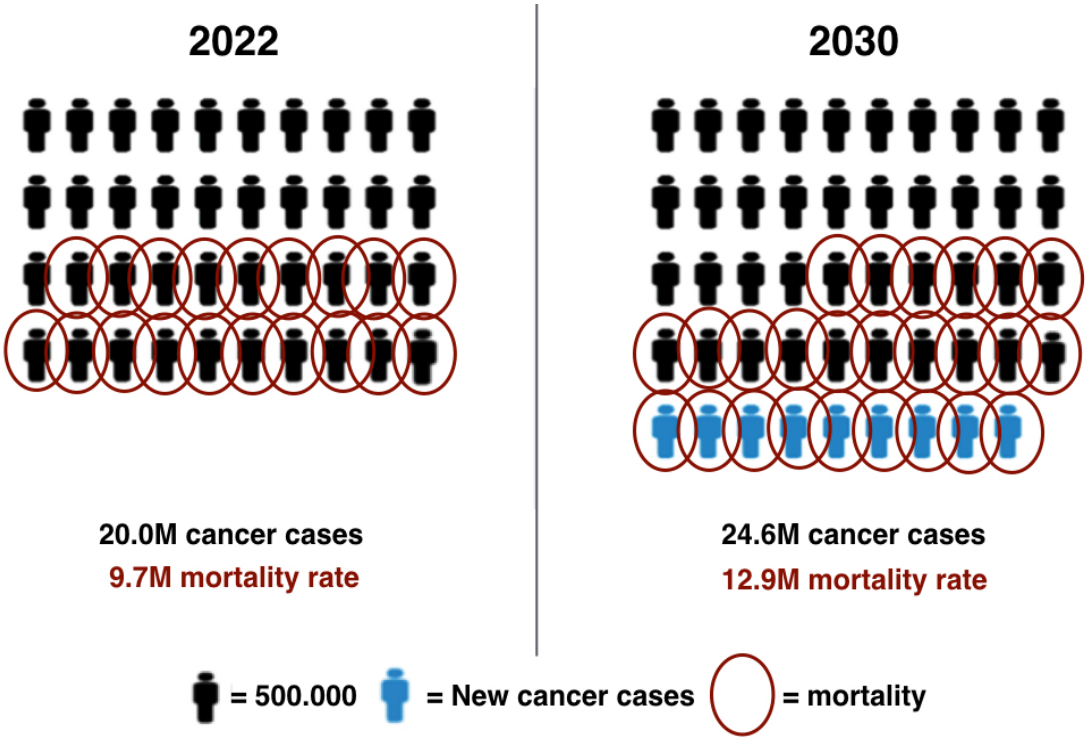


Figure 2. Image comparing the number of cancer cases worldwide in 2022 with the estimated number for 2030. Source: GLOBOCAN 2022.

1.2 Etiology

Cancer comprises a complex group of diseases marked by uncontrolled cell growth and division, which can invade and metastasize to other parts of the body. The process of transforming a normal cell into a tumour cell is influenced by both genetic and environmental factors, often interrelated, that lead to this uncontrolled proliferation of cells. As explained in Figure 3, these factors can broadly be categorized into genetic, environmental, and lifestyle-related influences, among others. The interplay between these factors is complex and can vary from person to person, making it important to understand the unique factors that may contribute to an individual's risk for cancer [8]. Preventative strategies, therefore, often focus on reducing exposure to known carcinogens, promoting a healthy lifestyle, and monitoring individuals with a high genetic risk. Early detection through screening is crucial for improving outcomes and survival rates in many cancer types.

A) Genetic Factors

1. Genetic Mutations: Mutations in certain genes are fundamental to cancer development. These mutations can be inherited (germline mutations) or acquired (somatic mutations). Key genes involved include oncogenes, tumour suppressor genes, and genes involved in DNA repair mechanisms [9].

- Oncogenes: Normally involved in cell division and growth, when mutated, they can become hyperactive and lead to cancer.

- Tumour Suppressor Genes: These genes normally inhibit cell growth and division, and mutations can inactivate them, losing their regulatory function.

- DNA Repair Genes: Mutations in these genes can impair the cell's ability to correct DNA damage, leading to further mutations.

2. Family History: A family history of certain cancers can indicate a hereditary risk, often due to inherited mutations in genes such as BRCA1 and BRCA2, which are linked to breast and ovarian cancer [10].

B) Environmental Factors

1. Carcinogens: Exposure to cancer-causing agents (carcinogens) in the environment can lead to genetic mutations. Common carcinogens include:

- Tobacco Smoke: Contains numerous chemicals that cause mutations leading to lung and other types of cancer [11].

- Radiation: ultraviolet (UV) radiation is also considered one of the most common environmental factors that can contribute to cancer development, particularly skin cancer (melanoma), with chronic and excessive exposure being a major risk factor [12]. Ionizing radiation, such as X-rays, is also considered an important carcinogenic factor that can cause tumours in all organs. Its ability to break chemical bonds and ionize atoms can lead to DNA damage and mutations, which can in turn lead to the development of cancer [13].

- Chemicals: Asbestos, benzene, and certain pesticides and industrial chemicals are known carcinogens.

2. Infections: Certain viruses, bacteria, and parasites have been linked to cancer [14,15]. Examples include [16,17]:

- Human Papillomavirus (HPV): Linked to cervical and other anogenital cancers.

- Hepatitis B and C Viruses: Associated with liver cancer.

- Helicobacter pylori: Linked to gastric cancer.

- Epstein-Barr Virus (EBV): Associated with Burkitt's lymphoma and other cancers.

C) Lifestyle-Related Factors

1. Diet and Nutrition: Diets high in red and processed meats, low in fruits and vegetables, and high in fat can increase cancer risk [18].

2. Physical Activity: A sedentary lifestyle is associated with higher risks of certain cancers, including colon and breast cancer. Regular physical activity is thought to reduce cancer risk by maintaining a healthy weight and hormonal balance [19].

3. Obesity: Being overweight or obese is linked to an increased risk of several cancer types, including breast, colorectal, pancreas and endometrial cancers [20].

4. Tobacco Use: Smoking is the leading cause of lung cancer and is also linked to many others, including cancers of the mouth, throat, esophagus, and pancreas. It is composed of more than 70 components known to be carcinogenic according to the International Agency of Research on Cancer (IARC) [11].

5. Alcohol Consumption: The consumption of alcohol has been shown to increase the risk of developing cancer in various organs such as mouth, throat, esophagus, liver, pancreas, breast or colon, among others, being the extent of the risk dependent on the level of alcohol consumption [21,22,14].

D) Hormonal Factors

Hormones can influence the development of cancer, particularly hormones like estrogen and testosterone. Prolonged exposure to these hormones, whether naturally or through hormone replacement therapy, can increase the risk of cancers such as breast and prostate cancer [23,34].

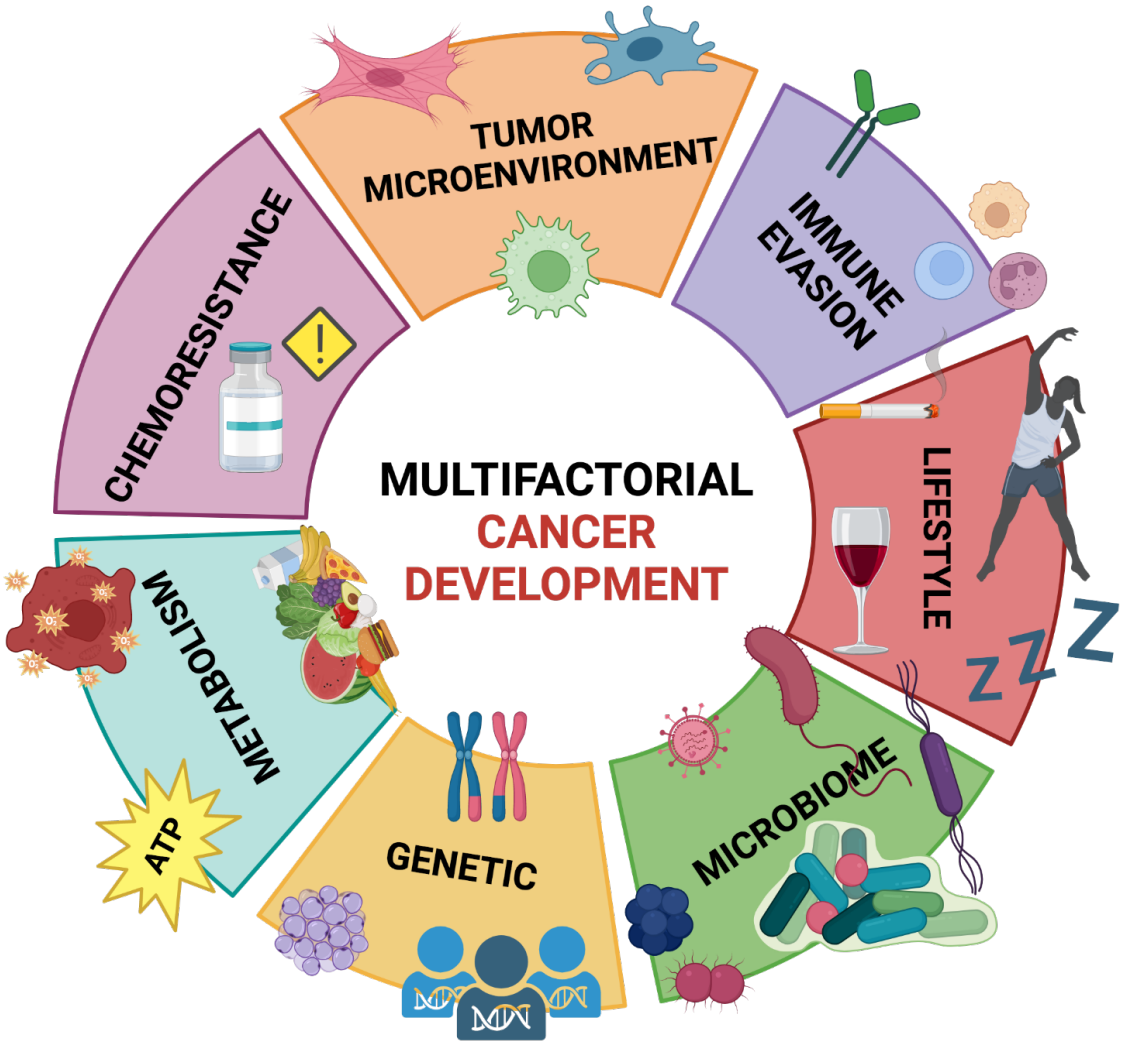


Figure 3. Main determining factors for the appearance and development of Cancer. Image created with Biorender.com.

1.3 Carcinogenesis

Cancer development involves a sequence of genetic and epigenetic changes in normal cells, resulting in metabolic alterations that drive uncontrolled cell growth, immune system evasion, and the invasion of distant tissues from the primary tumour site, ultimately leading to metastasis [25,26]. Hanahan and colleagues (2000) defined six different hallmarks in cancer that allow for tumour progression and metastasis, these were the first abilites of tumour cells studied [27] (Figure 4):

i) To maintain proliferative signaling: Normal cells require specific signals, known as mitogenic growth signals, to shift from a dormant state to an active, proliferative state. Tumour cells, however, can autonomously generate these signals, by passing the need for external cues from the tissue microenvironment and leading to uncontrolled proliferation.

ii) To evade growth suppressors: Cancer cells can avoid anti-proliferative signals that typically keep healthy cells in a quiescent state and prevent uncontrolled growth. By disabling these growth suppressor signals, cancer cells can continue to divide and grow unchecked.

iii) To activate invasion and metastasis: Tumour cells can develop the ability to escape from their primary site, migrate through the bloodstream, and colonize other organs, forming new tumours.

iv) To achieve replicative immortality: Unlike normal cells that have a limited replicative capacity, most tumour cells acquire an unlimited replicative potential during tumour progression. This allows the cancer cells to continue dividing indefinitely.

v) To induce angiogenesis: Tumour tissues, like normal tissues, require an adequate supply of nutrients and oxygen to grow and survive. Tumours can initiate angiogenesis, the formation of new blood vessels, to increase vascularization around them, ensuring a steady supply of essential nutrients and oxygen.

vi) To develop resistance to cell death: Apoptosis, the process of programmed cell death, is crucial for eliminating unnecessary or damaged cells and maintaining tissue homeostasis. Cancer cells can acquire mutations that confer resistance to apoptosis, allowing them to evade this regulatory mechanism and survive when they would normally be eliminated.

All these features contribute to the transformation of a normal cell into a tumour cell and initiate tumour progression. In 2011, Hanahan [28] expanded the list of hallmarks to include i) genome instability, ii) inflammation, iii) reprogramming of energy metabolism, and iv) immune system evasion. Finally, in 2022, Hanahan [29] introduced four additional hallmarks: i) non-mutational epigenetic reprogramming, which involves changes in gene expression without

alterations in the DNA sequence; ii) unlocking phenotypic plasticity, which enables tumour cells to switch between different cell types and acquire new functions; iii) senescent cells, which are aged and damaged cells that accumulate in tumours and can promote cancer growth; and iv) polymorphic microbiomes, referring to changes in the microbial communities within and around tumours that can influence cancer progression (Figure 4).

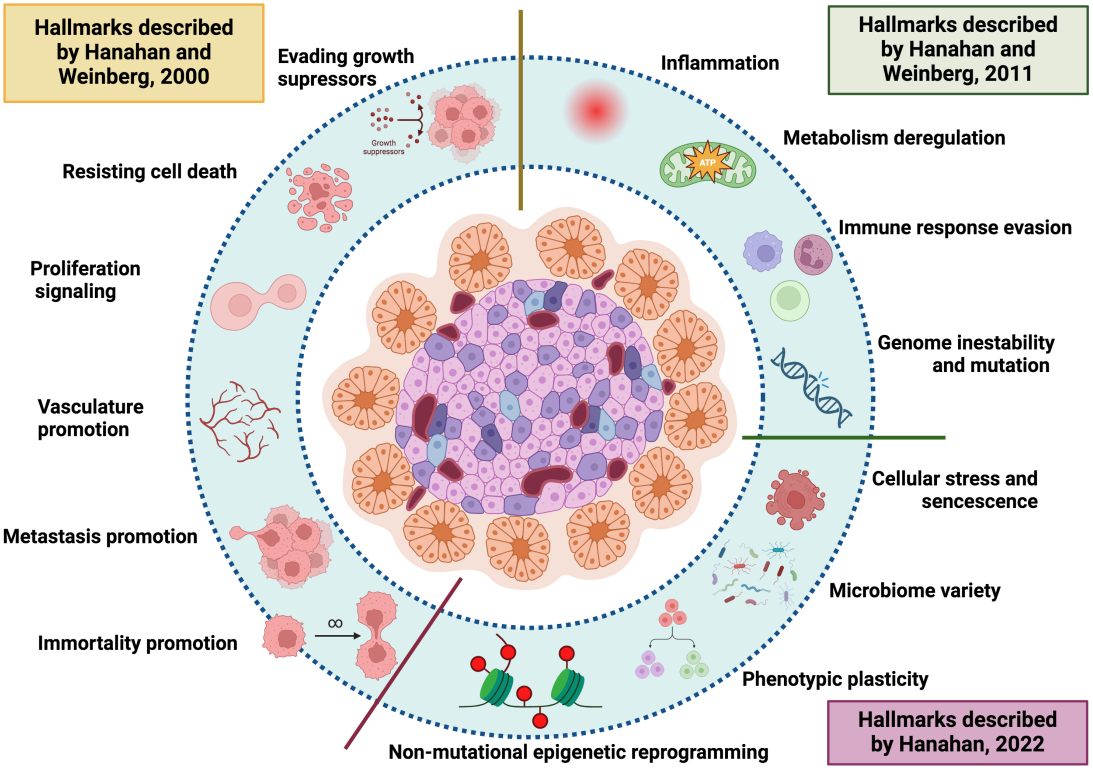


Figure 4. Overview of the cancer hallmarks updated from the studies conducted by Hanahan and colleagues. Image created with Biorender.com.

1.4 Tumour heterogeneity

One of the major challenges in cancer therapy is the immense tumour heterogeneity, which can be understood from a genetic perspective, as mutations play a crucial role in determining the disease's progression [30].

Intertumoural heterogeneity refers to the variability that can exist between tumours developing in the same organ. Different subtypes of tumours are characterized by their unique morphology, receptors, and molecular profiles.

Conversely, intratumoural heterogeneity describes the variation that exists within an individual tumour, where cells within the same tumour can express different markers and exhibit diverse behaviors [31].

A. Intertumoural heterogeneity

There are two main theories regarding intertumoural heterogeneity. The first is the genetic mutational model, which suggests that the variability seen in tumours within the same organ is due to both genetic and epigenetic mutations. This type of heterogeneity results in different tumour subtypes within the same organ, each distinguished by its metabolism, marker expression, and morphology (Figure 5A) [31].

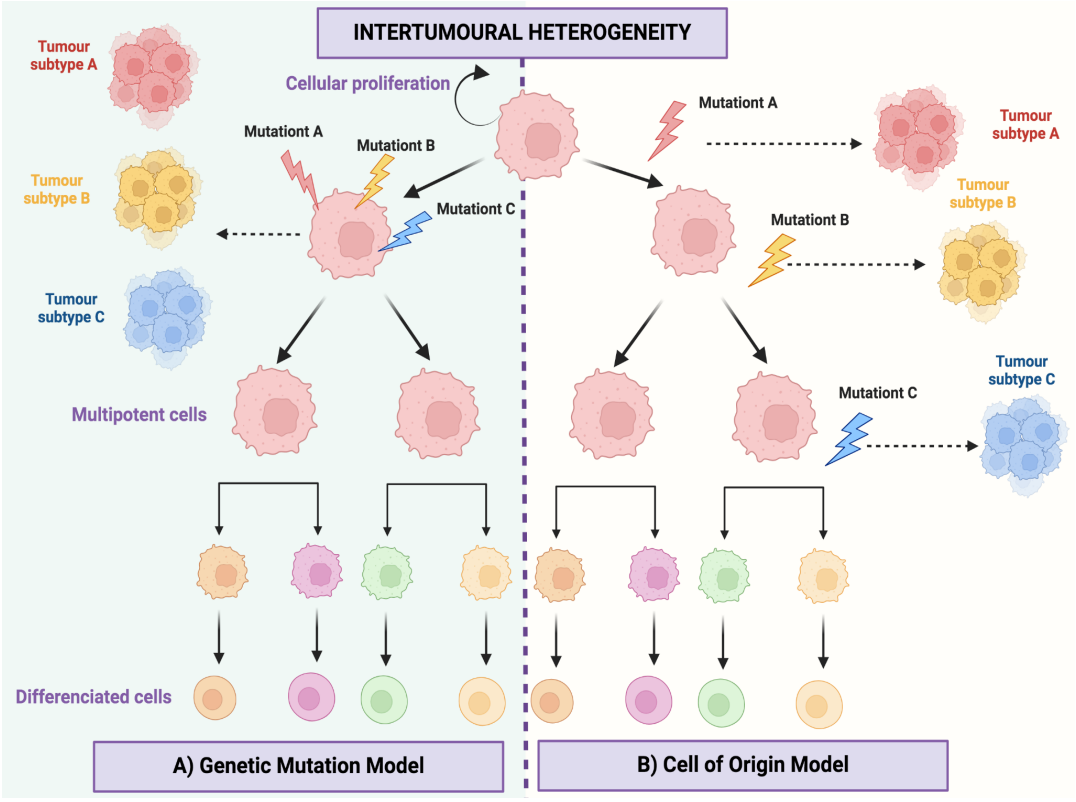


Figure 5. Intertumoural heterogeneity theories: Genetic mutational model (A) and Cell of origin model (B). Image created with Biorender.com.

The second model is the cell-of-origin model. According to this theory, various populations of cells at different positions in the lineage hierarchy can

serve as the initial cells of origin for the various cancer subtypes that develop within a particular organ or tissue. This implies that the unique molecular and phenotypic characteristics of different cancer subtypes are attributable to the specific cell population from which they originated (Figure 5B) [32].

B. Intratumoural heterogeneity

There are three different models proposed to explain intratumoural heterogeneity. The stochastic or clonal evolution model suggests that each cancer cell in a tumour has an equal chance of being the cell of origin, and all cancer cells contribute to maintaining the tumour, albeit with varying capacities. This variability in cancer cell activity is due to intrinsic stochastic factors such as genomic instability and epigenetic changes, as well as microenvironmental influences. This model explains intratumoural genetic heterogeneity through subclonal differences resulting from these factors (Figure 6A) [33].

The hierarchical model or cancer stem cell model posits that tumours have a hierarchical organization, where a healthy stem cell acquires specific mutations transforming it into a cancer stem cell (CSC), which subsequently gives rise to the rest of the tumour. The hierarchical organization and heterogeneity within the tumour, comprising all differentiated cells, are derived from the asymmetric division of the CSC population. These CSCs are believed to be responsible for the tumour's long-term progression, resistance to medical treatments, ability to metastasize, and tendency to recur after treatment (Figure 6B) [34].

The third model is the dynamic model, which suggests that the phenotype of CSCs is influenced by the tumour microenvironment (TME), making it more flexible than the hierarchical model. This model proposes that interactions between cancer stem cells and their surrounding microenvironment play a crucial role in regulating the properties of the cancer stem cells, including their self-renewal, differentiation, and invasive potential. The dynamic model also underscores the importance of plasticity in tumour cell behavior, contributing to intratumoural heterogeneity and having significant implications for cancer

treatment (Figure 6C) [35].

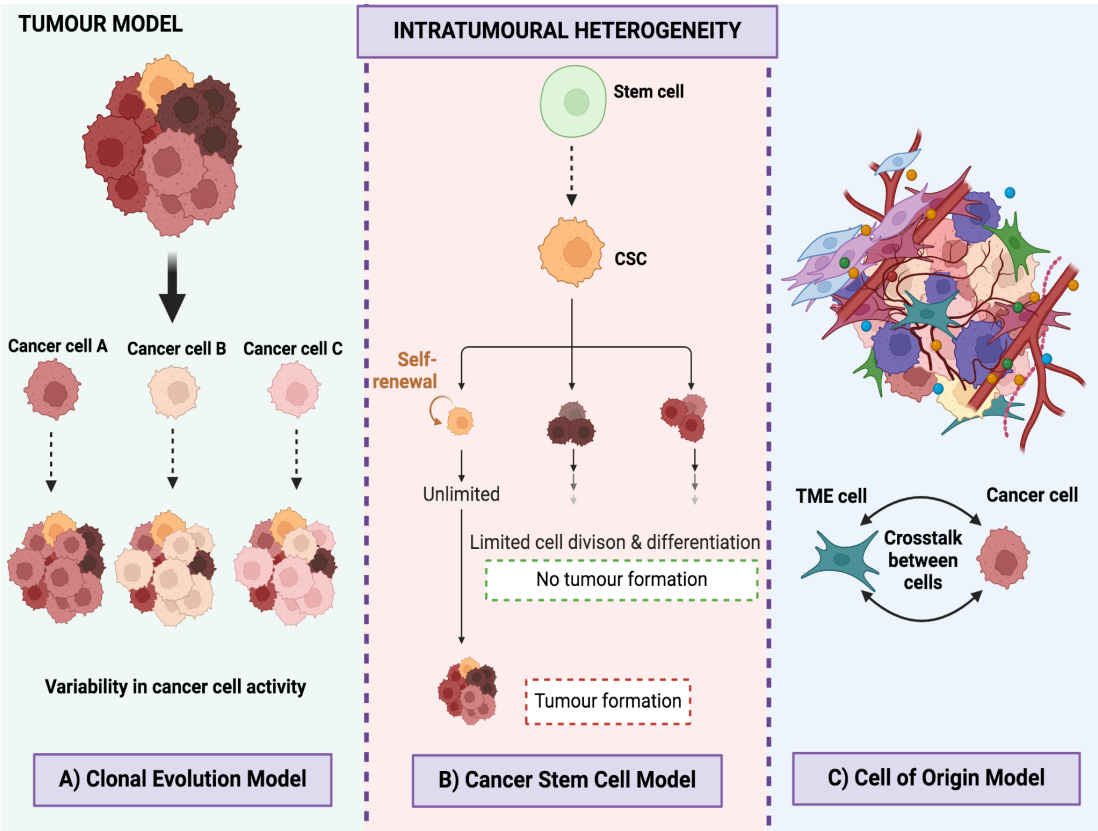


Figure 6. Intratumoural heterogeneity theories: Stochastic or clonal evolution model (A), hierarchical or Cancer Stem Cell model (B), and Dynamic or Tumour Microenvironment model (C). Image created with Biorender.com.

A novel perspective on tumour origins, proposed by Pablo Hernández in 2019 [36], offers a fresh viewpoint based on the dynamic model. According to this model, a small number of non-CSCs have the potential to initiate the formation of a primary tumour. As the tumour progresses, it induces the malignant transformation of surrounding cells, creating a TME. Various factors, including messenger RNA (mRNA) molecules secreted within this microenvironment, influence the phenotype of non-stem tumour cells at the tumour's edge. These cells revert to a less differentiated state, acquiring characteristics similar to CSCs. These newly transformed cells can then detach from the primary tumour, migrate to distant organs, and initiate the development of metastatic tumours. Consequently, the distinct origins of the primary and metastatic tumours account for the differences observed between them (Figure

7).

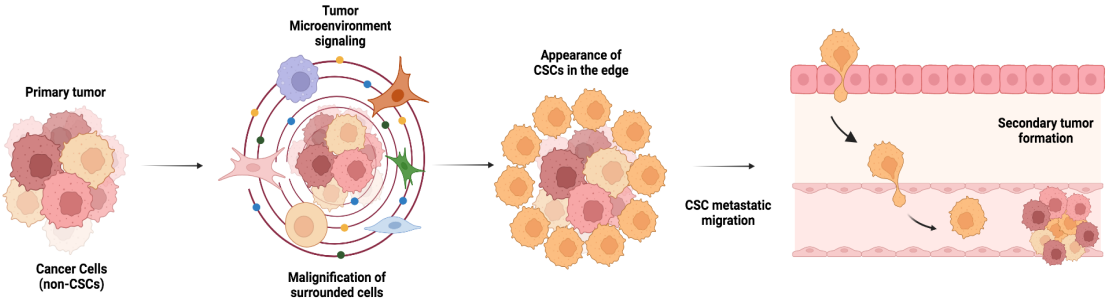


Figure 7. Dynamic Model based on Pablo Hernández's model from 2019. Image created with Biorender.com.

2. GASTROINTESTINAL CANCERS

Gastrointestinal (GI) cancers refer to a group of cancers that affect the digestive system. This group includes malignancies that occur in the esophagus, stomach, liver, pancreas, small intestine, colon, rectum and anus (Figure 8). These cancers can significantly impact digestion and absorption of nutrients, leading to a variety of health issues. GI cancers represent a diverse group of malignancies with varying risk factors, clinical presentations, and treatment strategies. Early detection through screening (e.g., colonoscopy for colorectal cancer, endoscopy for esophageal and gastric cancers) and addressing modifiable risk factors (such as diet and lifestyle changes) are critical in managing and preventing these cancers. Treatment options typically include a combination of surgery, chemotherapy, radiation therapy, and targeted therapy, tailored to the specific type and stage of cancer [37].

2.1 How do they happen?

GI cancers develop when cells in the digestive organs undergo malignant changes, leading to the formation of tumours. These changes often result from a combination of genetic, environmental, and lifestyle factors (mentioned above in Figure 3 and section 1.2):

1. Genetic Factors: Inherited mutations in certain genes can increase the risk of developing GI cancers. Family history of GI cancers can be a significant risk factor [38].

2. Environmental Factors: Long-term exposure to carcinogens such as tobacco smoke, certain chemicals, and radiation can damage the DNA in cells, causing cancer [39].

3. Lifestyle Factors: Poor diet, lack of physical activity, obesity, excessive alcohol consumption, and chronic infections (like *Helicobacter pylori or H. pylori* in the stomach or Hepatitis B/C in the liver) can contribute to the development of GI cancers [40].

2.2 Classification of GI cancers

Gastrointestinal cancers are classified based on the location within the digestive system where they originate (Figure 8). The major types include:

1. Oesophageal Cancer: Develops in the esophagus and is often linked to chronic irritation caused by smoking, heavy alcohol consumption, and acid reflux. Types include squamous cell carcinoma and adenocarcinoma.

2. Gastric (Stomach) Cancer: Arises in the stomach lining and is associated with factors such as *H. pylori* infection, dietary habits (e.g., high salt and smoked foods), and smoking. The most common type is adenocarcinoma.

3. Liver Cancer: Primarily includes hepatocellular carcinoma, originating in the liver cells, and is strongly associated with chronic liver diseases, such as hepatitis B and C infections and cirrhosis.

4. Pancreatic Cancer: Originates in the pancreas, which has both exocrine and endocrine functions. The most common type is pancreatic adenocarcinoma. Risk factors include smoking, chronic pancreatitis, and diabetes.

5. Small Intestinal Cancer: Although rare, it involves the small intestine. Types include adenocarcinoma, carcinoid tumours, and gastrointestinal stromal tumours (GISTs).

6. Colorectal Cancer: Affects the colon and rectum, often developing from pre-cancerous polyps. Risk factors include genetics, inflammatory bowel disease, diet, and lifestyle choices. Screening methods such as colonoscopy can help detect and remove polyps early.

7. Anal Cancer: Develops in the tissues of the anus, often linked to *HPV* infection. Types include squamous cell carcinoma and adenocarcinoma of the anal region.

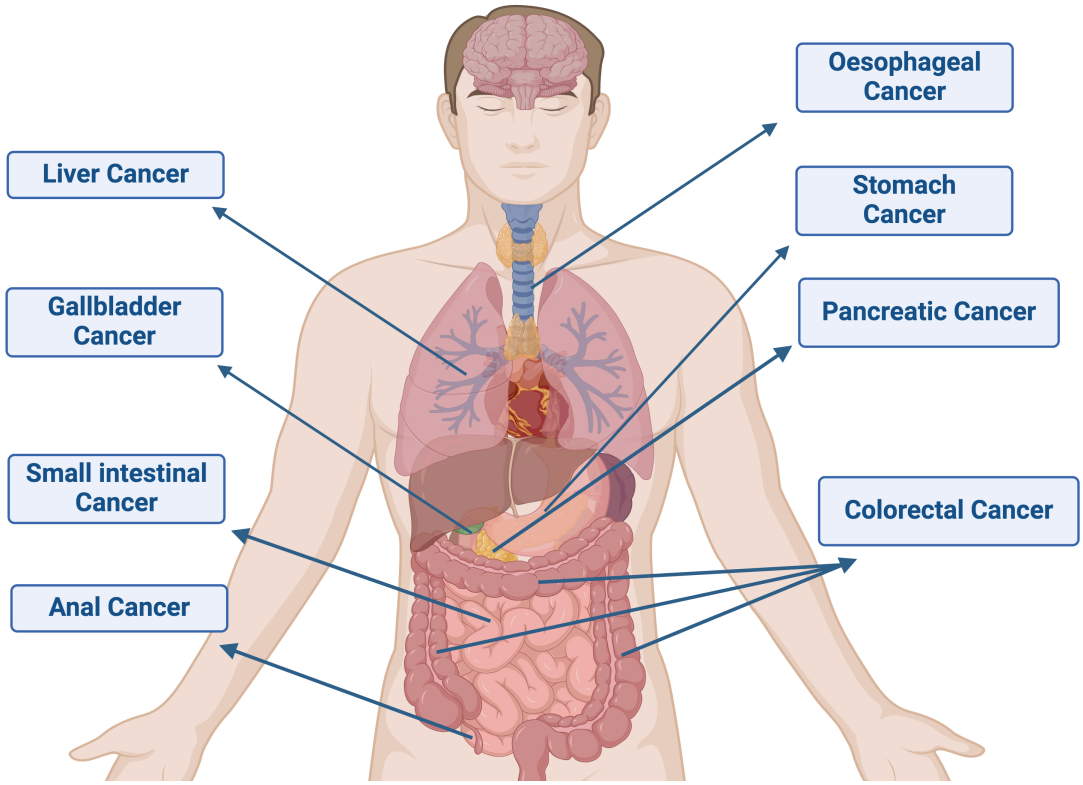


Figure 8. Gastrointestinal Cancers classification based on their location. Image created with Biorender.com.

2.3 Pancreatic Cancer

Pancreatic cancer (PC) is a highly aggressive malignancy that arises in the tissues of the pancreas. In 2022, there were 510.992 cases of pancreatic cancer diagnosed globally, making it the 12th most common cancer worldwide and accounting for approximately 2% of all new cases (Figure 9A). Additionally, in the same year 467.409 deaths were attributed to pancreatic cancer, ranking it as the 6th deadliest cancer (Figure 9B). This indicates that while pancreatic cancer is not among the most frequently diagnosed cancers, it is one of the leading causes of cancer-related deaths (Source: Globocan 2022).

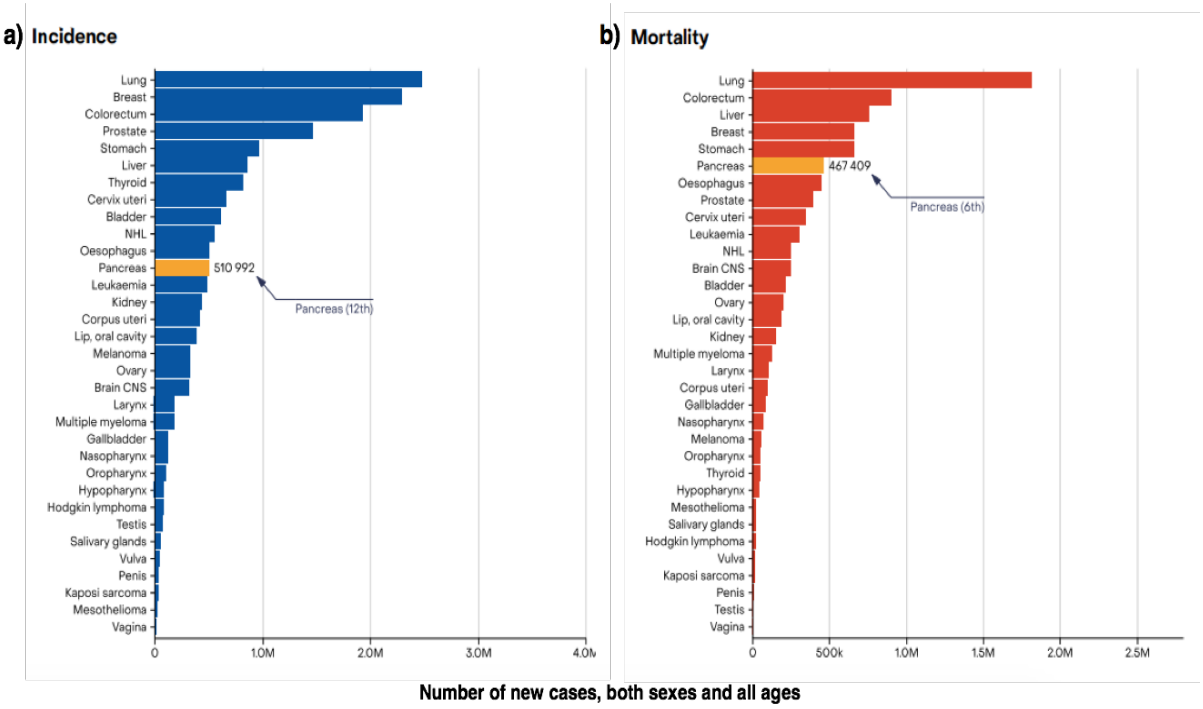


Figure 9. A) Bar chart showing the number of new cancer cases worldwide. B) Bar chart showing the number of deaths by cancer worldwide. The yellow bar in both represents pancreatic cancer. Source: GLOBOCAN 2022.

2.3.1 Risk factors in Pancreatic Cancer

Pancreatic cancer (PC) is a complex disease influenced by various risk factors that can be broadly categorized into demographic, environmental, hormonal, and genetic factors (Figures 3). Understanding these risk factors can help in prevention, early detection, and targeted interventions for this challenging cancer.

A) Demographic Risk Factors:

1. Age: PC risk increases with age, with the majority of cases diagnosed in individuals over 60 years old [41].

2. Sex: Men have a slightly higher risk of developing PC compared to women [42].

3. Race/Ethnicity: African Americans have a higher risk of PC compared to other racial/ethnic groups [42].

B) Environmental Risk Factors:

1. Tobacco and Alcohol consumption: Cigarette smoking and alcoholic drinks are some of the most significant risk factors for PC [43,44].

2. Obesity and Physical Inactivity: Being overweight or obese, as well as leading a sedentary lifestyle, are associated with an increased risk of PC [45].

3. Diet: A diet high in red and processed meats, saturated fats, and low in fruits and vegetables may raise the risk of PC [45].

4. Occupational Exposure: Certain occupational exposures to chemicals, such as pesticides or dyes, may contribute to an increased risk [46].

C) Hormonal Risk Factors:

1. Diabetes Mellitus: Individuals with long-standing diabetes have a higher risk of developing PC [47].

2. Chronic Pancreatitis: Prolonged inflammation of the pancreas is a risk factor for developing PC [48,49].

3. *H. Pylori* Infection: Infection with *H. pylori* may also contribute to the development of PC, with prevalence rates ranging from 4-25% of cases [50]

4. Reproductive Factors: Some studies suggest that factors like early menarche or late menopause may influence PC risk [51-54].

D) Genetic Risk Factors:

1. Family History: Having a first-degree relative with PC increases an individual's risk [55].

2. Hereditary Syndromes: Inherited genetic conditions such as hereditary breast and ovarian cancer syndrome (BRCA1/BRCA2 mutations) or Lynch syndrome can predispose individuals to PC [56].

3. Genetic Mutations: Mutations in genes such as KRAS, p16 or TP53 are associated with an increased risk of PC [57,58].

Figure 10 also shows that this disease can be preceded by several other diseases:

i) Intraepithelial neoplasm of the pancreas (PanIN): There are three subtypes of the

non-invasive epithelial neoplasm known as PanIN: PanIN-1, PanIN-2, and PanIN-3, all of which exhibit significant atypia [59]. KRAS and HER-2 gene mutations are frequently linked to this neoplasia [60].

ii) IPMN, or intraductal papillary mucosal neoplasm: IPMN is typically a small, asymptomatic tumour that is more prevalent in people who smoke. There are two subtypes to choose from: IPMN-MD (the type of main duct) and IPMN-BD (the type of branch duct) [60]. Mutations in the KRAS gene (present in 80% of cases) and the RNF43 gene (present in 75% of cases) are linked to this neoplasia [61].

iii) Mucinous Cystic Neoplasm, or MCN, The most uncommon variant, MCN, can be broken down into three subtypes: moderate, severe, and mild MCN [58]. KRAS (75 percent of cases) and RNF43 (40 percent of cases) are the associated mutations, just like IPMN [62].

These precursor diseases all include a relevant subpopulation of PC stem cells, which play a critical role in the progression and malignancy of pancreatic cancer [63].

2.5 Diagnosis and treatment algorithm in Pancreatic Cancer

It is currently difficult to diagnose a curable condition early. Early signs and symptoms (such as jaundice, sudden weight loss, or fatigue) are rare in patients (Supplementary 1).

The vast majority of patients are treated upfront with systemic therapy [66], and the analysis of multiple molecular profiles has emerged as an impartial strategy for the identification of novel drug targets and predictive biomarkers. Only 15% to 20% of patients are diagnosed in the early stages and are amenable to curative surgery [64,65]. The majority of people who have surgery will have a disease recurrence within a year [67]. The development of chemoresistant cells, undetected micrometastases, and poor therapeutic efficacy of adjuvant treatments are primarily to blame for these disappointing outcomes.

Additionally, this kind of tumour lacks specific, sensitive markers that can aid in its detection. Only a few precise molecular tools are available for

diagnosis, prognosis, and treatment selection. The only diagnostic biomarker for PC that has been approved by the FDA is carbohydrate antigen 19-9 (CA 19-9), which is a blood antigen that can be used with Computed Tomography (CT) scan and Magnetic Resonance Imaging (MRI). The evaluation of treatment response following surgery or chemotherapy can also be done by measuring blood CA 19-9 levels [68]. However, due to its low sensitivity and specificity, CA 19-9 should not be used in a screening setting. In fact, a false-positive response can be caused by elevated levels of CA 19-9 by other pancreaticobiliary diseases like chronic pancreatitis or biliary obstruction. Therefore, more specific and sensitive PC diagnostic markers, such as non-invasive biomarkers that can be detected in readily accessible biofluids like blood, urine, and saliva, are urgently required.

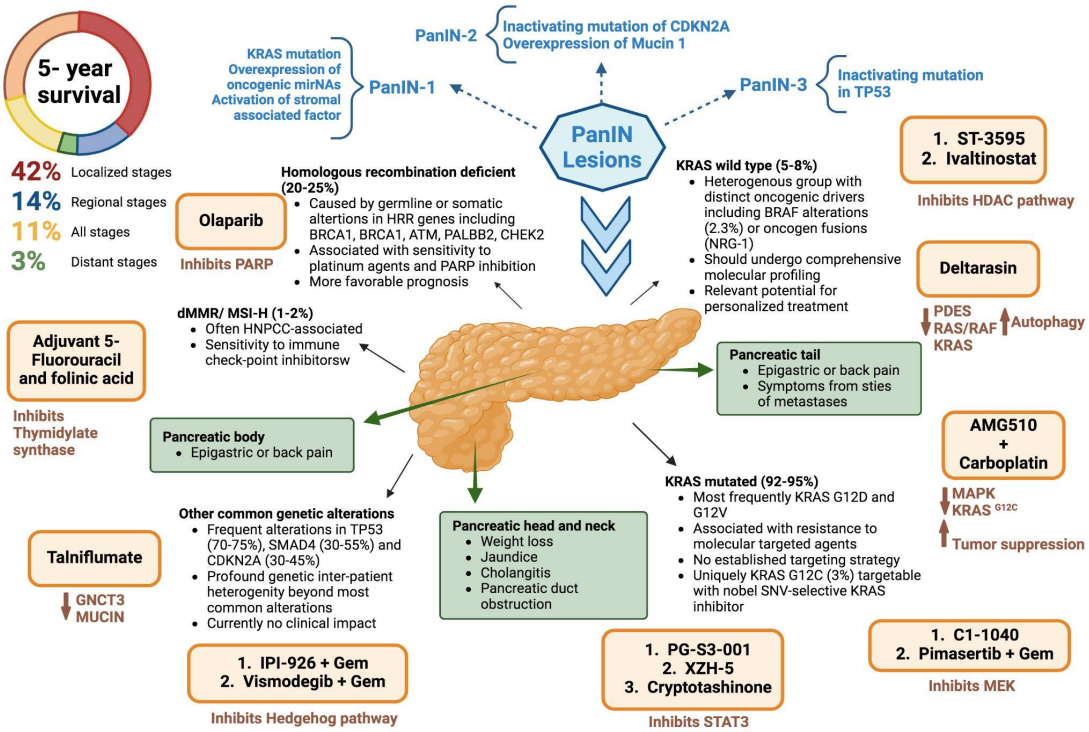


Figure 10. Representative diagram of the main characteristics of PDAC. In green, some of the symptoms that the patient suffering from PDAC may present are indicated. Treatments that have been or are being used against this cancer, as well as their targets, are highlighted in yellow. The main mutations responsible for the appearance of PDAC are highlighted in black. Characteristics of different PanIN lesions in blue. Image created with Biorender.com.

Liquid biopsies involve sampling and analyzing biofluids. These

procedures enable medical professionals to collect samples for routine diagnostic procedures and have also been used in the clinic to direct and monitor the treatments given to a number of cancer patients [69,70]. The ability of blood liquid biopsies to distinguish PDAC from healthy individuals is supported by a number of recent studies [71,72]. The connection between platelets in the blood and PDAC is the subject of new evidence. Traditionally, thrombocytosis and hypercoagulation have been linked to PDAC [73-75]. Armand Trousseau, a physician, was the first to describe spontaneous coagulation in cancer patients more than 150 years ago. He was diagnosed with gastric cancer after experiencing multiple migratory venous thrombosis, and he passed away a year later from pancreatic cancer [76]. However, there is no established connection between PDAC and blood platelets, and blood platelets have only recently received more attention as an alternative source of cancer biomarkers. As a matter of fact, platelets might go about as responders to the presence of malignant growth [77]. It has been described as RNA or proteins from apoptotic tumour cells that can instruct platelets by modifying their RNA profile through splicing [78,79]. Tumour educated platelet (TEP) is the name given to the resulting platelet [80]. It has been demonstrated that TEPs from cancer patients differ from those from inflammatory and other non-cancerous conditions. Because of this, the identification of TEPs may be a potential biomarker for the diagnosis of cancer [79,81].

In metastatic PC, conventional anticancer chemotherapy is still the standard of care (Table 1) (Supplementary 1). FOLFIRINOX (5-fluorouracil + irinotecan + oxaliplatin) or GEM + nab-paclitaxel are the most commonly used regimens. Both have an overall survival (OS) of less than one year (11 and 8.5 months, respectively), an overall response rate (ORR) of between 31.6% and 23%, and progression free survival (PFS) rates of 12% and 16% at 12 months, respectively [82,83]. The NALIRIFOX regimen (fluorouracil, liposomal irinotecan, and oxaliplatin) had a similar OS result at 11.1 months, but the PFS rate at 12 months was 27%, as opposed to 14% for the GEM + nab-paclitaxel combination [84] (Table 1).

Gemcitabine, also known as 2'-2'-difluorodeoxycytidine (dFdC), is an antiproliferative pyrimidine analog that works by transforming into active

triphosphorylated nucleotides. Ribonucleotide reductase is inhibited and DNA synthesis is disrupted by these nucleotides. It is frequently used to treat various hematological conditions, such as acute leukemia, as well as several solid tumours, such as those of the pancreas, breast, ovary, bladder, and non-small cell lung cancer (NSCLC) [85-87]. Gemcitabine monotherapy was the first-line treatment for metastatic PDAC up until a few years ago because it had better clinical benefits than 5-fluorouracil (5-FU). When compared to 5-FU, this medication has significantly increased one-year survival rates (18% vs. 2%) and median OS (5.65 vs. 4.41 months). Additionally, Gemcitabine's clinical efficacy response rate is approximately five times higher than 5-FU's (23.8 percent vs. 4.8 percent). As a consequence of this, a large number of studies have been carried out in an effort to improve the prognosis of patients by evaluating the effects of combining GEM with various other treatment options. Unfortunately, OS was only marginally improved when GEM was combined with capecitabine or cisplatin [88,89]. Additionally, no statistically significant differences were found when comparing various combinations of chemotherapeutic agents to Gemcitabine monotherapy. For metastatic pancreatic cancer, two combination regimens have emerged as gold standards in recent years: FOLFIRINOX, which consists of 5-fluorouracil (5-FU), leucovorin, irinotecan, and oxaliplatin [90]; Gemcitabine and nab-paclitaxel have been combined since 2011 [91-93]. Gemcitabine's efficacy has been the subject of a number of studies, all of which have had mixed results. When compared to Gemcitabine, patients with resected pancreatic cancer who received adjuvant therapy with a modified FOLFIRINOX regimen (oxaliplatin, irinotecan, leucovorin, and fluorouracil) had a significantly longer survival time [94]. The modified FOLFIRINOX group had a significantly longer median disease-free survival (DFS) than the Gemcitabine group (21.6 vs. 12.8 months). Additionally, the Gemcitabine group had a median OS of 35.0 months, while the modified FOLFIRINOX group had a OS of 54.4 months.

Gemcitabine and other therapeutic medications are effective for patients with advanced and metastatic pancreatic cancer; however, the emergence of chemoresistance significantly limits the effectiveness of Gemcitabine. Gemcitabine is unquestionably more resistant to pancreatic cancer cells than any other chemotherapeutic agent. The majority of studies on chemoresistance in

advanced pancreatic cancer have primarily focused on Gemcitabine [95-97] because research on the effects of alternative drugs is still in its early stages. This is explained further in the chemoresistance section.

The foundation of second or subsequent lines of treatment remains based on chemotherapy, and there are numerous options available with no clear advantage of any one over the others. As a result, patients' performance status, anticipated toxicities, and previous treatments are typically taken into consideration when tailoring treatment (Supplementary 1). OS typically ranges from three to nine months, and ORR varies from ten percent to twenty percent for combination treatments to less than ten for the monotherapies arm [98-101]. In the context of PC, target therapies have not produced significant success in terms of novel approaches. However, in the case of BRCA1–2 germ-line mutated patients who have not progressed during first-line platinum-based chemotherapy, there is an exception for maintenance therapy with the PARP inhibitor olaparib: This treatment was associated with a longer PFS (7.4 months compared to 3.8 months in the placebo arm) and a doubled PFS rate at 12 months (33.7 compared to 14.5 percent), but this advantage did not translate into an OS gain [102].

Erlotinib, an epidermal growth factor receptor (EGFR) inhibitor, was compared to GEM monotherapy to see if it improved OS and ORR by 3 weeks. However, this combination was not widely used in clinical practice [103]. Tyrosine Kinase Inhibitor (TKI) treatment for NTRK fusion-positive cases and immunotherapy for MSI-H cases can occasionally be used, despite the low rate of PCs with genomic alterations susceptible to agnostic therapies [104-106]. Numerous additional targets are or have been evaluated, but none are utilized outside of clinical trials [107] (Table 1).

Table 1. Treatment regimens in Pancreatic Cancer. *(Toledo, B et al., Exp. Rev. Clin. Pharmacol. 2024).*

Line	Regimen	Phase	N	Median OS (months)	Authors	Year
First line	5FU + Irinotecan + Oxaliplatin	II-III	342	11.1	Conroy T et al. [108]	2011
First line	GEM + Nab-paclitaxel	I-II	861	8.5	Von Hoff D et al. [94]	2013
First line	5FU + Liposomal Irinotecan + Oxaliplatin	III	770	11.1	Wainberg Z et al. [109]	2023
Second line	GEM + Nab-paclitaxel	II-III	57	8.8	Portal A et al. [110]	2015
Second line	Folfirinox + Irinotecan + Oxiplatin	II	48	9	Chung M et al. [111]	2018
Second line	5FU/Leucovorin (+ Oxaliplatin)	III	108	6.1	Gill S et al. [112]	2016
Second line	Nanoliposomal Irinotecan + 5FU + Folinic Acid	III	417	6.1	Wang-Gillam A et al. [113]	2016
Second line	Olaparib	III	3315	18.9	Golan T et al. [114]	2019
Second line	Erlotinib + GEM	III	569	6.24	Moore M et al. [115]	2007
Second line	Entrectinib	I-II	54	10	Doebele R et al. [116]	2020
Second line	Larotrectinib	I-II	159	44.4	Hong D et al. [117]	2020
Second line	Pembrolizumab	II	233	23.5	Marabelle A et al. [118]	2020

Abbreviations: N: number of patients, OS: overall survival, 5FU: Fluorouracil, GEM: Gemcitabine, PEGPH20: PEGylated recombinant human hyaluronidase, mFOLFIRINOX: oxaliplatin, leucovorin, irinotecan, and fluorouracil.

FOLFIRINOX is a treatment plan that includes oxaliplatin, 5-FU, irinotecan, leucovorin, and other chemotherapeutic agents. The PRODIGE-4/ACCORD-11 phase III clinical trial's favorable results have established this regimen as the standard treatment for metastatic PDAC [108]. FOLFIRINOX had a response rate of 26% (including 4% complete response) in 46 patients with

advanced PDAC in an initial phase II trial [98]. The median time to progression was 8.2 months, and the median OS was 10.2 months. In the aforementioned phase III trial, FOLFIRINOX was further evaluated due to its improved quality of life (QoL) and favorable safety profile. The randomized controlled trial included 342 patients with histologically and/or cytologically confirmed metastatic pancreatic adenocarcinoma who had not previously been treated with chemotherapy because oncologists lacked data on the efficacy and safety of FOLFIRINOX in comparison to GEM. The FOLFIRINOX group had a median OS of 11.1 months, while the GEM group had a median OS of 6.8 months (P 0.001), and the PFS was 6.4 months in the FOLFIRINOX group and 3.3 months in the GEM group (P 0.001). Additionally, the objective response rate for the FOLFIRINOX regimen was 31.6%. Sadly, 45.7% of patients have experienced neutropenia, 5.4% have experienced febrile neutropenia, 9.1% have experienced thrombocytopenia, 14.5% have experienced vomiting, and 12.7% have experienced diarrhea as a result of this regimen. In conclusion, FOLFIRINOX outperformed GEM in terms of survival rate but also in terms of toxicity [108].

In this setting, significant efforts have been made to reduce the harmful effects. A modified version of FOLFIRINOX (mFOLFIRINOX) was evaluated in a prospective phase II open-label study [99]. This study included a 25% reduction in the doses of irinotecan and the administration of bolus 5-FU every two weeks (until progression, unacceptable toxicity, or surgical resection) [99]. Compared to the full-dose FOLFIRINOX regimen, mFOLFIRINOX administered in conjunction with prophylactic pegfilgrastim had a comparable response rate and was more tolerable [99].

Mahaseth and colleagues proposed modifying the FOLFIRINOX regimen to exclude bolus 5-FU and include growth factor administration for all patients because 5-FU significantly contributes to toxicity. Consequently, each patient received a prophylactic subcutaneous injection of 6 mg of pegfilgrastim on the third day following chemotherapy. With a satisfactory response rate of 30%, this modified FOLFIRINOX regimen demonstrated an improved safety profile while maintaining efficacy in metastatic PDAC [100]. The incidence of grade 3-4 neutropenia was reduced to 3%. Patients receiving FOLFIRINOX at a dose

intensity of 80% and the usual use of growth factor support had comparable outcomes [101].

More recently, Vivaldi and colleagues employed the GONO-FOLFOXIRI regimen (irinotecan at 165 mg/m² over 1 hour, followed by oxaliplatin 85 mg/m² and leucovorin 200 mg/m² administered concomitantly over 2 hours through a Y-connector, on day 1, followed by a continuous infusion of fluorouracil 3,200 mg/m² over 48 hours starting on day 1) and a modified schedule (irinotecan at 150 mg/m² over 1 hour, followed by oxaliplatin 85 mg/m² and leucovorin 200 mg/m² administered concomitantly over 2 hours through a Y-connector on day 1, and followed by a continuous infusion of 5-FU 2,800 mg/m² over 48 hours starting on day 1) in 137 patients with stage III/IV PDAC. With a disease control rate of 72.2 percent, the entire patient population had one complete response (0.6 percent) and 52 partial responses (38 percent). The median OS and progression-free survival (PFS) were, respectively, 12 and 8 months. In terms of the toxicity profile, neutropenia was the most common hematologic grade 3-4 toxicity (35.7%), but only 0.7% of patients experienced febrile neutropenia. Grade 3 diarrhea was the most common non-hematologic adverse event in 11 patients (8%), followed by nausea in 10 patients (7.3%), stomatitis in 9 patients (6.5%), and liver toxicity in 6 patients (4.4%) [103].

Ultimately, the introduction of FOLFIRINOX may be regarded as one of the most significant advancements in PDAC treatment since the 1996 introduction of Gemcitabine. Despite careful patient selection and extensive growth factor support, this regimen has been shown to significantly improve OS, but it also comes with a significant increase in toxicity (described above). Even when current clinical criteria like young age, good performance status, and absence of a biliary stent are applied, individual patient clinical outcomes (in terms of both efficacy and toxicity) remain somewhat unpredictable.

2.6 Omics approaches in Pancreatic Cancer

As it has been mentioned, cancer is a complex and heterogeneous disease.

A wide variety of factors, such as genetic aberrations, epigenetic modifications, hijacked cell signaling, metabolic alterations, and environmental influences, can greatly affect whether tumour emergence and progression occurs one way or another [5,42].

Changes in epigenetics and genetics are frequently linked to cancer development. However, cancer is essentially a disease of proteins from a functional point of view, and the phenotype frequently results from malfunctioning signaling pathways [119]. As a result, large-scale data collections covering a variety of tumour stages and treatments are required, as well as multiple data layers (epigenomic, genomic, proteomic, metabolomic, etc.). and highlighting the various populations.

A comprehensive atlas of cancer data is already being developed by a number of consortia [120]. Even though there is a growing amount of molecular data for different types of tumours and matching normal tissues at different omics levels, downstream analysis is often difficult and left up to the researcher. The type of omics data, the number of samples, infrastructure capabilities, the availability of standardized pipelines, technical issues (such as low quality and error rates), and the kind of validation that is required all present challenges in data integration. Despite this, a path to a deeper comprehension of this intricate disease can be found through the successful integration of omics data (Figure 11).

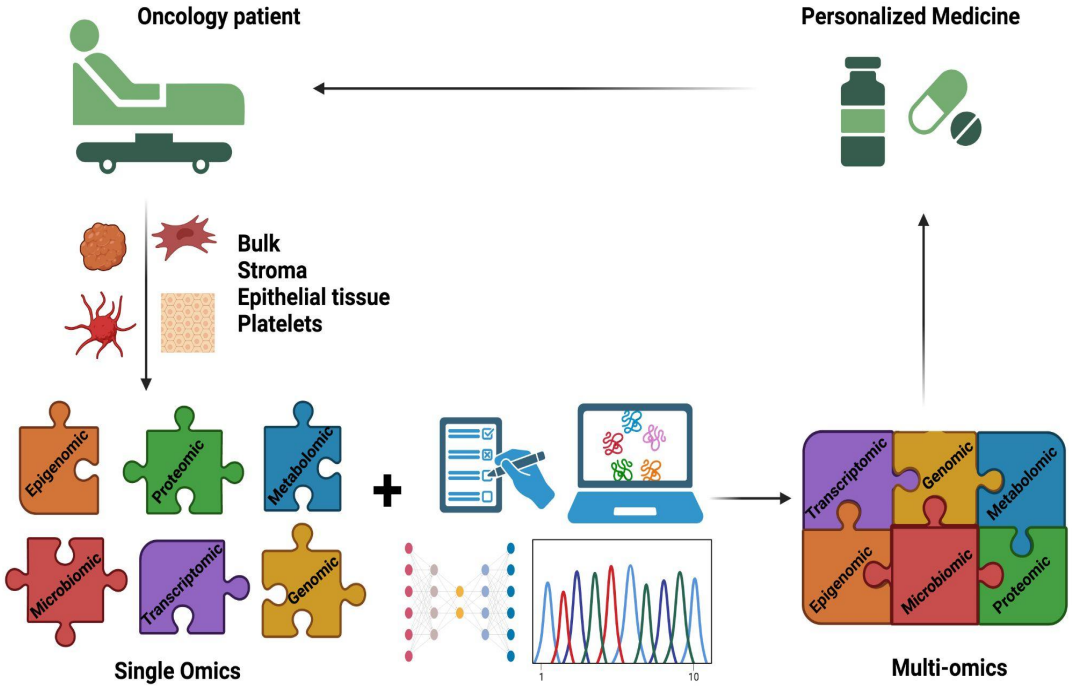


Figure 11. Contribution of multi-omics in cancer study. Platelets, epithelial tissue, tumour stroma or bulk are obtained from patients affected by cancer. Different omics analysis are carried out separately in order to identify the principal targets. The complexity of this pathology limits the success of this study, this is where multi-omics comes in since the integration of data from several different omics analysis, bioinformatic, databases study and algorithms lead to better results. The final diagnosis is therefore improved and a personalized treatment can be proposed for each patient. Image created with Biorender.com.

By integrating genomic, transcriptomic, and proteomic data, the sole focus of precision medicine has shifted from genomic profiling to a more comprehensive assessment of tumour biology [121,122]. The heterogeneity and complexity of cancer have been better understood through multi-omics analysis. Figure 11 demonstrates that the capability of omics data integration to trace the flow of information from upstream information (epigenomics and genomics) to downstream effects (proteomics, phosphoproteomics, and metabolomics) is one of its primary strengths.

The results of the Human Genome Project were anticipated to lead to an increase in diagnoses, including an increase in treatment and prevention for oncological diseases. Our understanding of the molecular basis of the majority of

tumours, including PDAC, has significantly improved as a result of advancements in genomic research. PDAC was stratified according to gene expression in a number of large-scale genomic studies [123-125]. The one-omic layer analysis, on the other hand, is insufficient to gain insight into the phenotype of cancer because these findings have not yet been implemented in clinical practice. As shown in Figure 11, the identification of specific PDAC subgroups and the personalized clinical management of PDAC patients can both be aided by further investigation and the integration of various data types (genomics, proteomics, epigenomics, and metabolomics).

Proteomics

In the field of cancer research, proteomic analysis has emerged as a potent tool that provides comprehensive insights into the protein changes that drive cancer development, progression, and therapy resistance. Since genetic changes alone may not be sufficient to comprehend complex and diverse tumours, the proteome is an essential addition to genomic data. It provides direct insight into cellular signaling states and the disease-causing driver molecules, as well as valuable information about the consequences of an abnormal genome. In a recent comparison of colorectal, breast, and ovarian cancer transcriptomics and proteomics data, proteomics outperformed transcriptome profiling in predicting gene function [126]. To develop more efficient diagnostic and therapeutic approaches, a comprehensive comprehension of the proteomic profile of PDAC is required because of its diverse and complex landscape. As a result, researchers can gain a more complete understanding of cancer biology by combining proteomic data with genomic and transcriptomic data, paving the way for more tailored and effective treatments (Figure 12).

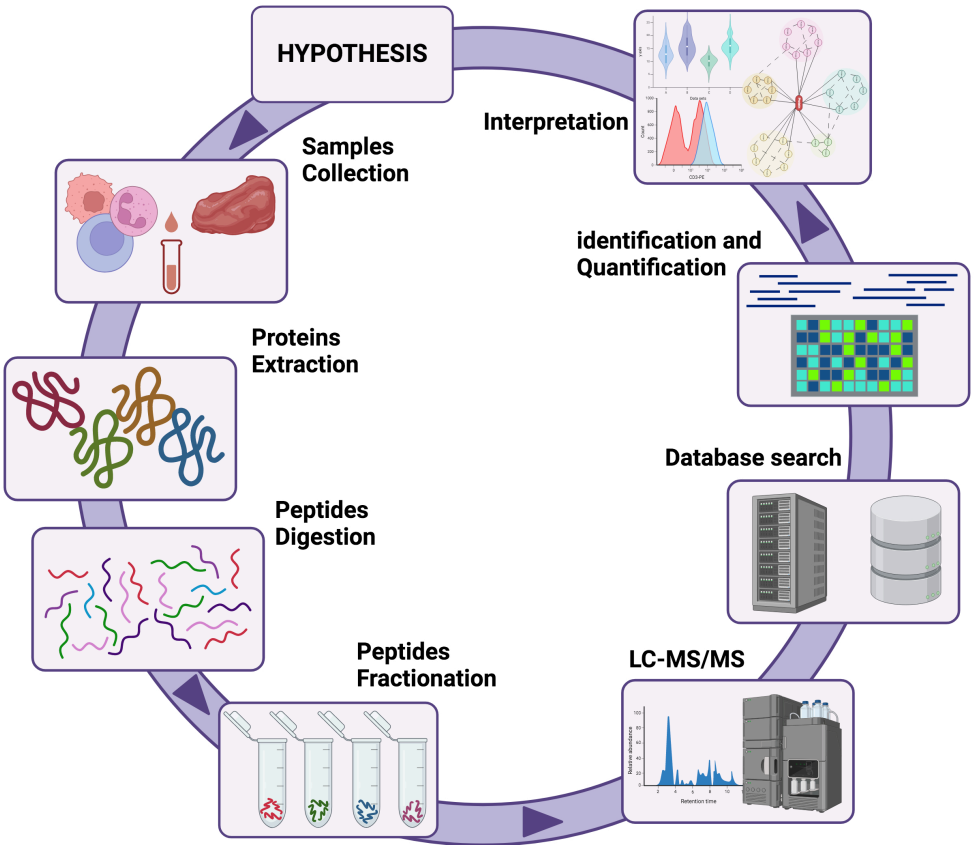


Figure 12. Proteomics analysis protocol. Image created with Biorender.com.

1. Early Detection and Diagnosis:

Proteomic studies in pancreatic cancer aim to identify specific protein biomarkers that can detect the disease at an earlier stage. Early detection is critical in PDAC due to its rapid progression and late presentation in most patients [42].

2. Tumour Microenvironment:

The TME in PDAC, particularly the dense stromal component, plays a crucial role in tumour progression, immune evasion, and treatment resistance (as explained later). Proteomic analysis can reveal the dynamic interactions between cancer cells and their microenvironment, identifying proteins involved in these interactions [127].

3. Molecular Subtyping:

Like other cancers, PDAC is not a single disease but consists of multiple molecular subtypes with distinct proteomic profiles. Classifying PDAC into these subtypes can inform prognosis and guide personalized treatment

approaches [128].

4. Drug Target Discovery:

Proteomic analysis can uncover novel drug targets in PDAC, including those involved in critical signaling pathways, metabolic processes, and immune regulation. Targeting these proteins could lead to the development of new therapeutic agents to improve patient outcomes [129].

5. Mechanisms of Resistance:

One of the most significant challenges in treating PDAC is its resistance to chemotherapy and radiotherapy. Proteomic studies can elucidate the mechanisms behind this resistance, such as alterations in drug metabolism, efflux, and repair pathways [130].

Transcriptomics and RNA splicing

Several studies have evaluated the transcriptional profiles of PDAC to stratify patients for personalized treatment strategies [125,131-134]. Transcriptomics and RNA splicing have emerged as essential fields of study that provide deep insights into the gene expression profiles and post-transcriptional modifications that drive cancer progression and resistance.

A) Transcriptomics is the comprehensive study of RNA transcripts produced by the genome under specific conditions. High-throughput technologies like RNA sequencing (RNA-seq) have revolutionized transcriptomics, enabling researchers to analyze the entire transcriptome of PDAC cells (Figure 13) [135,136].

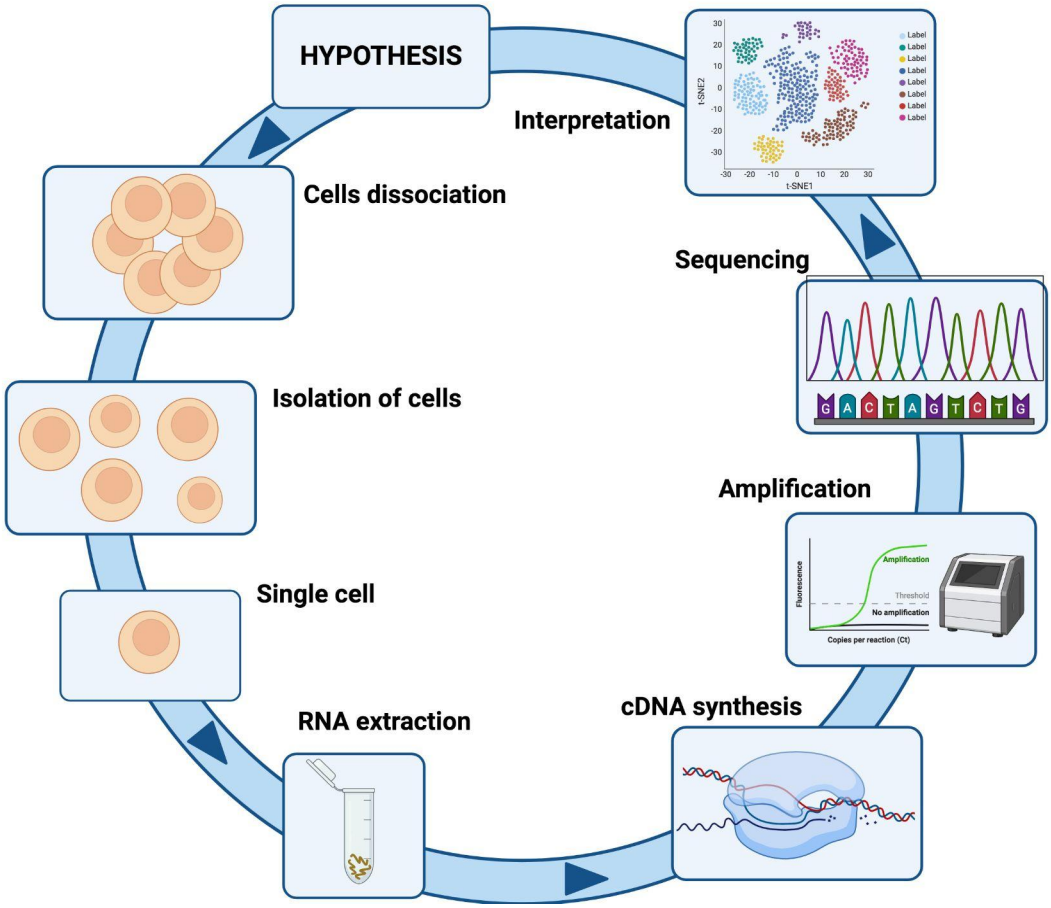


Figure 13. Transcriptomics analysis protocol. Image created with Biorender.com.

1. Gene Expression Profiling:

Transcriptomic analysis of PDAC tissues and cell lines reveals differentially expressed genes compared to normal pancreatic tissues. Oncogenes such as KRAS and MYC are often upregulated, while tumour suppressors like TP53 and CDKN2A are downregulated. This information is crucial for understanding the molecular drivers of PDAC [137].

2. Identification of Molecular Subtypes:

RNA-seq data have helped identify distinct molecular subtypes and heterogeneity of PDAC, each with unique gene expression profiles and clinical outcomes. These subtypes include classical, basal-like, and squamous, with the classical subtype generally having a better prognosis [138].

3. Signaling Pathways and tumour Microenvironment:

Transcriptomics sheds light on the signaling pathways active in PDAC, such as the KRAS, TGF-β, and Wnt pathways. Moreover, it provides insights

into the interaction between PDAC cells and the TME, highlighting the roles of stromal cells, immune cells, and extracellular matrix components [139,140].

4. Identification of Biomarkers:

Differentially expressed genes identified through transcriptomics serve as potential biomarkers for early detection, prognosis, and therapeutic targets in PDAC, such as the overexpression of genes like CA19-9 and CEACAM1 [141,142].

B) RNA splicing is a critical post-transcriptional process where introns are removed, and exons are ligated to produce mature mRNA transcripts. Aberrant splicing events contribute to cancer progression by generating protein isoforms that support tumour growth, metastasis, and resistance to therapy (Figure 14) [143].

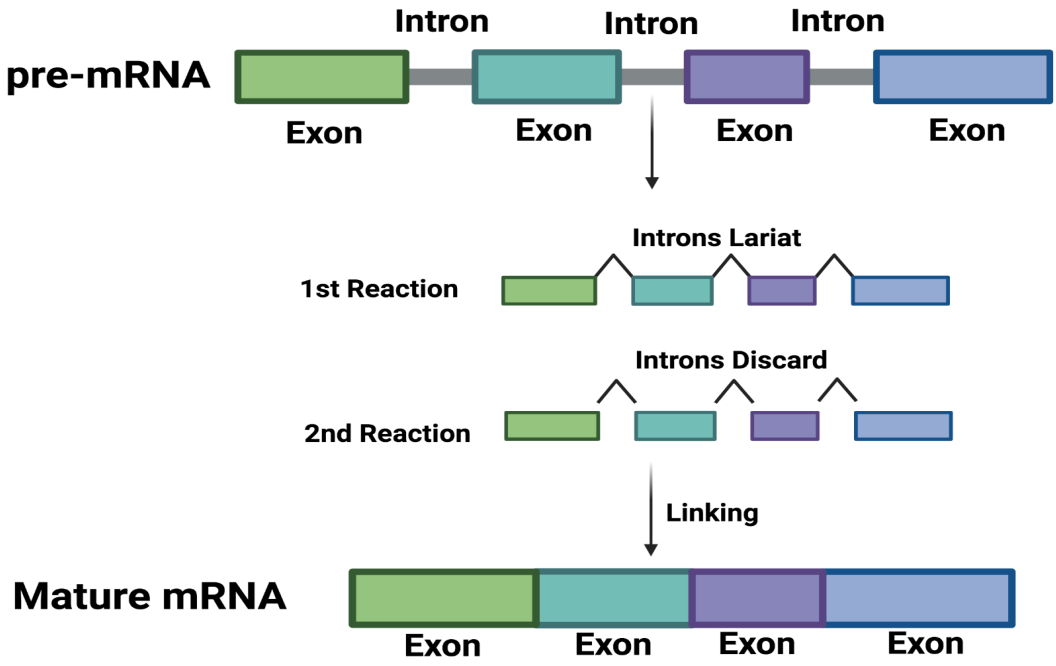


Figure 14. RNA splicing process. Image created with Biorender.com.

1. Spliceosome Components and Splicing Factors:

In PDAC, dysregulation of splicing factors such as SRSF1, SRSF3, and SF3B1 has been observed. These factors alter the function of the splicing

machinery, leading to abnormal splicing patterns of genes involved in key cancer pathways [144].

2. Alternative Splicing Events:

Alternative splicing can produce multiple mRNA isoforms from a single gene. In PDAC, this often results in the generation of splice variants that promote oncogenesis. For example, alternative splicing of the CD44 gene produces variants associated with increased tumour invasiveness and poor prognosis [145].

3. Therapeutic Implications:

Targeting splicing dysregulation offers a novel therapeutic approach for PDAC. Spliceosome inhibitors and antisense oligonucleotides (ASOs) are being investigated to correct aberrant splicing events and restore normal gene function [146].

4. Splicing and Drug Resistance:

Abnormal splicing contributes to therapeutic resistance in PDAC by altering the expression of genes involved in drug metabolism, efflux, and DNA repair. For instance, alternative splicing of the BCL2 family of genes can produce isoforms that inhibit apoptosis, leading to resistance to chemotherapeutic agents [147].

The molecular landscape of PDAC can be better understood with the help of transcriptomics and RNA splicing. Comprehensive transcriptomic analyses shed light on the pathways that drive tumour progression and metastasis, revealing the differentially expressed genes and distinct molecular subtypes of PDAC. In addition, the mechanisms by which aberrant splicing contributes to oncogenesis and therapeutic resistance can be better understood by comprehending RNA splicing changes in PDAC. Researchers can identify new biomarkers and therapeutic targets by integrating transcriptomic and splicing data, paving the way for more personalized and effective PDAC treatments.

Bioinformatics advances in data integration and drug combination

Numerous studies in recent years have demonstrated that proteogenomic integration prioritized known genomics targets and produced novel findings for

the generation of therapeutic hypotheses [148-153]. Mun and colleagues' recent study of early-onset gastric cancer [152] demonstrated that cancer subtype stratification profiles from transcriptomics, proteomics, phosphoproteomics, and N-glycoproteomics were successfully integrated. The data from proteomics and phosphoproteomics primarily identified two subtypes that were linked to the best and worst survival, according to the study. Furthermore, phosphoproteome data were used to predict drug sensitivity. Data integration ensures that biological insight for diagnostic, prognostic, and predictive markers will be maximized, advancing precision medicine (Figure 11).

Utilization of machine learning algorithms is pertinent to drug combination. Several machine learning techniques are currently being used to evaluate drug combination therapy for HIV, hypertension, infectious diseases, and cancer [154] for this purpose. High-throughput testing of drug combinations for each cell line is frequently the foundation of current predictive models for the effects of drug combinations. The molecular characteristics that are predictive of therapy response are then identified using these data [155]. On a pan-cancer scale, the AstraZeneca Drug Combination Prediction Challenge, a pan-cancer DREAM community challenge, revealed these results [156] (Figure 15). It is becoming possible to predict synergy using models of drug interactions. The complexity of these computational models ought to be high enough to accurately predict the effect of combination therapies on a cellular and signaling level. Deep learning and other more advanced machine learning techniques may be required to enable adequate modeling due to the high complexity.



Figure 15. PanCancer Project. This idea aims to gather and analyze four thousand genomes corresponding to patients from all over the world with different types of tumour with the aim of identifying and understanding the causes of tumour processes. Source: Pemb.cat (Pancancer project).

3. TUMOUR MICROENVIRONMENT

According to the "seed and soil" hypothesis, a suitable host microenvironment (the soil) is necessary for the best growth of tumour cells (the seed) [157]. Emerging data suggest that tumours are made up of tumour parenchyma and stroma, two separate but interdependent components that intersect to support tumour growth. CAFs are the most prevalent type of stromal cell in tumours [158,159] and are derived mainly from endogenous fibroblasts, apart from another great variety of cells, which are essential in maintaining homeostasis, controlling the growth and differentiation of neighboring epithelial cells, and regulating wound healing processes in healthy tissues [160] Over the past 40 years, traditional perspectives often portrayed cancer cells as the sole drivers of malignancy, relegating the stromal compartment to a passive, supportive role [159,160]. However, recent studies have revealed the reciprocal communication and dynamic crosstalk between the tumour and stromal elements, indicating that both elements are equally involved in the tumourigenic process [161,162]. These findings have led to a paradigm shift in therapeutic approaches, moving beyond targeting cancer cells alone to address the intricate interactions within the tumour microenvironment. The importance of the TME in tumour initiation, maintenance, and progression is now widely acknowledged [163-165], especially in tumours with high fibrotic content, such as PDAC [166] (Figure 16).

It is becoming more and more obvious that every tumour is different in some way, and that the way a tumour interacts with its surroundings has effects that appear to vary between tumour kinds and even between individuals with the same tumour type. There are still many unsolved issues about the connection between CAFs and the tumour, despite intriguing findings showing that CAFs depletion causes delayed tumour growth and enhanced response to treatment [161]. Do they originate as physiological barriers to fight against the aggression of the onset of the tumour mass? Are they allies of the tumour even before tumour cells begin to proliferate? Or are fibroblasts enlisted in the cancer crusade only after the call-up by tumour cells? Do normal fibroblasts react to cancer

aggression as if it were a wound to heal within the tissue, and then become CAFs? We have reviewed the latest findings about the TME and carcinogenic events with the aim of discussing novel therapeutic approaches to fight cancer.

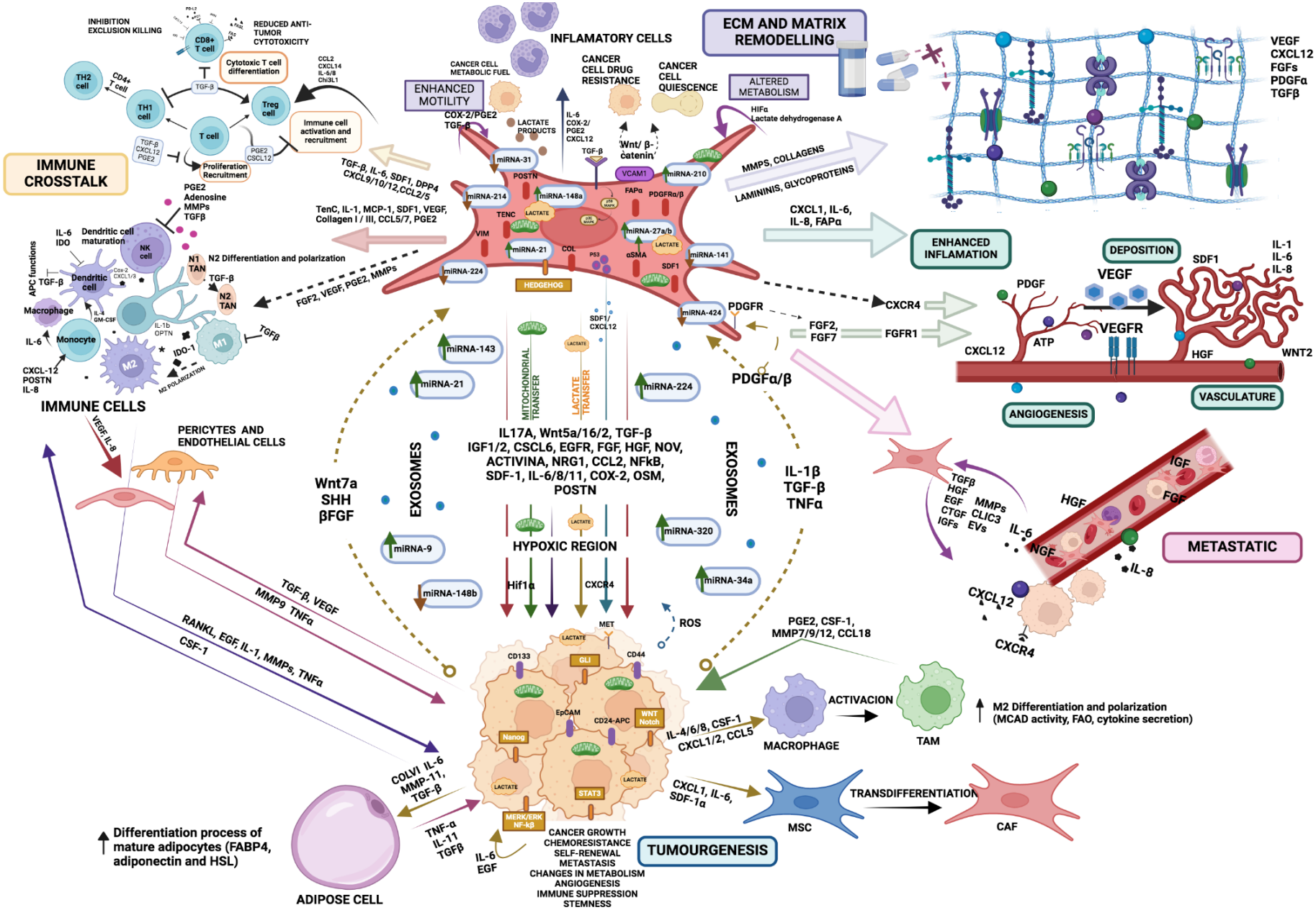

IMMUNE CROSSTALK
INHIBITION EXCLUSION KILLING
REDUCED ANTI-TUMOR CYTOTOXICITY
CD8+ T cell
Cytotoxic T cell differentiation
TH2 cell
CD4+ T cell
TH1 cell
Treg cell
T cell
Immune cell activation and recruitment
Proliferation Recruitment
TGF-β
PGE2
CSCL12
CCL2
CXCL14
IL-6/8
Ch3L1
PGE2
Adenosine
MMPs
TGFβ
IMMUNE CELLS
IL-6
IDO
Dendritic cell maturation
NK cell
Dendritic cell
APC functions
N1 TAN
N2 Differentiation and polarization
N2 TAN
Macrophage
IL-4 GM-CSF
IL-1b OPTN
IL-6
Monocyte
M1
M2
IDO-1
M2 POLARIZATION
CXCL-12
POSTN
IL-8
VEGF, IL-8
PERICYTES AND ENDOTHELIAL CELLS
ADIPOSE CELL
Differentiation process of mature adipocytes (FABP4, adiponectin and HSL)
TGF-β, VEGF
MMP9 TNFα
RANKL, EGF, IL-1, MMPs, TNFα
CSF-1
COLVI IL-6
MMP-11,
TGF-β
TNF-α
IL-11
TGFβ
IL-6
EGF
INFLAMATORY CELLS
ENHANCED MOTILITY
CANCER CELL METABOLIC FUEL
CANCER CELL DRUG RESISTANCE
CANCER CELL QUIESCENCE
ALTERED METABOLISM
COX-2/PGE2
TGF-β
LACTATE PRODUCTS
IL-6
COX-2/
PGE2
CXCL12
Wnt/ β-catenin
TGF-β
HIFα
Lactate dehydrogenase A
TGF-β, IL-6, SDF1, DPP4
CXCL9/10/12,CCL2/5
TenC, IL-1, MCP-1, SDF1, VEGF,
Collagen I / III, CCL5/7, PGE2
FGF2, VEGF, PGE2, MMPs
miRNA-31
POSTN
VCAM1
miRNA-210
miRNA-214
miRNA-148a
FAPα
PDGFRα/β
TENC
LACTATE
VIM
miRNA-27a/b
miRNA-21
COL
P53
αSMA
miRNA-141
SDF1
miRNA-224
HEDGEHOG
PDGFR
miRNA-424
CXCR4
FGF2,
FGF7
FGFR1
PDGFα/β
ECM AND MATRIX REMODELLING
MMPS, COLLAGENS
LAMININIS, GLYCOPROTEINS
CXCL1, IL-6,
IL-8, FAPα
ENHANCED INFLAMATION
DEPOSITION
VEGF
SDF1
IL-1
IL-6
IL-8
PDGF
VEGFR
CXCL12
ATP
HGF
WNT2
ANGIOGENESIS
VASCULATURE
miRNA-143
miRNA-21
MITOCHONDRIAL TRANSFER
SDF1/
CXCL12
LACTATE TRANSFER
miRNA-224
IL17A, Wnt5a/16/2, TGF-β
IGF1/2, CSCL6, EGFR, FGF, HGF, NOV,
ACTIVINA, NRG1, CCL2, NFkB,
SDF-1, IL-6/8/11, COX-2, OSM,
POSTN
Wnt7a
SHH
βFGF
EXOSOMES
EXOSOMES
IL-1β
TGF-β
TNFα
miRNA-9
HYPOXIC REGION
miRNA-320
miRNA-148b
Hif1α
CXCR4
miRNA-34a
ROS
PGE2, CSF-1,
MMP7/9/12, CCL18
MET
CD133
GLI
CD44
EpCAM
CD24-APC
WNT Notch
Nanog
STAT3
MERK/ERK NF-kβ
CANCER GROWTH
CHEMORESISTANCE
SELF-RENEWAL
METASTASIS
CHANGES IN METABOLISM
ANGIOGENESIS
IMMUNE SUPPRESSION
STEMNESS
TUMOURGENESIS
IL-4/6/8, CSF-1
CXCL1/2, CCL5
MACROPHAGE
ACTIVACION
TAM
M2 Differentiation and polarization
(MCAD activity, FAO, cytokine secretion)
CXCL1, IL-6,
SDF-1α
MSC
TRANSDIFFERENTIATION
CAF
TGFβ
HGF
EGF
CTGF
IGFs
MMPs
CLIC3
EVs
IGF
HGF
FGF
IL-6
NGF
IL-8
CXCL12
CXCR4
METASTATIC
VEGF
CXCL12
FGFs
PDGFα
TGFβ

Figure 16. Crosstalk between CAFs and the tumour Microenvironment. The interaction between cancer cells and CAFs creates a unique TME that fosters cancer progression. CAFs, a subtype of stromal cells within the TME, actively communicate with cancer cells through various signaling pathways. One crucial aspect is their secretion of growth factors (TGF-β, FGF, HGF, IGF), cytokines, extracellular matrix components (collagens, glycoproteins, proteoglycans, and MMPs), and other molecules (CD81, CD9, FAP, miRNAs, exosomes, transposable RNAs, and metabolites), which influence the behavior of neighboring cancer cells, promoting their proliferation, invasion, and metastasis.
Extracellular matrix components secreted by CAFs are involved in ECM degradation and the formation of pores or tunnels through which cancer cells can pass. Matrix remodeling not only facilitates tumour cell evasion from the primary tumour but also represents a critical event in the formation of the pre-metastatic niche, a key step in the metastatic process, as well as in facilitating immune suppression.
Another characteristic is that CAFs can orchestrate an immunosuppressive TME by interacting with the immune microenvironment in tumours. Through the secretion of multiple chemokines, cytokines, and other effector molecules such as TGF-β, IL-6, CXCL12, CCL2, SDF-1, VEGF, along with IDO and PGE2, CAFs modulate immune cells' antitumour immunity through various pathways: promoting the trans-differentiation or polarization of immune cells such as TAMs, TANs, MCs, DCs, and T lymphocytes into certain protumourigenic cell subsets; facilitating the activities of immune inhibitory cells in terms of recruitment, activation, and immunosuppressive effects, including M2-type TAMs, N2-type TANs, rDCs, Treg cells, and MDSCs; and restricting the cytotoxic activity and cytokine production of effector immune cells like NK cells and CTLs. Notably, several infiltrating immune cells such as TAMs, TANs, MCs, and DCs can, in turn, exert a promoting effect on CAFs' activation and function, thereby contributing to the formation of immune suppressive loops. Moreover, CAFs can upregulate the expression of immune checkpoint molecules such as PD-1/PD-L1 and CTLA4/B7 in both themselves and other cells in the TME to induce T-cell dysfunction.
CAFs also play a crucial role in promoting angiogenesis, the formation of new blood vessels, by secreting VEGF, FGF, and PDGF, which enhances the proliferation and migration of endothelial cells. By influencing angiogenesis, CAFs enhance the vascular supply to the tumour, facilitating its growth and providing potential routes for metastasis.
Reciprocally, cancer cells can stimulate the activation and transformation of fibroblasts into CAFs by secreting molecules including TGF-β, ITGB4, p53, mRNAs, and miRNAs into the surrounding fibroblasts and CAFs. These cancer-derived molecules confer specific phenotypes, such as mitophagy and glycolysis, myofibroblast features, matrix remodeling, and inflammatory gene expression, creating a supportive niche for tumour appearance and growth. Image created with Biorender.

3.1 Cancer, a non-healing wound

Dormant or resting fibroblasts are detected in areas of interstitial stroma between functional parenchymal layers in adult and well-differentiated tissues. They are described as thin, elongated cells with frontal and posterior extensions and fusiform shapes. From a molecular point of view, quiescent fibroblasts remain poorly defined [162,163]. In response to tissue injury, quiescent fibroblasts are activated into normal activated fibroblasts (NAFs), facilitating tissue repair and regeneration. NAFs were first described in wound healing and were predominantly identified by their expression of Alpha Smooth Muscle Actin (αSMA) and vimentin (VIM), along with enhanced collagens (type I, III, IV, and V), laminins, and fibronectin production, which remodel the extracellular matrix (ECM) [164,165]. Matrix metalloproteinases (MMPs) are also primarily produced by activated fibroblasts or myofibroblasts, highlighting their critical function in controlling ECM turnover and preserving ECM homeostasis [166]. In order to bind the borders of the wound, myofibroblasts constrict the ECM. They also produce matrix proteins, which fix any residual tissue flaws and draw epithelial cells to complete the healing process. Once this is finished, activated fibroblasts reduce in number and undergo a specific type of programmed cell death known as nemosis, which is fuelled by macrophages and fibroblasts that resemble macrophages [167,168]. As tumours are regarded as “non-healing wounds" [169,170] (due to the similarity of the process), in this regard, myofibroblast populations persist and initiate a chronic response known as cancerous or stromal fibrosis where tissue fibroblasts are fooled so that instead of undergoing apoptosis, the fibroblasts enhance proliferation and become a functionally diverse population known as fibrosis-associated fibroblasts (FAFs) or CAFs [171] (Figure 17).

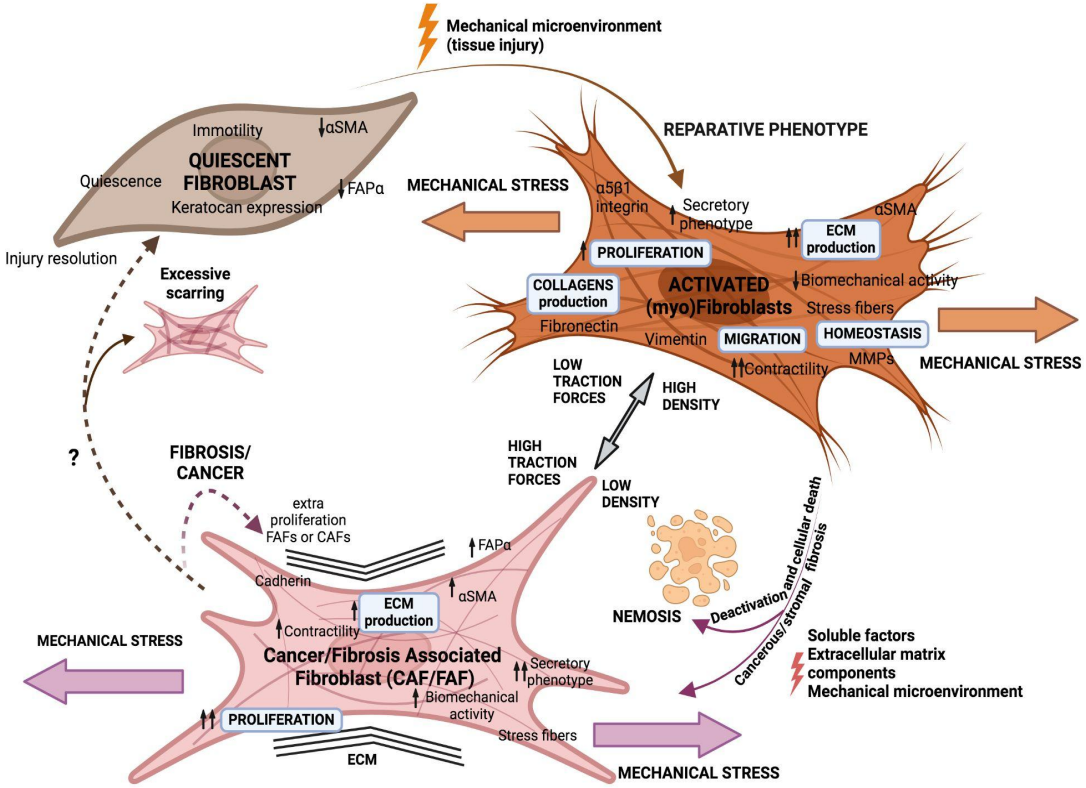


Figure 17. Fibroblasts' phenotype adapts in response to external aggression. Activation of quiescent fibroblasts to a myofibroblast phenotype to repair tissue damage by wound healing is triggered by microenvironmental signals. Myofibroblasts should disappear by apoptosis (nemosis) after punctual aggression, but if fibroblasts are subjected to constant stress, they could evolve into a FAF or CAF phenotype. (*Toledo, B et al., Int. J. Mol. Sci. 2022*).

Tumour stroma, in fact, shares many similarities with non-healing wounds, including inflammation, tissue formation, and remodeling [172]. In fact, inflammation is important in cancer progression because an inflammatory microenvironment can increase error and mutation rates while also promoting the proliferation of mutated cells [173]. Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) (which are highly reactive chemicals formed from O2 and N2, respectively, and can easily react with other molecules in a cell) produced by activated inflammatory cells cause deoxyribonucleic acid (DNA) damage and genomic instability [174,175]. Furthermore, inflammatory

cytokines such as Transforming Growth Factor β (TGF-β), among others, induce genomic instability and increase changes in tumour cell plasticity [176]. Inflammation also produces growth factors, cytokines like interleukins (IL-6), and a protein complex like NF-kB that can give tumour progenitors a stem cell-like phenotype or stimulate stem cell expansion [177,178]. Moreover, chronic inflammation has been proven to benefit the creation of a TME that attracts myeloid cells and supports tumour growth and angiogenesis [179-181] . In fact, epidemiological and clinical research has suggested that inflammation and tissue fibrosis in some organs, such as the liver, lung, and pancreas, may increase the risk of carcinoma development [182,183]. The conception that inflammation, or other forms of tissue aggression, can end up initiating tumour progression alters the generally accepted idea that tumour cells induce fibroblasts malignification and not vice versa (Table 2). In fact, an *in vivo* study demonstrated that injection of non-tumourigenic cells previously cocultured with CAFs led to the formation of large tumours and, in contrast, no tumour formation was observed when injected cells were co-cultured with NAFs [184,185].

Table 2. Tumour cell's secreted factors and their functions over CAFs. Green stands for CAFs' activators factors; red for CAFs' inactivators factors; and yellow for factors with dual function (activators and inactivators). (*Toledo, B et al., Int. J. Mol. Sci. 2022*).

Factors	Mode of Action	References
Cytokines	CAF activation/inactivation	[158,176–178,186–192,193,194–197,198,199]
IL-1	CAF activation: Promotes cell growth and invasion by NF-κB and IL-1β/IL-1R pathway	[162,200,201,202]
TGF-beta	CAF activation: Promotes cell proliferation and migration by TGF-β/Smad pathway	[176,203,204,205–209,193–196 210-218]
IL-6	CAF activation: Promotes cell chemoresistance, cancer progression, cell metastasis and cell adhesion by STAT3/NF-kβ , JAK2/STAT3, ERK1/2, STAT3/Notch, and STAT3/PD-L1 pathways	[177,178,200,219,190,193,220,221,216,222]

PDGF	CAF activation	[203,204,193,194, 223–236]
EGF	CAF activation	[204,237-239]
IGF	CAF activation: Promotes cell chemoresistance, cell survival, and cell growth by IGF-/AKT, IGF/IGFR, and PI3K/AKT/mTOR pathways	[204,240–243]
JAK2/STAT3	CAF activation	[194,244]
YAP1/TEAS1	CAF activation	[245,246]
p53	CAF inactivation	[247]
VEGF	CAF activation	[248-250,233,234]
CXCL12	CAF activation: Promotes cell metastasis, cell invasion, EMT and cisplatin resistance and cancer progression by PI3K/AKT, TGF-β, Wnt/β-catenin, and CXCL12/CXCR4 pathways	[186–188,251–258,241,228]
TIMP-1	CAF inactivation	[259–262]
OPN	CAF activation	[198,263–271]
MAPK	CAF activation	[198]
AKT	CAF activation	[198,257]
ERK1/2	CAF activation	[269,270]
c-Ski	CAF inactivation	[272]
IL-33	CAF activation: Promotes cell migration and invasion	[273]
TNF	CAF activation	[274]
SHH	CAF activation	[275]
miRNAs	CAF activation/inactivation	[164,276–279]
miR-200s	CAF activation: Invasion, metastasis via transcription factors Fli-1 and TCF12	[228]
miR-21	CAF activation: miR-21 and Smad7 induce CAF by TGF-β1 signaling regulation. Motility and invasion by MMP inhibitor RECK	[280,281]
miR-155-5p	CAF activation: Angiogenesis by SOCS1/JAK2/STAT3 signaling pathway	[282]

miR-1247-3p	CAF activation: Stemness, EMT, chemoresistance, and tumourigenicity by IL-6/8; lung metastasis by β1-integrin-NF-κB pathway	[283]
miR-211	CAF activation	[284]
lncRNA	CAF activation/inactivation	[285-287]
LPA	CAF activation	[288]
GPE	CAF activation: Promotes cell proliferation by GPER/EGFR/ERK pathway	[289]
DAPT	CAF inactivation	[290]
Tumour-derived exosome	CAF activation	[291]
Mitocondrial transfer	CAF activation	[292]
SMAD	CAF activation/inactivation	[281,287,293,294]
Smad2/3	CAF activation	[294,295]
Smad4	CAF activation	[287,293,294]
Smad7	CAF inactivation	[281]

3.2 Stromal cells are recruited to the battlefield by cancer cells

The cancer cell secretome, which includes factors such as TGF-β, platelet-derived growth factor (PDGF), and fibroblast growth factor 2 (FGF2), recruits stromal cells during tumourgenesis [248,296] (Table 2). The most abundant cells within the stroma are fibroblasts, which automatically activate the proliferative and carcinogenic Hedgehog and Notch pathways in response to tumour cell signaling [297,298] (Figures 18 and 19). However, this mechanism is not universal, as the loss of Notch signaling can equally promote CAF phenotypes in squamous cell carcinoma [299]. Nonetheless, other mechanisms by which malignification of stromal cells is also achieved have been described, such as inflammatory modulators like IL-1 act through the NF-κB pathway and IL-6 activate transcription of STAT factors [162,300] (Table 2), the positive feedback process involving Janus kinase (JAK)-STAT signaling, the presence of a contractile cytoskeleton and alterations in histone acetylation [194,195], or the

secretion of Galectin-1 during the epithelial–mesenchymal transition (EMT) process may also promote fibroblast transformation and induction of glioma cell migration [223] (Table 2). In addition, physical changes in the ECM [224], heat shock factor 1 (HSF1), which responds in part to protein misfolding [196,225], loss of suppressor genes such as PTEN, caveolin-1 (CAV-1), p53 and p21 [197,226,251], or physiological and genomic stresses such as double-stranded DNA breaks that induce the production of IL-6 and the TGF-β family ligand activin A can also favour this process [301]. It has been demonstrated that stromal fibroblasts exposed to carcinoma cells' conditioned medium were more capable of promoting tumour growth [302,303]. According to Toullec and colleagues (2010), stromal cell-derived factor 1 (SDF-1) is a key factor involved in the malignancy and action of resident fibroblasts in human adenocarcinomas [252]. It is likely that carcinoma cells not only initiate the transformation of stromal fibroblasts into myofibroblasts but also contribute to their activation *in vivo* (Figures 18 and 19).

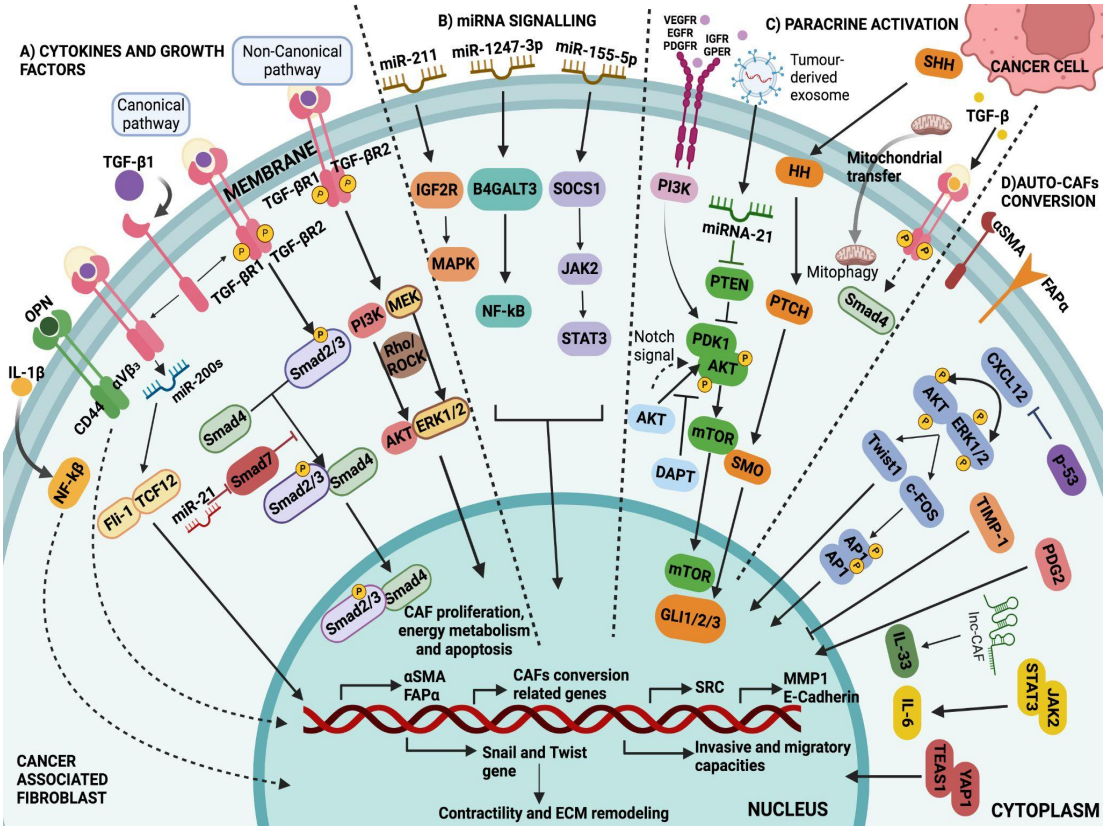


Figure 18. Major pathways involved in CAF development. (A) Cytokines and growth factors: Regulation of CAF-specific gene expression through TGF-β signaling via Smad-dependent and Smad-independent pathways. In the canonical pathway, Smad2/3-Smad4 translocate to the nucleus and bind to a specific DNA sequence, being possibly inhibited by Smad7. In the non-canonical pathway, TGF-β promotes the activation of several signaling pathways, other than Smad, including PI3K kinases, MEK, and Rho-Rock, among others. (B) miRNAs signaling: miRNAs and lncRNAs transform NAFs into CAFs through downstream signaling involving JAK/STAT, NF-κβ, and MAPK cascades. (C) Paracrine activation: Through the PDK1/AKT signaling pathway, in which tumour exosome miRNA-21 inhibits it and PI3K promotes it. Notch signaling via AKT signaling pathway controls AKT phosphorylation and mTOR activation. As a result, mTOR regulates the expression of targeted genes associated with differentiation into CAFs; in addition, expression of tumour cell-derived SHH has been confirmed to modulate CAFs via paracrine activation of HH signaling, and the overexpression of SMO in CAFs contributes to HH signal transduction and GLI1 activation. Fibroblasts can also be metabolically reprogrammed via cancer cell-derived mitochondrial transfer and the TGF-β signaling pathway derived from cancer cells. (D) Auto CAFs-conversion: A self-propelled conversion induced by changes in cellular homeostasis, which controls the activation of cytoskeletal proteins and the secretory phenotype via the YAP1/TEAS1 and JAK2/STAT3 signaling pathways. EMT is facilitated by an activated CXCL12 signal via the ERK/AKT-Twist1-MMP1 pathway. TIMP-1 is related to collagen contractility and CAF proliferation and migration through the ERK1/2 signaling pathway in CAFs (Table 2). (*Toledo, B et al., Int. J. Mol. Sci. 2022*).

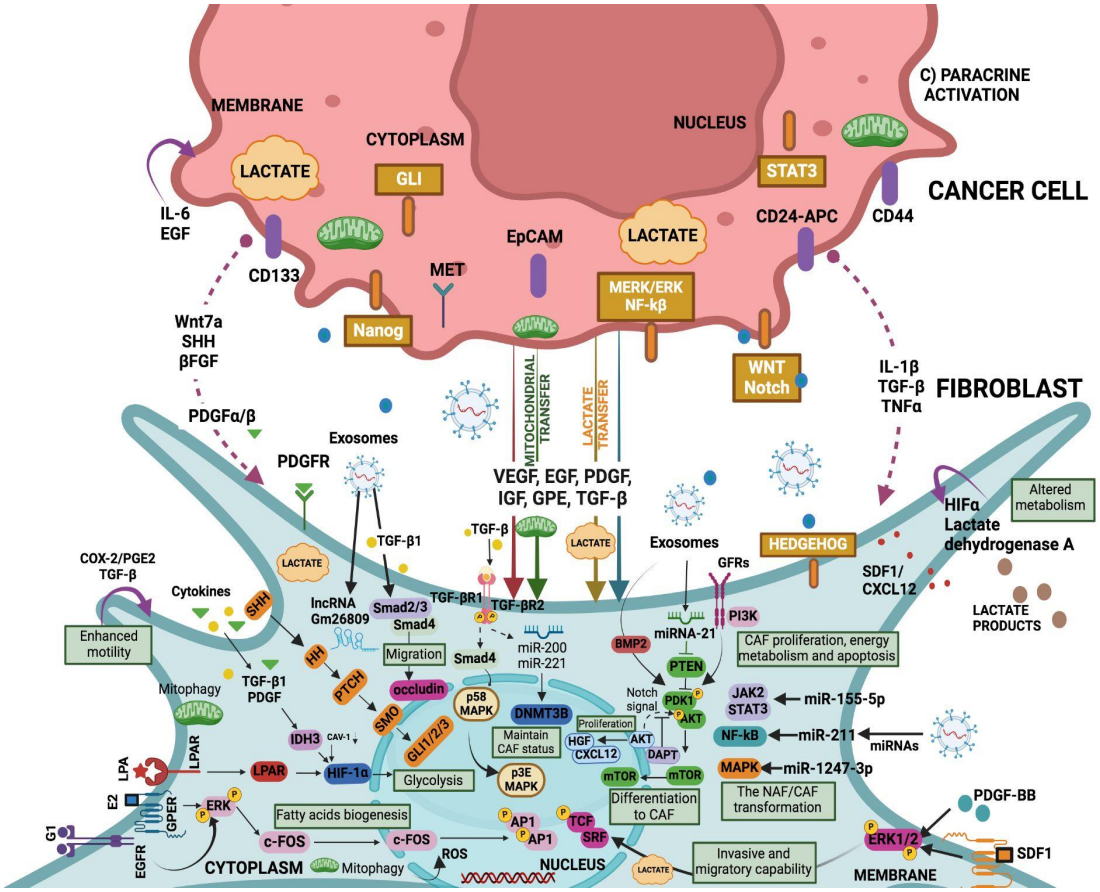


Figure 19. CAF "education" by the cancer-derived factors (extension of section C) in Figure 17). Cancer-derived exosomes carry elements such as miRNA and lncRNA that promote the transformation of NAF to CAF through downstream signals that include cascades such as TGF-β/Smads, JAK/STAT, NF-κB, and MAPK. NAF-CAF conversion may also be driven by the reprogramming of glucose metabolism and the HIF-1α signaling pathway involved in glycolysis. Both the canonical TGF-β signaling pathway (TGF-β/Smads) and the non-canonical pathway (with activation of TGF-β but not Smads) are actively involved in the malignancy of NAFs. In CAFs, while miRNA-21 can attenuate the inhibition of PTEN on PDK1/AKT, the receptor–ligand binding-activated PI3K can promote it. As a result, through the PDK1/AKT signaling cascade mTOR protein is transported into the nuclei, and subsequently, the mTOR protein regulates the expression of targeted genes associated with CAFs differentiation. Notch signaling pathway is also involved in CAF differentiation via AKT. In turn, CAF-mediated PI3K/AKT signaling pathway regulates cell proliferation, migration, and stemness in cancer cells. EGFR/ERK signaling in CAFs is stimulated by E2 and G1 and upregulates fatty acids metabolism. Further, PDGF-BB and SDF-1 stimulate a higher invasive and migratory capability of CAFs via ERK1/2 phosphorylation (Table 2). (*Toledo, B et al., Int. J. Mol. Sci. 2022*).

Signals from other cells in the TME, as well as tumour cells, may instruct CAF function (Figure 18); for example, granulin produced by macrophages promotes the activation of a fibrotic environment in liver metastases. These

mechanisms, which are not directly dependent on the presence of cancer, may contribute to the protumourigenic environments found in inflammatory conditions and are linked to an increased risk of cancer [253]. CAFs also cause tumour cells to secrete factors that stimulate them, such as hepatocyte growth factor (HGF), connective tissue growth factor (CTGF), epidermal growth factor (EGF), insulin-like growth factor (IGF), nerve growth factor (NGF), FGF, and Wnt family members [159,296]. In addition, CAFs secrete cytokines like CCL7, CXCL12, and CXCL14 that favour tumour progression [203-206] . An example of the "conversation" between tumour cells and CAFs comes from PDAC, IL-6 derived from CAFs was modulated by retinoic acid, which was in turn associated with the EMT process of the tumour cells [207]. The positive activation of HSF1 in CAFs, with the consecutive enhanced production of TGF-β and SDF-1 in a non-autonomous manner is related to the malignancy of cancer cells [200,244] (Figure 19) (Table 2).

Furthermore, the fibrotic environment offers benefits for optimal tumour development, such as the formation of a physical barrier to immune surveillance, which allows for tumour growth [254]. CAFs also promote the development of a surrounding desmoplastic environment by synthesizing fibrillar collagens, which stiffens tissue and alters homeostasis, resulting in the collapsing of nearby blood vessels, which promotes hypoxia and thus results in more aggressive cancer phenotypes [255,256]. Furthermore, CAFs engage in paracrine communication with other cell types in the cancer ecosystem, inducing EMT in neoplastic cells [304] increasing tumour invasiveness [305] and metabolic reprogramming towards a reverse Warburg phenotype (Supplementary 6).

Pioneer Otto Warburg was the first to uncover that metabolic distinctions exist between malignant tumour cells and neighboring normal cells. Neoplastic diseases are characterized by persistent and often uncontrolled cell proliferation, leading to corresponding adjustments in energy metabolism to support cell growth and division. Even in the presence of oxygen, cancer cells shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis, resulting in a high rate of glycolysis followed by lactic acid fermentation (Supplementary 6). Consequently, cancer cells favor glycolysis over mitochondrial respiration, even

under aerobic conditions. This metabolic shift, named the "Warburg Effect" in honor of Otto Warburg, illustrates this phenomenon [306]. Although aerobic glycolysis is less efficient in generating energy, cancer cells exploit this unique metabolic adaptation to increase the uptake and incorporation of nutrients (such as nucleotides, amino acids, and lipids) into their biomass, giving them the advantage of quickly obtaining all the necessary elements to produce new cells [307]. Notably, the Warburg Effect is a metabolic characteristic of the primary tumour, but when cancer cells start to spread from the original tumour to other organs or tissues in the body, their energy requirements change [308]. Recent data suggest that metabolic flexibility is essential for the success of metastatic dissemination and is crucial for efficient colonization of distant sites. Cancer cells develop different metabolic strategies depending on their metastatic location [309]. For example, breast cancer cells enhance their metastatic ability to the liver by adopting a dominant metabolic phenotype, while metastases in the lungs and bones rely more on OXPHOS [310] (Supplementary 6). Metabolic targeting strategies that have been preclinically validated in lung cancer models could also represent a valid method to effectively target K-Ras in other tumour types. Notably, a recent study conducted on PDAC cell lines revealed that tumour-associated macrophages (TAMs) derived *in vitro* exhibit Warburg metabolism, which promotes tumour growth and dissemination. Specifically, this shift in macrophage metabolism supported angiogenesis and enhanced the extravasation of tumour cells in a VEGF-dependent manner. Moreover, it induced EMT in a TGF-β dependent manner, facilitating tumour spread to secondary sites such as the lungs and liver. Conversely, inhibiting glycolysis in TAMs using a competitive inhibitor of HK2, 2-deoxyglucose (2DG), was sufficient to disrupt this pro-metastatic phenotype, reversing the observed increases in TAM-supported angiogenesis, extravasation, and EMT [311] (Supplementary 6). In a phase I dose-escalation trial, the combination of 2-DG and docetaxel was tested in 34 patients with metastatic or advanced solid tumours. The results indicated that the recommended dose of 2DG in combination with weekly docetaxel is 63 mg/kg/day with tolerable adverse effects [312]. In particular, further studies on the use of anti-cancer agents targeting the Warburg Effect in PDAC by KRAS are warranted.

It was evaluated and demonstrated by De Wever that the pro-invasive activity of human CAFs *in vitro* using human colon cancer cells and fibroblasts isolated from surgical fragments of colon cancer, further establishing the idea that cross-signaling between cancer cells and fibroblast-like stromal cells may contribute to the progression of human solid tumours [313] (Figure 19).

Although all fibroblasts present in a tumour are defined as CAFs, they come from diverse cell sources [314]. We have already described above how resident fibroblasts are activated to become CAFs, but other cell types within the tissue also undergo a transformation that ends in an induced cell differentiation toward CAF-like cells. It is becoming evident that the origin of CAFs may vary between different tumour histotypes, and within different areas of the same tumour, and this origin may be critical in determining the degree of CAFs malignancy [159,315,316] (Figure 20).

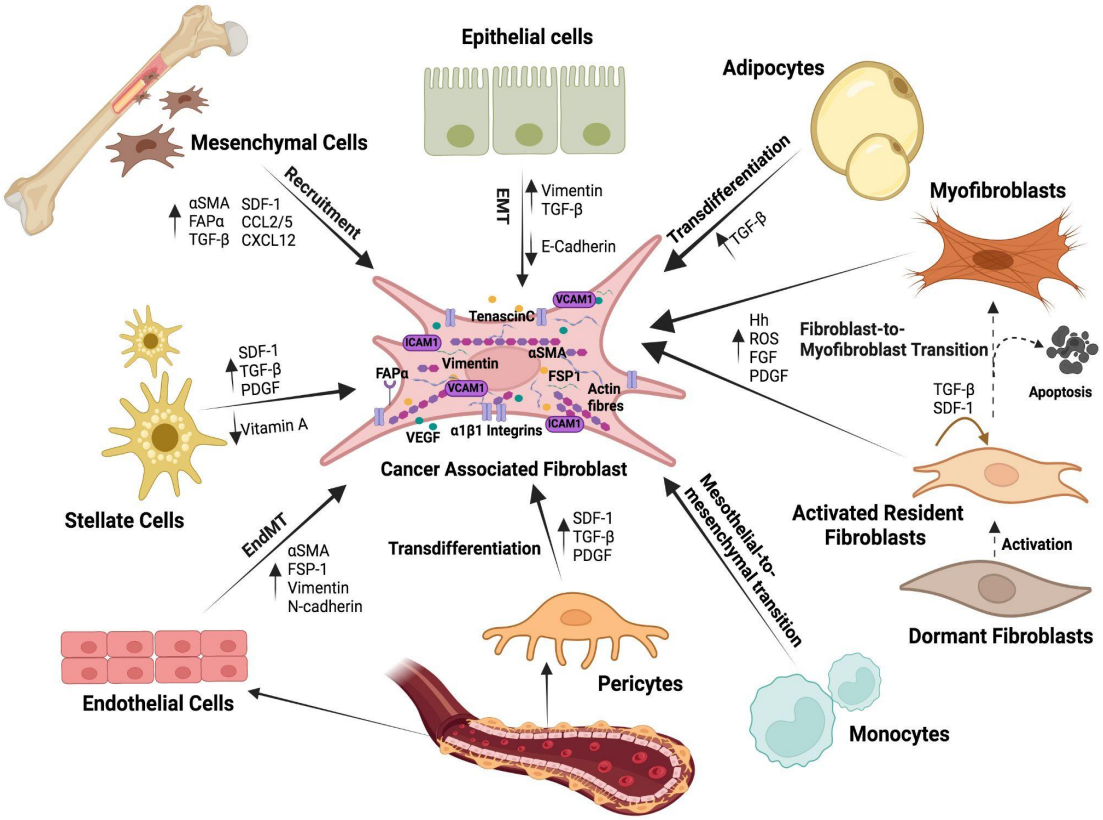


Figure 20. Heterogeneous cellular origin of CAFs. Within the TME, CAFs can be derived from a wide variety of cells: activated resident fibroblasts, bone marrow-derived

mesenchymal stem cells, endothelial cells, epithelial cells, adipocytes, pericytes, and stellate cells. Depending on the origin, CAFs have distinctive characteristics, functions, and locations. Image created with Biorender.com. (*Toledo, B et al., Int. J. Mol. Sci. 2022*).

Many studies support the idea that mesenchymal stem cells (MSCs) are recruited and induced by the developing tumour to enhance proliferation and to acquire a CAFs-like phenotype [317]. MSCs malignification is driven by cytokines and chemokines released in the TME such as TGF-β, PDGF, and IL-6, among others [158,245] (Table 2). For instance, bone marrow (BM)-derived human MSCs exposed to tumour-conditioned medium differentiate and express myofibroblast markers αSMA and fibroblast activation protein-alpha (FAPα) [318]. Based on studies in animal models, such as mouse gastric cancer, it has been estimated that BM-derived MSCs can account for up to 15–25% of the CAFs population [319]. In another study, after 28 days of implantation of pancreatic carcinoma cells in a murine tumour xenograft model, BM cells accounted for nearly 40% of the total stromal myofibroblast population within the tumour [320]. Intriguing studies in patients who had previously received a BM transplant from an opposite sex donor revealed that a high percentage of the tissue fibroblasts were of donor origin [321]. In contrast, Arina et al. (2016) recently showed that CAFs were derived primarily from local fibroblasts, not from cells in the BM, thus supporting the theory of the multiple origin of CAFs (Figure 20).

A recent study found that loss of E-cadherin in tumour buds increased VIM expression and CAFs activation, all of which are signs of EMT and are associated with more aggressive tumours [322]. CAFs, on the other hand, can develop from endothelial cells via the endothelial-to-mesenchymal transition (EndMT). They undergo partial transdifferentiation, acquiring fibroblastic markers like αSMA, FSP-1, VIM, and N-cadherin while retaining endothelial markers like VE-Cadherin, CD31, and Tie1/2. Furthermore, the absence or reduction of adipocytes in pathological tissue could be due to activated fibroblasts interfering with their differentiation process. Adipocytes may also play a role by interacting with cancer cells and providing metabolic support,

whether or not they are converted to CAFs [257] (Figure 20).

3.3 CAFs Biomarkers: a mixed bag

We can agree that a clear correlation exists between tumour initiation, fibroblast corruption, and tumour progression, so identifying CAFs markers could help to determine the stage of tumour development and the initiation of the pre-metastatic niche (Figure 21). However, a vast variety of proteins associated with CAFs that present issues when it comes to being biomarkers with potential clinical applicability have been described. In fact, CAFs markers are expressed at different intensities at different cancer stages and this great heterogeneity has led to the realization that there are different CAFs subpopulations with key roles in the processes of tumour development and spread [258,323]. Here, we review CAFs markers that are commonly accepted and their potential therapeutic implications.

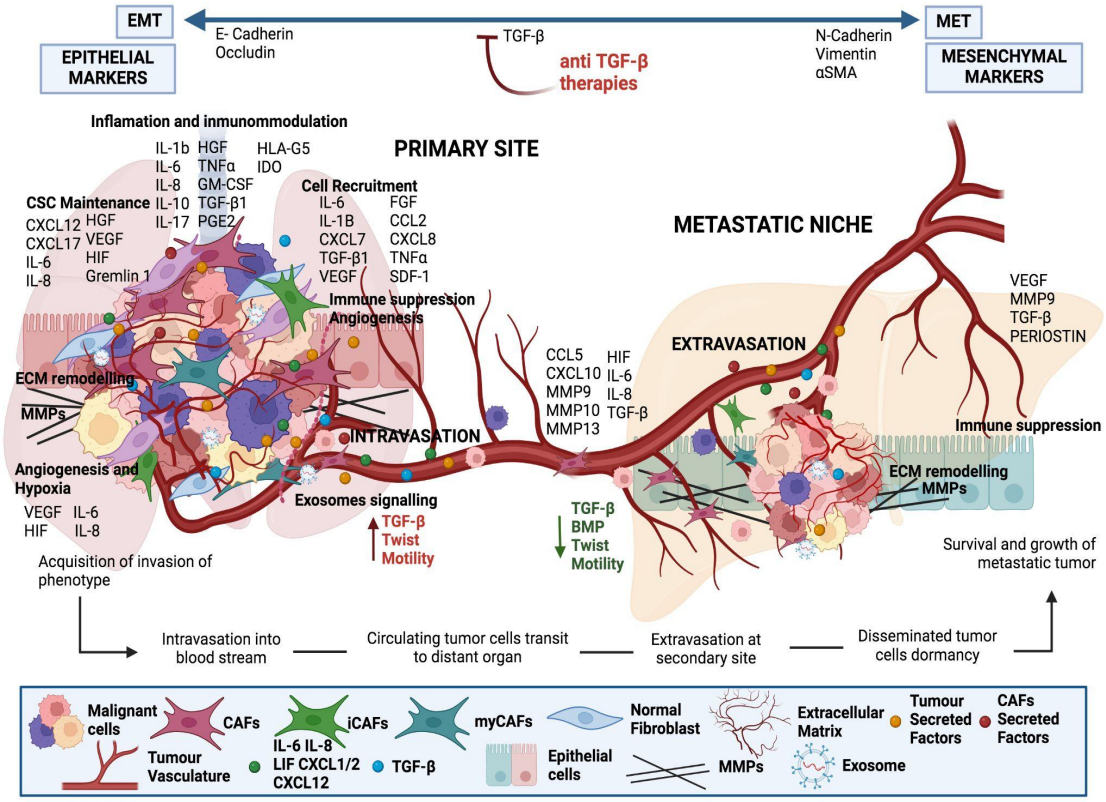


Figure 21. Crosstalk between tumour cells and CAFs at the primary tumour and metastatic site. Factors derived from cancer cells activate local fibroblasts to become

CAFs. CAFs, in turn, produce interleukins to activate signaling in cancer cells increasing their metastatic potential. CAFs are chaperons of tumour cells as they support their survival and extravasation to the metastatic site, as well as preparing the premetastatic niche. (*Toledo, B et al., Int. J. Mol. Sci. 2022*).

3.3.1 Cytoskeleton and Cytoplasmic Proteins

In the first place, we identify αSMA, a cytoskeleton protein associated with TGF-β production, with a highly contractile phenotype [324], which is considered a robust marker of CAFs possessing myofibroblast morphology [325]. Nevertheless, although αSMA is a marker commonly used to determine the presence of CAFs, the fact that it is also present in normal fibroblasts, even with expression levels comparable with those of CAFs [326-328], and in pericytes or myocardiocytes [329], implies a certain degree of confusion (Figure 20). To introduce more complexity, the degree of expression of αSMA seems to be correlated with different CAFs subtypes. Tuveson and colleagues distinguished two groups of CAFs in co-cultures of murine pancreatic stellate cells (PSCs) and human PDAC organoids. CAFs that were located next to tumour cells showed a contractile matrix-producing phenotype and a high expression of αSMA (myofibroblast-like CAFs or myCAFs). On the other hand, they noted that when CAFs were found in regions far from the tumour focus, the cells presented an inflammatory-like phenotype (iCAFs). Those iCAFs showed an immunomodulatory secretome with lower expression of αSMA and a high IL-6 secretory capacity through which they activate JAK/STAT [330]. Interestingly, CAFs subpopulations could transform into each other depending on the spatial or biochemical niche where they were cultured. For example, iCAFs grown in monolayers reverted to a myCAFs phenotype and expressed myCAFs markers [273]. CAFs plasticity implies powerful therapeutic implications, as it could mean that an active fibroblast may revert to an inactive form. JAK inhibitors can suppress tumour growth as well as the switch between iCAFs and myCAFs, whereas TGF-βR inhibition could partially attenuate myCAFs function without influencing tumour growth [331-333]. Nevertheless, both subtypes seem to influence tumour progression and hamper common therapies. While iCAFs appear to promote tumours by producing chemokines and cytokines [246], being

markers of malignancy in pancreatic tumourigenesis [332], myCAFs increase ECM deposits impeding drug delivery [331].

Another highly accepted marker is the fibroblast-specific protein 1 (FSP-1 or S100A4). This marker is also not only limited to CAFs but can be found in epithelial cells undergoing EMT [334,335] and BM-derived cells, such as liver macrophages, that respond to tissue injury [336]. Subpopulations of S100A4+ fibroblasts present in TME have been shown to facilitate malignant progression, for instance a study with FSP-1-deficient mice showed reduced tumour growth and attenuation of metastatic potential, whereas injection of FSP-1+ CAFs partially reversed this effect [337]. S100A4+ CAFs promote tumour metastasis through secretion of vascular endothelial growth factor (VEGF)-A and Tenascin-C (TN-C) [338].

Further, VIM, a type III intermediate filament protein that maintains cell integrity and is involved in cell migration, motility, and adhesion [339,340], which has been described as a CAFs marker. In fact, it has been shown that VIM plays a functional role in lung adenocarcinoma invasion and metastasis [341]. However, VIM's efficacy as a CAF-specific marker is hampered by its widespread expression in both the general fibroblast population and in a variety of other cell types, including macrophages, neutrophils, lymphocytes, and any other cells of mesenchymal origin [342].

3.3.2 ECM-related Components

Tenascin-C (TN-C), a member of ECM glycoproteins, is markedly up-regulated in pathologic tissues that are undergoing remodelling, such as those that are suffering inflammation, wound healing, or tumour progression [343,344]. In fact, negative prognosis of breast, colon, liver, and oral squamous cell carcinoma lymph node metastases cancer patients have been correlated with TN-C expression [345-347]. New results suggest that TN-C promotes multiple events in tumour progression and metastasis and, in particular, in angiogenesis [348]. Thus, specifically targeting TN-C could be a possible way to prevent the establishment of pre-metastatic niches [201].

Periostin (POSTN), a matricellular N-glycoprotein, is implicated in the EMT process, and is crucial for the collagen fibrillogenesis process [349,350] and wound healing [351,352]. Cellular survival, motility, and adhesion are facilitated by POSTN-activated signalling pathways, which are essential for tumour growth, angiogenesis, invasion, and metastasis [353-355]. Studies have shown that POSTN is predominantly expressed in tumour stroma [356-358]. Therefore, this protein could be a suitable CAFs marker.

The tissue inhibitor of metalloproteinase-1 (TIMP-1) has been shown to co-express in fibroblasts with α-SMA, and thus is a candidate to become a CAFs marker [295]. In fact, TIMP-1 released by CAFs promotes the migration and spread of cancer cells [359]. In lung adenocarcinoma, it was demonstrated that SMAD3/TIMP-1 in CAFs and CD63 in cancer cells were necessary to drive tumour progression *in vitro* and *in vivo* [360]. In another study, CAF-derived TIMP-1 promoted the migration of a colon cancer cell line, whereas TIMP-1 neutralization inhibited the enhanced migration, and TIMP-1 secretion was higher in CAFs co-cultured with cancer cells than in monocultured CAFs [361] (Table 2).

Osteopontin (OPN), a chemokine-like phosphorylated glycoprotein released intracellularly, is typically elevated in a variety of human malignancies [362]. It is essential for cell migration, EMT, ECM invasion, and in maintaining the stemness of tumour cells by either binding to integrins and CD44 or by activating the NF-Kb, MEK/MAPK, PI3K/Akt, and FAK pathways [247,363-365] (Figures 18 and 19). Additionally, it has been shown that OPN plays a significant role in the differentiation of myofibroblasts into cardiac and dermal fibroblasts in culture, which may be pertinent to the fibrotic process [366,367]. Breast cancer cell-derived OPN promotes the activation of resident fibroblasts into CAFs by the association with CD44 and αvβ3 integrin on the fibroblast cell surface, which mediates signalling through Akt and ERK to induce Twist1-dependent gene expression [368,369] (Figures 18 and 19). Furthermore, tumour-derived OPN also transforms MSCs into CAFs through the transcription factor, myeloid zinc finger 1 (MZF-1)-dependent TGF-β1 production, promoting tumour growth and metastasis [187] (Table 2).

3.3.3 Receptors and Membrane-Bound Proteins

The next candidate is FAPα, a type II integral membrane serine protease of the prolyloligopeptidase family. FAPα has been reported to be overexpressed in CAFs in many types of carcinomas, including colorectal, pancreas, ovarian, breast, bladder, and lung carcinomas [370,371]. FAPα overexpression has been associated with poor prognosis as occurs, among others, in rectal [240], hepatocellular [241], colon [242], and pancreatic cancers [243]. Although FAPα is not solely specific to CAFs, as it has also been observed in bone, BM stem cells, and skeletal muscle [372,373], it could be one of the most promising therapeutic targets. FAPα silencing has been shown to inhibit stromagenesis, tumour growth, and angiogenesis in murine models of pancreatic cancer [374] and to suppress cell proliferation, migration, and invasion of ovarian squamous cell carcinoma cells *in vitro* and *in vivo* after inactivation of phosphatase and tensin homolog (PTEN), phosphoinositide-3-kinase (PI3K), and serinethreonine kinase. The use of immune checkpoint antagonists that promote T-cell function, such as anti-cytotoxic T lymphocyte-associated protein 4 (α-CTLA-4), and anti-programmed cell death 1 (α-PD-L1), has revealed an unexpected benefit in PDAC therapy based on FAPα+ CAFs depletion [372].

Platelet-derived growth factor receptors (PDGFR) are cell surface tyrosine kinase receptors for members of the PDGF family. PDGFRα and PDGFRβ are high molecular weight membrane proteins (around 180 kD) which dimerize non-covalently upon ligand binding. PDGFRα is a marker for fibroblasts in some tissues like skin [375], whereas PDGFRβ is expressed in fibroblasts, pericytes, and CAFS in multiple tissues and tumour types [376]. A blockage of PDGFR signalling has been shown to suppress angiogenesis and tumour growth and a case in point is Dasatinib, a PDGFR inhibitor, which can partially reverse the pro-tumourigenic effect of CAFs in lung adenocarcinoma and may be a potential treatment strategy [377]. Based on their ability to promote the growth of co-injected pancreatic tumour cells in immunocompromised mice, PDGFR+ CAFs have protumourigenic properties *in vivo* [378] (Table 2).

3.3.4 Growth Factors and Cytokines

SDF-1 is a homeostatic chemokine that is also known as CXCL12 (Figures 18 and 19). Fibroblasts in invasive breast carcinomas participate in tumour development primarily by secreting SDF-1 [379]. High levels of SDF-1 in endothelial cells and fibroblasts within ischemic injury have been shown to attract circulating CXCR4-expressing progenitor cells to injury sites [249,380]. Thus, elevated expression of CXCR4 by carcinoma cells favours the primary tumour in murine xenograft models [381], whereas deletion of this marker in breast carcinoma cells inhibits tumour growth [382]. Further, over-expression of CXCL14 and CXCL12 chemokines by myofibroblasts enhances epithelial cell proliferation and invasion, demonstrating the important paracrine effect of stromal cells on carcinoma cells [382,383].

Finally, Prostaglandin E2 (PGE2), known to be produced by immune cells, is also produced by malignant stromal cells like CAFs, and it promotes angiogenesis, induces metastasis, and increases tumour cell survival and proliferation [384-386]. It has been shown that PGE2 stimulated VEGF-A production in stromal fibroblasts generated from mice's stomach cancer [302]. In addition, PGEP2 and PGEP4 are expressed in lung fibroblasts [387,388] and in CAFs from CRC to stimulate the development and expression of genes associated with inflammation and carcinogenesis [389].

3.4 Fibroblasts: Induced and Inductors

Normal fibroblasts appear to have a double effect on cancer cells. Fibroblasts act against malignant progression as early as during tumourigenesis [227,228], but as cancer progresses, fibroblasts are subverted to promote tumour growth (so-called CAFs) [383] (Figure 22). In fact, during the early phase of tumourigenesis, non-CAF fibroblasts have a protective role against tumour progression, enhancing intercellular induction of tumour cell apoptosis through four different pathways mediated by ROS (NO/peroxynitrite pathway, HOCl pathway, NO consumption, and nitrogen chloride pathway) [390].

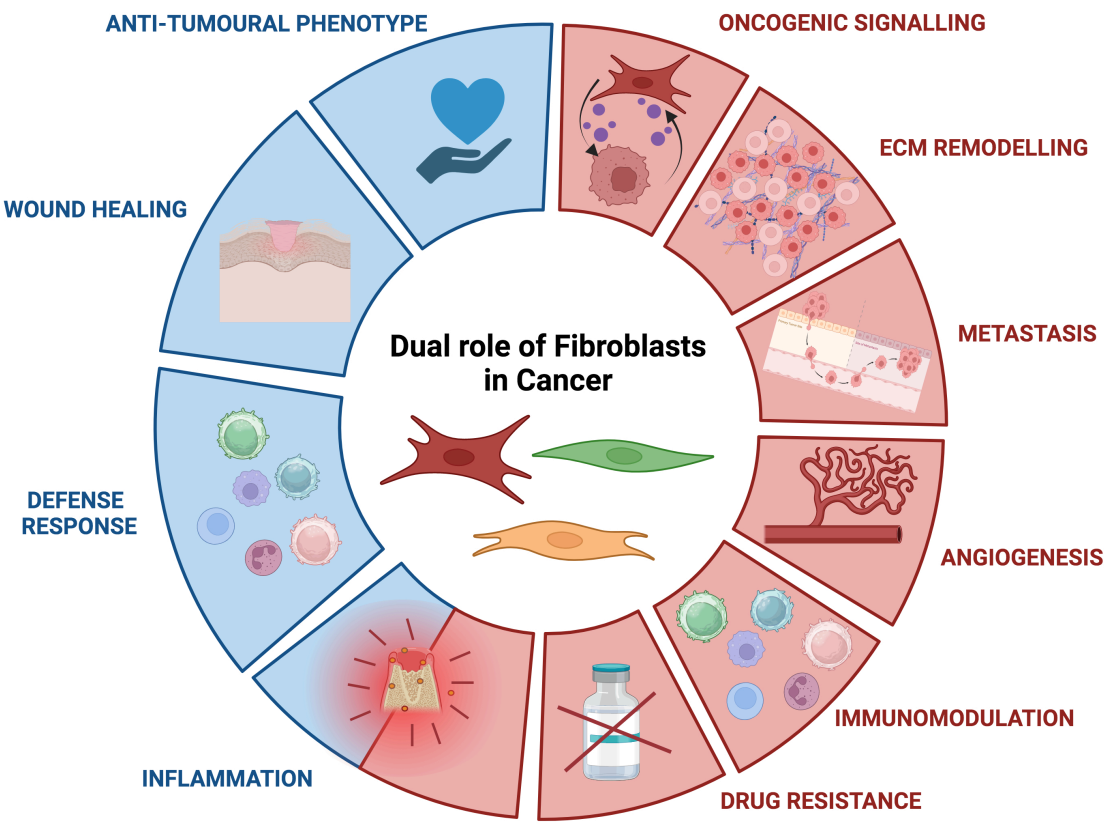


Figure 22. Main anti- (green) and pro-tumoural (red) roles that fibroblasts develop in the human body. The fact that the phenotype goes towards one (good) or the other (evil) side will depend on some factors such as: the signals it receives, the state of stress, the environment in which it receives and the condition of the patient themselves. Image created with Biorender.

However, if tumour cells escape this fibroblast "first line of defense" mediated by ROS, they proceed to malignify stromal elements and to favour a cross-communication between fibroblasts and tumour cells, which leads to tumour progression. Although the reasons for CAFs remaining activated indefinitely are unknown, it is clear that fibroblasts engage in reciprocal and elaborated feedback with cancerous epithelium and the dual action of fibroblasts during tumour development [391]. Interestingly, a recent study found that CAFs are capable of reverting lung cancer spheroids from a dysplastic to a hyperplastic state by knocking out Sox2 activity. Therefore, CAFs are capable of modifying intrinsic oncogenic characteristics and influencing tumour development [221]. SLITs are a family of secreted ECM proteins that act as tumour suppressors under normal conditions and play an important role in cell migration, tissue development, and

vascular network establishment. At first, when a fibroblast is in the early stages of activation, SLIT2 increases and inhibits the expression of CXCL12, leading to the prevention of tumour growth and metastasis. However, CAFs decrease SLIT2 production, and subsequently, up-regulate CXCL12, promoting metastatic behaviour of tumour cells, which supports the theory of aforementioned functional duality in fibroblasts [392,393].

Injecting a teratoma cell line into an early mouse embryo demonstrated that healthy cells have tumour suppressor properties; the resulting postnatal animals were derived from both host cells and transplanted tumour cells, but the mouse presented normal development with no sign of specific features of tumour cells [394,395]. Dormant fibroblasts were assimilated to tumour suppressor cells because they inhibited the growth of *in vitro*-transformed baby hamster kidney cells and *in vivo*-transformed keratinocytes [396]. Tumour growth suppressor factors, such as the WAP Four-Disulfide Core Domain 1 (WFDC1) protein, were found to be highly expressed in resting fibroblasts but downregulated in CAFs [397], implying that resting fibroblasts can indeed inhibit tumourigenesis. It is unclear whether these fibroblast subtypes are normal fibroblasts resistant to CAF conversion or distinct subpopulations of antitumour CAFs. Tumour suppressor fibroblasts have also been found in established tumours, according to research [274]. PDAC is known for having a large amount of fibrotic tissue within the tumour mass, as the majority of the tumour volume is made up of αSMA+ fibroblasts [274]. The removal of these fibroblasts was thought to reduce the aggressiveness of PDAC, but clinical studies using this strategy revealed accelerated disease progression [255]. Further investigation in mice revealed that deletion of αSMA+ fibroblasts resulted in invasive tumours with increased EMT and CSCs sub-population, resulting in a decrease in survival. This result was mainly due to decreased immune surveillance and increased regulatory T (Treg) cells infiltration. Thus, it seems that the PDAC-associated fibrotic response is a primary host defence mechanism against tumour invasion which then undergoes a malignification process [244] (Figure 22).

In solid tumours, not only do CAFs remain persistent and autocrine activated, but they also stimulate neoplastic cells in a paracrine manner [398,399], accompanying tumour cells throughout the carcinogenic process [400,401]

(Figure 21). CAFs regulate epithelial proliferation as a result of their direct paracrine interactions with tumour cells, having an indirect effect on inflammatory processes in which CAFs serve as mediators. Interestingly, induction of CAFs autophagy has been shown to promote tumour cell survival through processes involving the downregulation of CAV-1 and subsequent stabilization of Hypoxia Inducible Factor 1-alpha (HIF1-α) [251].

CAFs increase the secretion of MMPs as observed in studies of head and neck squamous cell carcinoma (HNSCC), highlighting their crucial role in cancer progression and early stages of tumour growth [296]. For example, MMP-13 promotes tumour angiogenesis and leads to tumour invasiveness [199]. TME stromal collagen accumulation is associated with poor prognosis in primary tumours and metastases [402-404]. Desmoplasia reduces microvascularity, limiting chemotherapy treatment penetration [405-407]. In fact, in a mouse model of pancreatic adenocarcinoma, lowering CAFs improved chemotherapy efficacy, implying that CAFs play an important role in intratumoural drug delivery in function of its ECM secretion [238]. Attempts to target the stroma, either directly or by enzymatically digesting ECM, have resulted in reduced tumour growth and improved chemotherapy response [407].

The role of tumour stromal fibroblasts in carcinogenesis was studied using an immunodeficient mouse tumour xenograft model in which tumour cells were implanted alongside CAFs, homologous fibroblasts extracted from non-cancerous tissue from the same individual, or normal fibroblasts extracted from healthy donors [408]. Tumours formed from carcinoma cells injected with CAFs grow faster than tumours formed from any of the other fibroblast types.

In addition, CAFs support invasion and metastasis by modelling the ECM [409], increasing tumour spread and therapeutic resistance [410] (Figure 22). Indeed, in primary tumours, IL-11 secretion by CAFs favours neoplastic GP130/STAT3 signalling, reprogramming MSCs into CCL5-secreting CAFs, and finally leading to cell invasion and promotion of metastasis [219] (Figure 21). In addition, tumour progression is enhanced by CAFs IL-8 secretion [411], CAFs exosomes autocrine induction of Wnt-11 [412], potentiation of the neoplastic EMT process for tumour extravasation [304], or by CAFs MMPs secretion to trigger the COX2-NF-B cascade, as seen in prostate cancer cells and promotion of

ROS accumulation and invasion [413] (Table 2).

Interestingly, the key role of CAFs as chaperons in metastasis is becoming more widely accepted. CAFs facilitate intravasation and extravasation and escort and sustain neoplastic cells within the bloodstream on their way to metastatic sites [239,275,337] (Figure 21). Recent *in vivo* studies have shown that metastatic cells have greater viability when they are accompanied by their own stromal elements, while when CAFs are depleted, there is a marked decrease in the number of lung metastases [414]. Invasive tumours in colon cancer patients have also been shown to be enveloped by a layer of CAFs during invasion [211]. Aiello et al. demonstrated that fibroblasts appear in distant metastases with 6-7 cells and that the goal of the final metastasis is to reach a stromal volume of the dimensions of the primary tumour [212].

Furthermore, major mediators of breast cancer metastasis to the lung include metastasis-associated fibroblasts (MAF) expressing FSP-1, TN-C, and VEGF-A [415]. The activation of liver-resident fibroblasts to enhance angiogenesis is necessary for melanoma spread to the liver [213]. Loss of this protein in FSP1 mutant mice affects fibroblast motility and is linked to decreased metastasis [214]. For CAFs extracted from prostate carcinoma samples, a similar pro-metastatic impact was also shown [304], where CAFs induce lung metastasis after heterotopic co-injection of cancer and stromal cells. Additionally, CAFs can boost the rate of successful colonization by choosing neoplastic clones that have a particular advantage over metastatic spread while still present in the initial lesion. CXCL12 and IGF secretion serve as mediators in this process [416] (Figure 21) (Table 2).

Finally, CAFs' support to CSCs has also been proven. Chen et al. recently found that CAFs, through IGF-II and IGF1-R intercommunication, can improve lung cancer root plasticity through mechanisms dependent on Nanog [220], a transcription factor involved in CSC pluripotency. Giannoni et al. reported that CAFs isolated from prostate carcinoma samples, through the activation of the EMT epigenetic program, promote CSC generation [304], and increase the expression of recognized CSC markers (CD133+ and high CD44/CD24 ratio) and the creation of nonadherent spheres as an associated property [417]. Others were able to demonstrate increased cell proliferation and elevated EGFR expression in

HNSCC tumour 3D spheroids when co-cultured with CAFs, in addition to an EMT and CSC-like phenotype, and decreased sensitivity to the cytotoxic agents, cisplatin and cetuximab (Mustafa Magan et al., (2020)) (Table 2).

3.5 CAFs and tumour Immunity

CAFs are beginning to take the lead rather than play a supporting role in immune modulation in recent years. The CAFs secretome may vary dynamically during the evolution of cancer by supporting tumour immunity at various stages of the tumour, but it does not contribute to a state of prolonged activation of the defensive level [215,216] (Figure 23).

CAFs can affect the host immune response both directly and indirectly, according to Knops et al.'s (2020) study. By sustaining an abnormal pro-tumour inflammatory milieu, they can hinder or abolish the cytotoxic actions of T and NK cells [418,419]. In order to help create and maintain an immunosuppressive milieu that impairs adaptive immunity against the tumour, CAFs promote the recruitment, development, and survival of regulatory T cells [217,420]. Cytotoxic T lymphocytes that are specific for an antigen are eliminated as a result of the presentation of Human Leukocyte Antigen (HLA)-class I antigens by stromal fibroblasts and the production of PD-L2 and FASL [218]. Cytotoxic T cell activity was restored, and fast tumour necrosis took place in genetically engineered Lewis lung cancer (LCC) mice when FAPα+ stroma was specifically removed by diphtheria toxin [276]. The doxorubicin-based chemotherapy reduced the invasion of immune-suppressive cells like Treg cells and further stimulated the anti-tumour immune response when this deletion was used with it [277].

The subtype known as CAFs-S1, which is identified by the expression of FAPα, αSMA, PDGFR, and CD29 markers, was found to be associated with the recruitment, retention, and differentiation of Treg cells, enhancing their capacity to inhibit CD4+CD25+ T-cell proliferation *in vitro*. This was revealed by a FACS-based analysis of CAFs in human breast tumours. Additionally, it was able to prolong T-cell life and trigger their differentiation into regulatory lymphocytes with the CD4+CD25+FOXP3+ phenotype [193]. This process was also revealed to be dependent on CXCL12's differential regulation by miR-200/141 in human high-grade serous ovarian tumours (HGSOC) [421].

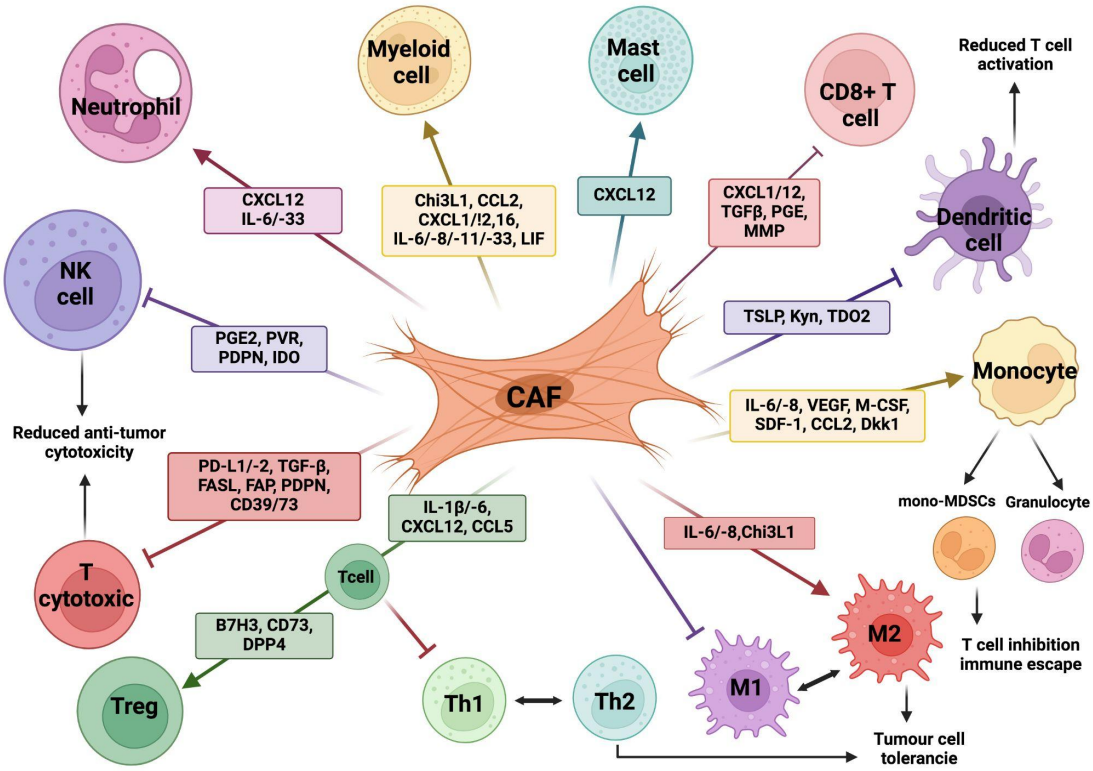


Figure 23. Crosstalk between CAFs and immune cells during tumour progression and immunosuppression. Image created with Biorender.

More recently, it has been observed that CAFs can lead to activation of CD4+ T cells and suppression of CD8+ T cells [278]. Targeting FAPα+ CAFs demonstrated antitumour effects through intratumour recruitment of T lymphocytes in genetically engineered animal models and orthotopic cancer transplant trials in immunocompetent mice [279,422]. The absence of FAPα+

CAFs cells appears to restore immune detection and tumour destruction [277] (Figure 23).

CAFs-derived CXCL14 affects macrophage recruitment to the tumour [423] and shunting of their differentiation status toward the M2 subtype is associated with a bad prognosis in cancer. In a mouse model of lung adenocarcinoma, Hegab et al. also noted that CAFs promoted the conversion of TAMs to the M2 phenotype [424] (Figure 23). Additionally, it has been demonstrated that the production of the chitinase-like protein 3 (Chi3L1) by mammary CAFs induces the recruitment of macrophages with an M2-like phenotype and reduced CD8+ T lymphocyte infiltration [425]. Additionally, in numerous animal cancer models, including colorectal, prostate, and breast carcinomas, CAFs have been demonstrated to recruit macrophages in TME [426]. Additionally, tumour-educated CAFs (derived from MSCs) attracted CD11b+Ly6C+ monocytes, F4/80+ macrophages, and CD11b+Ly6G+ neutrophils via the CCL2-CCR2 axis to promote tumour immunity in a mouse model of spontaneous lymphoma [427] (Figure 23).

In a mouse model of transplantable colorectal carcinoma (CRC), it was found that fibroblasts expressing high levels of FAPα recruited myeloid cells via CCL2, which resulted in resistance to anti-PD-1 immune checkpoint therapy that was eliminated by targeting the FAPα. This is significant because it indicates that myeloid cell recruitment by CAFs is also associated with resistance to therapy. These results were confirmed in tissue samples from human CRCs, where the presence of fibroblasts with high FAPα+ concentration was positively connected with myeloid cell infiltration and negatively correlated with T-cell infiltration [428,429]. Similar effects were shown when FAPα was pharmacologically targeted in a transplanted pancreatic cancer model, where there was a decrease in macrophage recruitment and an increase in T-cell infiltration [430]. Additionally, in transgenic and transplanted mice models of melanoma, targeting FAPα+ fibroblasts by immunization with an adenoviral vector eliminated the recruitment and function of immunosuppressive cells, including monocytic and polymorphonuclear MDSCs, within the TME [431]. Human prostate tumour CAFs were discovered to secrete SDF1 to draw monocytes into primary cultures

in vitro. Additionally, the production of the immunosuppressive cytokine IL-10 increased due to the M2-like polarization of circulating monocytes that was induced by these SDF1-producing CAFs [432]. These results are in line with the functional role that CAFs-derived SDF1 has been shown to play in supporting tumour development and immunosuppression. In addition to secreting metabolic reprogramming molecules such as indoleamine 2,3-dioxygenase 1 (IDO1), arginase 2 (Arg2), and galectin, the αSMA+ CAFs (myCAFs) also induce T-cell anergy and limit CD8+ T-cell proliferation [229,230,433] (Figure 23).

3.6 Tumour Stroma and Therapeutic Resistance

Tumour stroma is not only implicated in all stages of cancer development and its immune resistance, but it can also function as an interstitial barrier, preventing effective drug delivery and promoting radioresistance [231,232]. Fibroblasts can be of influence by either (i) sustaining specific subtypes of neoplastic cells, such as CSCs [233], (ii) rewiring the signalling networks in cancer cells [434], or (iii) modulating immune cells [234]. All this leads us to the conclusion that both the presence and the amount of CAFs will be determinants in the final outcome in terms of tumour progression, dissemination, and chemoresistance [235].

CAFs' immunological regulation, proangiogenic effects, and metabolic reprogramming might promote cancer cell survival and make it easier for the disease to evade the effects of therapy. For the first time, it was discovered in 2009 that breast cancer patients with high stromal gene expression did not respond well to neoadjuvant chemotherapy because of an elevated intratumour interstitial fluid pressure that subtly reduced antineoplastic medication uptake [236]. Notably, adhesion of tumour cells to CAFs, through homotypic binding of N-cadherin, can induce EMT progression, leading to therapeutic resistance, among other methods [222,435]. In turn, cancer cells promote fibroblast proliferation through oxidative stress and fibroblasts protect tumour cells from apoptosis through autophagic mechanisms [436]. Worryingly, standard chemotherapy has been shown to transform mammary NAFs into CAFs, resulting in a hypoxic, glycolytic, autophagic, and proinflammatory TME that in turn stimulates Sonic

hedgehog (SHH)/GLI signalling, an antioxidant response, and interferon-mediated signalling in breast cancer cells [437].

Additionally, CAFs' released soluble components have been linked to resistance to anticancer treatment therapy. One example is the HGF, which has been linked to prostate cancer and is thought to play a critical role in CAF-mediated resistance to therapy with tyrosine kinase inhibitors or EGFR receptors [434,438] (Figure 24). Colorectal cancer resistance is mediated by this factor, which then activates the HGF receptor c-Met [274]. According to Crawford et al. (2009), CAFs play a critical role in the resistance to anti-angiogenesis therapy because PDGF expression is also positively regulated in tumour CAFs that are resistant to anti-VEGF therapy and administration of a PDGF-neutralizing antibody was able to change this anti-VEGF resistance (Figure 24). Additionally, TGF-β1 promotes resistance to docetaxel in prostate cancer through induction of the anti-apoptotic gene Bcl-2 by acetylation of KLF5 and to oxaliplatin in human colorectal cancer through EMT [188-190]. It can also reduce a tumour's response to α-PD-L1 blockade by limiting CD8+ T cell accumulation in TME [191]. In colorectal cancer, TGF-β2 and HIF1 produced by CAFs can also activate GLI2, a mediator of the Hedgehog signalling pathway, to encourage the growth of cancer cells and chemotherapy resistance [192]. Finally, NSCLC is less sensitive to cisplatin when IL-6 produced by CAFs stimulates well-studied pro-survival signalling cascades and enhances TGF-β1-driven EMT in lung cancer cells [439].

3.7 Therapeutic Strategies

The idea that there are several factors involved in chemo/radiotherapy resistance that go beyond the tumour cells is on the rise. The physical barrier implied by the TME, with not only the proteins that form the stroma but with the interactions of the different cells that play a role on it, is a key example in which CAFs are protagonists (Figure 21). Thus, a detailed and personalized characterization of CAFs and stromal profiles is essential for the detection of novel targets to optimize therapeutic strategies while maintaining the survival of healthy cells and preventing the emergence of resistance [161]. Since the stromal and epithelial components of a tissue, malignant or healthy, act in an integrated

and reciprocal manner, combination therapy might be an effective treatment choice. In conclusion, general CAFs depletion is probably not the best strategy for anti-CAFs therapies, while the reversal of the CAFs phenotype to a "non-CAFs" phenotype or inhibition of CAFs protumourigenic signals might instead offer better therapeutic options [440,441] (Figure 24).

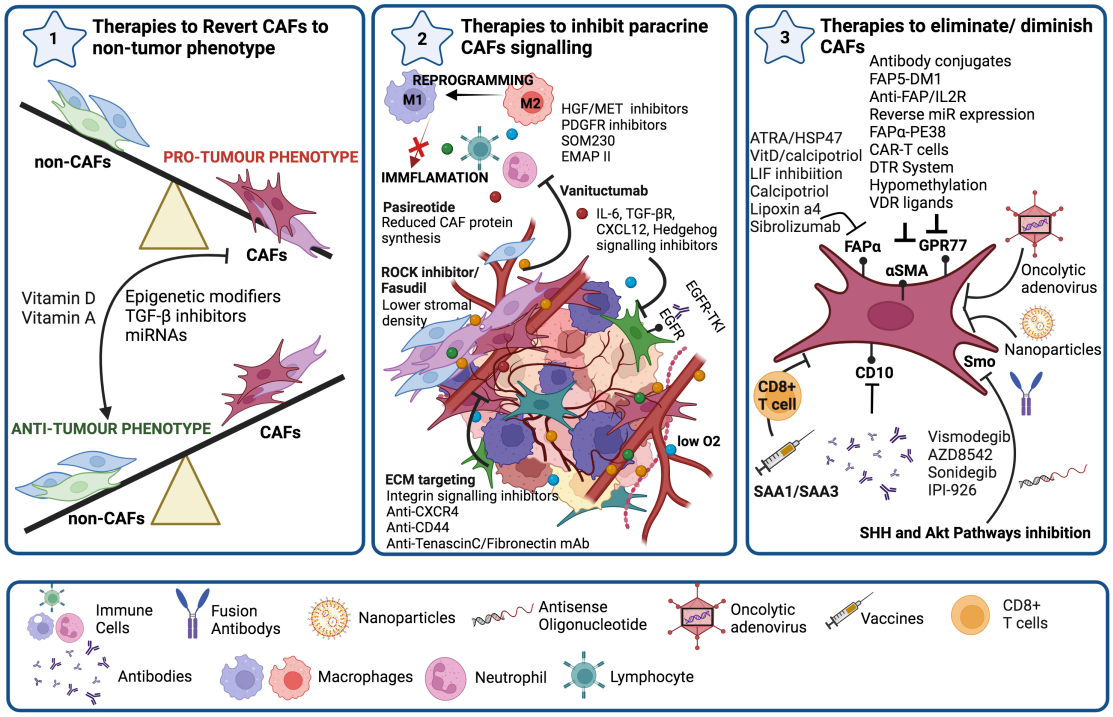


Figure 24. Strategies for CAF-targeted anticancer therapy. (1) Reprogramming to a non-CAF phenotype using epigenetic modifiers or TGF-β inhibitors. In addition, the use of anti-miRNAs, miRNA mimetics, or siRNA administration to reverse CAFs phenotype. (2) Blocking all signalling emitted by CAFs either by targeting crucial molecules (IL-6, TGF-β, CXCL12) or signalling pathways such as CCL2 and CCR2 signalling axis, JAK-STAT3, TGF-β, and the Hedgehog signalling pathway, or FAK signalling pathways to restrict ECM remodelling along with TN-C, MMPs, and HA. (3) Targeting αSMA or membrane markers such as FAPα, GPR77, or CD10 to eradicate CAFs populations (*Toledo, B et al., Int. J. Mol. Sci. 2022*).

Recently, new therapeutic strategies aimed at "re-educating" CAFs into a non-tumour associated status have been developed (Figure 24). The use of epigenetic modulating agents such as the demethylating agent 5-Azacytidine (5-AZA) has been shown to transform an aggressive PDAC model into a less aggressive and drug-sensitive phenotype, slowing tumour growth *in vivo*, after the engraftment of treated transformed cells. GEM was then added to 5-AZA-treated

cells, and this had a noticeable growth inhibition effect on GEM-resistant pancreatic cancer cells [442] (Figure 24).

Other approaches consist of the use of TGF-β inhibitors. The dual role of TGF-β in tumour progression is clear, acting as a suppressor at early stages and as a promoter later on. This functional duality could be demonstrated by comparing the effect of TGF-β signalling pathways in tumour cells of transgenic mice where it suppressed initial tumourigenesis but promoted the final metastatic process [244]. Detailed above is how the presence of TGF-β in TME promotes tumour growth by enhancing stromal support through the stimulation of CAFs collagen production (also preventing T-cell access to the tumour site), angiogenesis, and altering immune surveillance [443]. Thus, the use of molecules that suppress TGF-β action is a therapeutic option that is being studied. Currently, antibodies against TGF-β, TGF-β inhibitors (such as LAP or TGF-β kinase inhibitors), or TGF-β antisense oligonucleotides have been developed, many of which are in phase II trials. Preliminary results show a reduction of blood vessels and blood lacunae in tumours, and a decrease in microvessel density together with wet weight of treated tumours [444].

Recent research has examined the use of miRNAs as a potential method to deactivate CAFs [259,260] (Figure 24). MiR-19a-19b-20a was shown to be up-regulated in pulmonary fibrosis as the condition worsened. This miR inhibited TGF-β-induced fibroblast activation, which may indicate that healthy fibroblasts try to prevent themselves from becoming activated to a malignant phenotype [261].

On the other hand, miR-145, an inhibitor of the TGF-β pathway, was upregulated in both CAFs produced by TGF-β signalling and CAFs from primary oral cancer, but not in healthy fibroblasts. Prior to TGF-β therapy, normal oral fibroblasts were transfected with miR-145, which inhibited their activation to the CAFs phenotype. After being converted to CAFs by TGF-β, they were transfected with miR-145, and this resulted in the restoration of their quiescent phenotype, indicating that normal fibroblasts may already have a built-in negative feedback loop to guard against CAF conversion [262]. Another study found that when compared to fibroblasts from healthy breast tissue, CAFs from human breast tumours showed lower levels of the tumour suppressor miRNA Let-7b. It was

shown, in a murine breast cancer model, that Let-7b miRNA inhibition, in normal mammary fibroblasts, increased their malignification, which in turn potentiated EMT induction and enhanced tumour growth. The opposite was observed when Let-7b was over-expressed in human breast CAFs, which ended in a reduction in cancer promotion [445].

Another therapeutic approach has been focusing on therapies to inhibit paracrine signalling of CAFs, for example, inhibitors of the HGF/MET signalling pathway, hedgehog, or angiotensin II receptor showed a reduction in the expression of CAFs and ECM content, thus enhancing drug delivery and inhibiting tumour growth and metastasis [446] (Figure 24). HGF is secreted by fibroblasts to activate c-Met in cancer cells [447]. MET kinase inhibitors have been shown to produce a beneficial effect in NSCLC [448,449]. Other preclinical studies focused on the use of NK4, a competitive Met antagonist [446] (Figure 24). Phase I and II clinical trials using innovative small compounds that target MET tyrosine kinase have been carried out in this regard, including tivantinib combined with GEM [450], cabozantinib [451], and crizotinib [452], concluding that HGF/MET-targeted therapy could be a promising therapeutic option [453].

The stromal tumour feedback requires the overexpression of PDGF, as was already mentioned. Additionally, as shown by gene targeting experiments, this signalling also supports angiogenesis in an autocrine and paracrine manner [331,454], and its expression is correlated with increased vascularity and vascular wall maturation [455]. It has been demonstrated in experimental models that PDGF inhibition decreases interstitial fluid pressure in tumours and improves the impact of chemotherapy [456,457]. As a result, PDGFR-specific antibodies may be utilized to prevent angiogenesis in tumours that do not respond to anti-VEGF therapy [458] (Figure 24). A powerful inhibitor of the VEGFR, PDGFR, and FGFR families, the indolinone derivative BIBF1120 is currently being used in clinical settings [459]. For instance, blocking PDGF receptor activation in CAFs in a mouse model of cervical carcinogenesis prevents the formation of premalignant lesions and chemoresistance [460,461].

Inhibiting the mTOR pathway in pancreatic CAFs with the somatostatin analogue SOM230 results in reduced secretion of IL-6, which stimulates chemoresistance, mediated by CAFs, and potentiated pancreatic tumour drug

sensitivity [462]. Other therapeutic targets have also demonstrated the reduction of chemoresistance (Figure 24). Targeting tumour stromal elements necessary for tumour progression, such as fibronectin, can also successfully slow down tumour development and spread while improving antitumour medication delivery. In fact, according to recent research, fibronectin depletion causes the secreted protein acidic and cysteine rich (SPARC) to switch from boosting cancer cell proliferation to inhibiting growth and inducing death [463]. Additionally, in a pancreatic cancer model, endothelial monocyte activating polypeptide II (EMAP II) inhibits fibronectin-integrin angiogenesis signalling, which results in a significant reduction in tumour development and a decrease in microvessel density and proliferative activity [280].

On the other hand, more drastic strategies have been proposed with the aim of eliminating or diminishing CAFs population. In this regard, FAPα inhibitors have shown promising preclinical results in reducing tumour progression and promoting an antitumour immune response [281]. In xenograft models of pancreatic, head and neck, and lung cancer *in vivo*, FAP5-DM1, a maytansinoid-conjugated monoclonal antibody, was demonstrated to suppress and produce complete regression of tumour growth [282]. Oral DNA vaccines against FAPα that have demonstrated efficacy in mice models of breast or colon carcinomas are also being tested [161], along with other immunotoxins targeting FAPα such FAP-PE38 [283] (Figure 24). Furthermore, it has been demonstrated that increasing CD8+ T cell antitumour responses using a chimeric antigen receptor (CAR) T-cell specific for FAPα can suppress the growth of a variety of subcutaneously implanted tumours in mice [284]. However, in mouse models of transgenic PDAC (KPC mice) and transplantable colon carcinoma (C26 cells), depletion of FAPα+ cells using the Diphtheria Toxin Receptor (DTR) system caused severe systemic toxicity, including cachexia and anaemia. This likely reflects the significance of FAPα+ stromal cells in maintaining normal muscle mass and haematopoiesis [372]. Given that fibroblasts play a significant role in numerous essential physiologic processes in humans, it is crucial to stress that completely eliminating entire fibroblast populations is quite difficult in patients. Furthermore, biomarkers like αSMA or FAPα are not only expressed by CAFs, which makes it more difficult to use them realistically.

GPR77 is an additional cell surface marker that has been researched in pre-clinical models. It has been demonstrated that this protein, along with CD10, can be used to pinpoint the fibroblast subpopulation that results in chemoresistance. It has been demonstrated that blocking GPR77 with a neutralizing antibody lowers the number of CSCs in tumours and improves the response to chemotherapy [233] (Figure 24). Furthermore, since these fibroblasts were discovered in the tumour before treatment, their simultaneous targeting with chemotherapeutic regimens may be advantageous for the patient.

These findings suggest that rather than eliminating CAFs entirely, effective anti-CAFs medicines for cancer would likely need to re-educate them towards a NAF phenotype or decrease CAFs number. Another factor to consider is the heterogeneity of CAFs subpopulations and, hence, current studies should aim at differentiating CAFs subtypes in order to therapeutically affect pro-tumoural CAFs and support antitumour CAFs. Notably, Olive et al. investigated whether co-delivery of the synthetic experimental medication IPI-926 (Figure 24), which suppresses stromal development by blocking Smo, a crucial step in the Hh pathway, could enhance the efficacy of GEM drug administration [285]. Reduced αSMA+ fibroblasts and pancreatic stromal depletion in the treated tumours improved GEM administration and lengthened life [285]. However, in a colon cancer model, activating Hh signalling in fibroblasts significantly decreased tumour burden and disease progression [286]. As a result, Hh-signalling CAFs appear to serve as both tumour promoters and suppressors, and it is still uncertain whether particular determinants predict this behaviour in various cancer types [287].

Furthermore, an enzymatic degradation of the ECM to decompress the tumour and to normalize vasculature has been proposed to promote a more efficient chemotherapeutic delivery to cancer cells in solid tumours. In this respect, hyaluronic acid (HA) degradation or anti-angiogenic therapies are under consideration. Examples include, among others, the angiotensin inhibitor losartan [288] and enzymatic therapies such as PEGPH20, a pegylated hyaluronidase enzyme designed to degrade HA produced by CAFs in TME. However, although freeing the stroma from its restrictive properties could improve the delivery of therapeutic compounds, PEGPH20 administration could also alter antitumour

responses in TME, and while hyaluronidase treatment has shown promising results in animal models and early clinical trials [289,290], PEGHP20 in combination with nab-paclitaxel/GEM did not meet the criterion in phase III clinical trials in pancreatic cancer patients (Hakim et al., 2019).

MMPs derived from CAFs have also demonstrated their importance in inducing tumour growth, metastasis, and angiogenesis in animal model experiments [291]. Therefore, MMPs inhibitors could be another approach to therapeutically target MMPs and reduce enzymatic activity, but the reality is that MMPs inhibitors also do not meet phase III criterion at present [292,293].

CAFs reprogramming, as well as short-term inhibition of CAFs-predominant signalling pathways, have been recognized as better strategies than complete ablation or chronic inhibition. Indeed, several studies have found that strategies aimed at CAF ablation are ineffective [294,440].

Finally, the diverse therapeutic approaches emerging from basic and applied research are promising and encouraging. Unfortunately, because of the variability in the role of CAFs in each tumour, as well as their specific tissue source, they must be targeted at each tumour histotype. As a result, any future therapy targeting CAFs will be more effective after extensive research on their taxonomy and a precise and detailed understanding of the stroma–tumour relationship.

4. PDAC CHEMORESISTANCE

Chemoresistance in PDAC significantly complicates treatment and contributes to the poor prognosis of the disease. Understanding this mechanism is essential for developing effective therapeutic strategies. Targeting chemoresistant cells, inhibiting EMT, modulating the TME, and using combination therapies are critical approaches to overcoming chemoresistance and improving the prognosis for PDAC patients [464,465].

4.1 Chemoresistance acquisition

The development of a chemoresistant phenotype in PDAC cells is multifactorial, involving genetic mutations, epigenetic alterations, cellular adaptations, and interactions with the TME [130]:

1. Genetic Alterations

a. Oncogene Activation: Mutations in oncogenes such as KRAS lead to uncontrolled cell proliferation and survival. KRAS mutations are almost ubiquitous in PDAC and contribute to resistance by activating downstream signaling pathways that promote cell survival, proliferation, and resistance to apoptosis [466].

b. Tumour Suppressor Gene Inactivation: Mutations or deletions in tumour suppressor genes like TP53, CDKN2A, and SMAD4 impair the cell's ability to undergo apoptosis or repair DNA damage, contributing to drug resistance and genomic instability [467-469].

2. Epigenetic Modifications

a. DNA Methylation: Hypermethylation of promoter regions in tumour suppressor genes can silence their expression, aiding in the development of chemoresistance. This modification can lead to reduced sensitivity to chemotherapeutic agents [470].

b. Histone Modification: Post-translational modifications of histones, such as acetylation and methylation, can alter chromatin structure and gene expression, promoting resistance by activating survival pathways and suppressing pro-

apoptotic genes [471].

3. Cancer Stem Cells

a. Properties: CSCs have self-renewal capabilities, are quiescent, and possess high drug efflux capacity, making them inherently resistant to standard chemotherapy [472].

b. Survival Mechanisms: CSCs can activate DNA repair mechanisms, resist apoptosis, and maintain a low proliferative state, all of which contribute to their survival during chemotherapy [473].

4. Epithelial-to-Mesenchymal Transition (Figure 25)

a. EMT Process: EMT is a biological process where epithelial cells gain mesenchymal, fibroblast-like properties, giving them enhanced migratory and invasive abilities. This transition is associated with increased resistance to chemotherapy [474].

b. EMT Pathways: Activation of signaling pathways such as TGF-β, Wnt, and Notch during EMT contributes to chemoresistance by promoting cell survival, invasion, and dissemination [474].

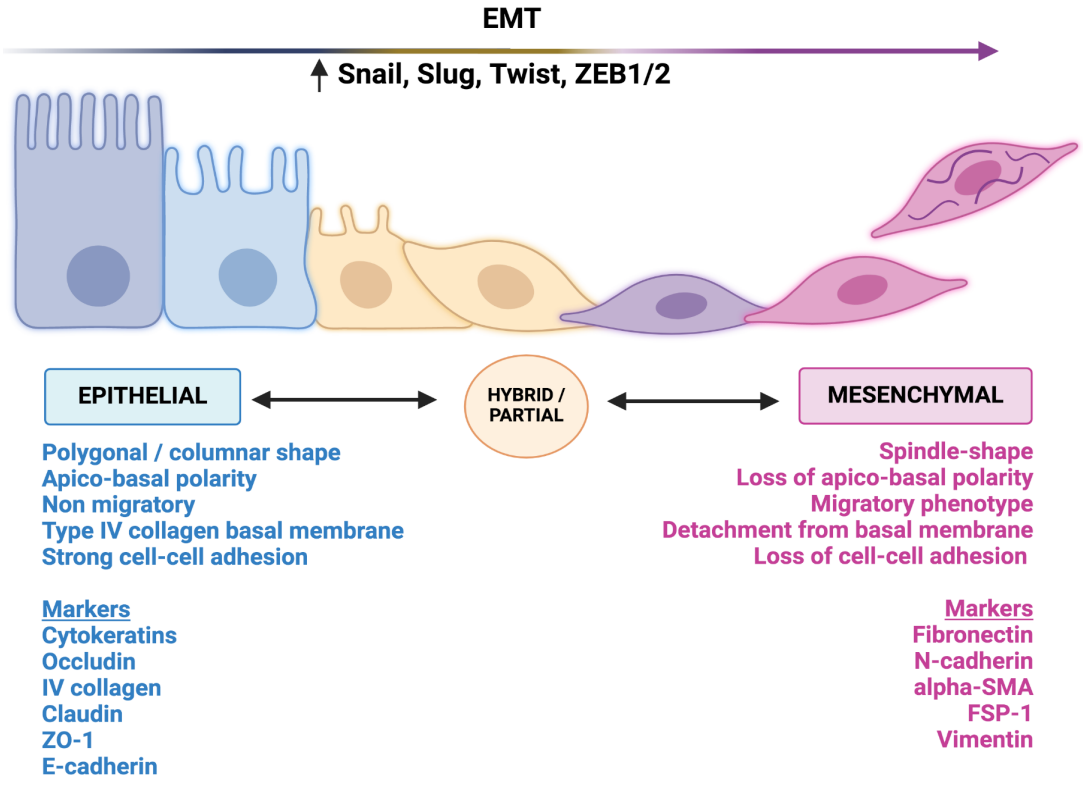


Figure 25. Epithelial to mesenchymal transition (EMT) process. EMT is a crucial process in cancer progression, wherein epithelial cells lose their cell polarity and adhesion properties, transforming into mesenchymal cells with enhanced migratory, apoptosis-resistance and invasive capabilities. This transition is pivotal for cancer metastasis, as it enables epithelial cancer cells to disseminate from the primary tumour site, invade surrounding tissues, and form secondary tumours at distant sites. The EMT process is marked by a series of well-defined stages, each characterized by specific biomarkers. In the initiation phase, epithelial markers such as E-cadherin, cytokeratins, and tight junction proteins are downregulated, signifying the loss of epithelial characteristics. Concurrently, mesenchymal markers like N-cadherin, vimentin, and fibronectin are upregulated, indicating the acquisition of a mesenchymal phenotype. Additionally, transcription factors such as Snail, Slug, Twist, and ZEB1/2 orchestrate the EMT process by regulating these markers. The intermediate stages of EMT exhibit a hybrid state where cells express both epithelial and mesenchymal markers, reflecting partial transitions that confer plasticity and adaptability to the cancer cells. Image created with Biorender.

5. Drug Efflux and Metabolism

a. Overexpression of Efflux Pumps: PDAC cells can upregulate ATP-binding cassette (ABC) transporters like P-glycoprotein, which actively pump chemotherapeutic drugs out of the cells, reducing their intracellular concentrations and effectiveness [475].

b. Altered Drug Metabolism: Changes in enzymes involved in drug metabolism can either enhance the inactivation of drugs or decrease their activation, thereby reducing the therapeutic efficacy [476].

6. Tumour Microenvironment

a. Cancer-Associated Fibroblasts: CAFs secrete growth factors, cytokines, and extracellular matrix components that create a protective niche for tumour cells. This microenvironment supports cancer cell survival and resistance to chemotherapy [127].

b. Immune Cells: TAMs and other immune cells in the TME can secrete factors that promote tumour survival and chemoresistance [477].

c. Desmoplastic Stroma: The dense stromal environment in PDAC acts as a physical barrier to drug delivery and provides biochemical support for tumour cells, further enhancing resistance [478].

7. Activation of Survival Pathways

a. PI3K/AKT/mTOR Pathway: This pathway is often upregulated in PDAC, promoting cell growth, survival, and metabolism. Its activation confers resistance to apoptosis and enhances cell survival under chemotherapy [479].

b. NF-κB or TGF-β Pathways: These pathways are frequently activated in PDAC and are involved in promoting inflammation, cell survival and resistance to apoptosis [480,481].

4.2 Chemoresistant cells properties

1. Self-renewal: Like normal stem cells, chemoresistant cells can divide and produce more cells, perpetuating the pool of chemoresistant cells within the tumour. Chemoresistant cells have the capability to divide in two specific manners. One method involves creating differentiated tumour cells, whereas the other entails division to yield identical cell types, referred to as self-renewal. This distinct characteristic of chemoresistant cells plays a crucial role in their capacity to maintain the tumour's existence and stimulate its proliferation (Figure 26) [482].

2. Plasticity: Chemoresistant cells (specifically CSCs) have the ability to differentiate into non-stem cancer cells that make up the bulk of the tumour, contributing to tumour heterogeneity. In normal tissues, progenitor cells may exhibit some plasticity and reversibility towards a stem cell-like state. In the tumour hierarchy of CSCs, research has shown that only long-lived stem cells, not differentiated cells, have the ability to generate tumours. The concept of CSC plasticity suggests that cancer cells can dynamically and reversibly switch between a CSC and a non-CSC/differentiated state, leading to the diverse makeup seen within tumours. Various factors, including genetic and epigenetic changes, as well as microenvironmental influences like inflammation, injury, and senescence, can impact the plasticity of cancer cells (Figures 26 and 27) [36,483].

3. Tumour initiation: Chemoresistant cells are thought to be responsible for initiating and maintaining tumour growth after a failed chemoresistant treatment, as they have the capacity to regenerate the entire tumour cell population (Figure 26) [130].

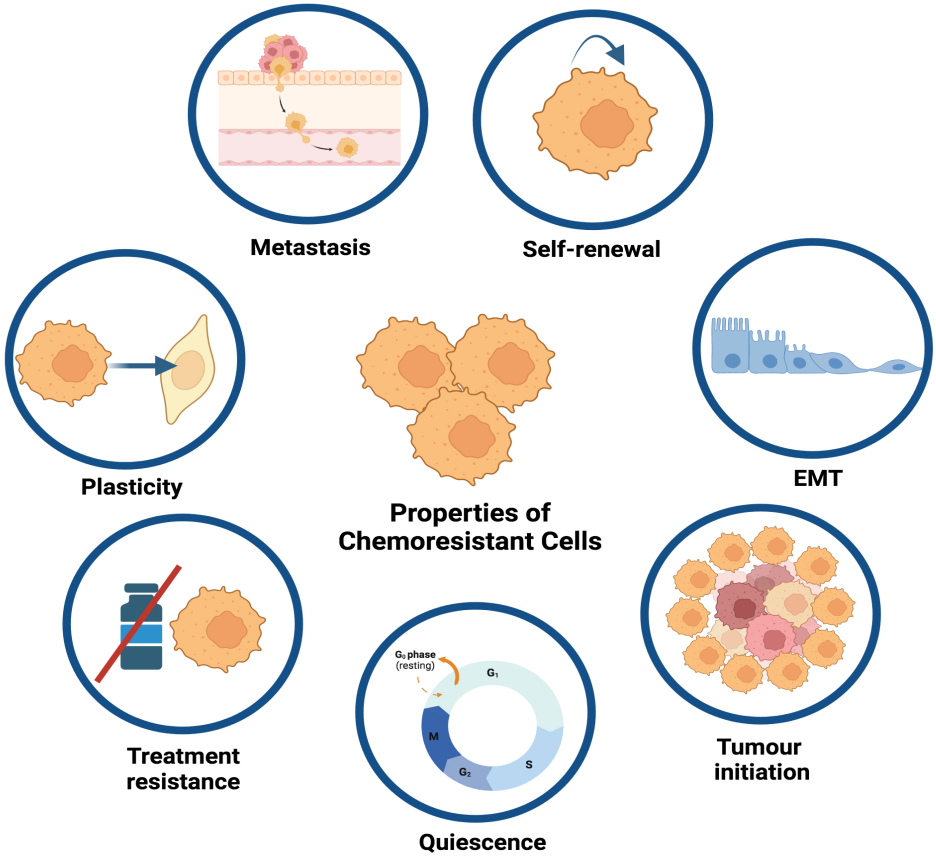


Figure 26. Main properties of chemoresistant cells in PDAC. Image created with Biorender.com.

4. Epithelial to Mesenchymal transition: This process involves the transformation of a cell from an epithelial phenotype to a mesenchymal phenotype. Studies have illustrated that cancer cells utilize the reactivation of EMT as a mechanism to disseminate and establish colonies in distant organs during tumour progression. The correlation between EMT and chemoresistant cells is apparent, as signaling pathways crucial for maintaining stemness, like Wnt, have been associated with specific aspects of EMT. Recent research indicates that Wnt signaling collaborates with TGFß to induce and uphold the mesenchymal state of cancer cells in an autocrine manner, thereby enhancing the metastatic potential of them (Figures 25 and 26) [484-489].

5. Quiescence: refers to a transient state of inactivity that cancer cells may undergo, leading to a halt in the cell cycle triggered by particular signaling pathways. This dormant state is reversible, enabling the cell to recommence

regular cell division. Nonetheless, while in quiescence, chemoresistant cells have the capacity to escape detection by the immune system and develop resistance to standard treatments, increasing the likelihood of tumour relapse (Figure 26) [490].

6. Treatment resistance: Chemoresistant cells are often more resistant to chemotherapy, radiation therapy, and other cancer treatments, leading to treatment failure and disease recurrence [465]. They employ active mechanisms to elude the effects of chemotherapy or radiotherapy. For example, they exhibit heightened expression of the enzyme aldehyde dehydrogenase (ALDH), a factor contributing to their resistance to cytotoxic therapies. Additionally, they have been linked to multidrug resistance (MDR) through the overproduction of ABC transporters. The relatively slow growth rate of them, stemming from their quiescent nature (mentioned before), renders them less vulnerable to chemotherapy (Figure 26) [491-497].

CSCs isolation

Chemoresistance research, evaluation, and treatment typically focus on CSCs, a type of chemoresistance cell. The prognosis and diagnosis of cancer patients can benefit greatly from the identification of specific CSCs markers (Figure 27). Although various proteins have been described as CSC markers, CD44, CD133, CD34, CD166, and CD19 are the most commonly accepted markers for solid tumours. However, the organ in which the tumour has developed also influences the overexpression of each of these markers. For instance, overexpression of CD133+, CD44+, and CD24+ has been observed in pancreatic cancer (Figure 27) [498], whereas CD44+ overexpression and CD24- downregulation have been observed in breast cancer [499]. Overexpression of CD133+ and CD20+ markers is common in melanoma [500]. The prognosis and diagnosis of patients who exhibit chemoresistance to oncological chemotherapy can benefit from an understanding of specific CSC markers.

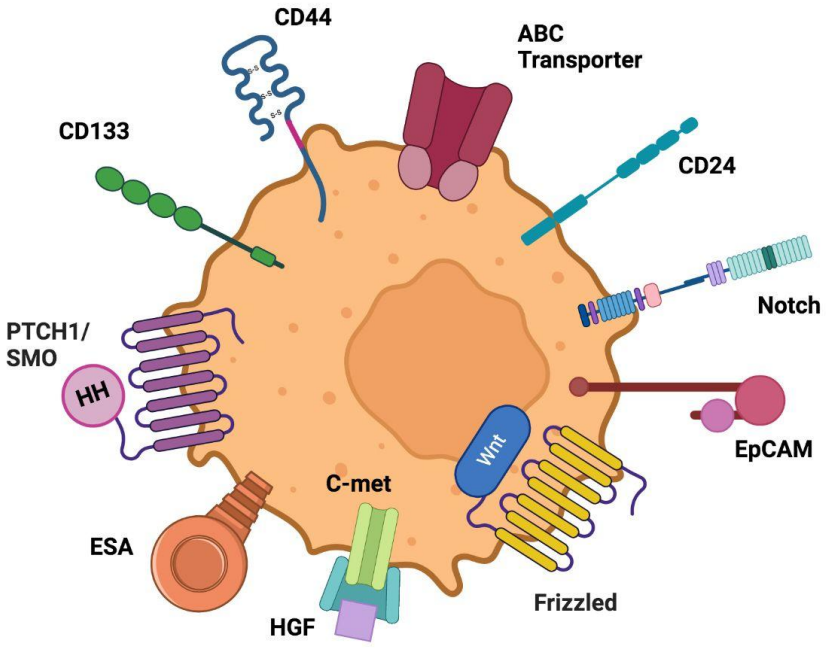


Figure 27. CSCs biomarkers in PDAC. Image created with Biorender.com.

4.3 Chemoresistant cells isolation

Currently, chemotherapy is still considered an irreplaceable front-line therapeutic strategy to combat almost all types of cancers, but multidrug resistance represents a common hurdle that deeply compromises clinical outcomes. Therefore, it is key to identify new resistance biomarkers and to analyze their predictive potential in order to guide treatment regimens [501]. Furthermore, a better understanding of the underlying drug tolerance mechanisms may be critical in achieving alternative therapies to improve oncological patients' prognosis. However, it is challenging since a high heterogeneity of chemoresistance markers expression profiles has been observed between different tumours and even between different cell lines of the same tumour type [502]).

In this regard, the development of drug-resistant cell lines is essential to study chemoresistance mechanisms. Figure 28 summarizes the procedure to obtain *in vitro* chemoresistant cell lines by mimicking the conditions experienced by cancer patients during chemotherapy. The use of increasing concentrations of chemotoxic agents represents a common experimental procedure with the aim of establishing stable, drug-tolerant tumour cell lines *in vitro* [503]. Although this method is very reliable and reproducible, some potential limitations should be

kept in mind: first, since only a small fraction of the bulk population of cells will show chemoresistance, it is recommended to start with a large population of cells (a minimum of 10^6 cells); second, it is very important to use the same freshly prepared drug stock for long-term use; third, it is crucial to start the protocol by determining the IC_{50} in each individual cell line.

In general, it is key to choose a cell line that is resistant to chemotherapy variations that may occur when using a new batch of cells (Figure 28). In general, it is crucial to with a relatively low referenced IC_{50} value for the drug of interest; finally, it is mandatory choose a cell line that is resistant to chemotherapy with a relatively low referenced IC_{50} to avoid any contamination, including *Mycoplasma*, since it has been proved that value for the drug of interest; finally, it is mandatory to avoid any contamination, including microbiota can increase chemoresistance [504].

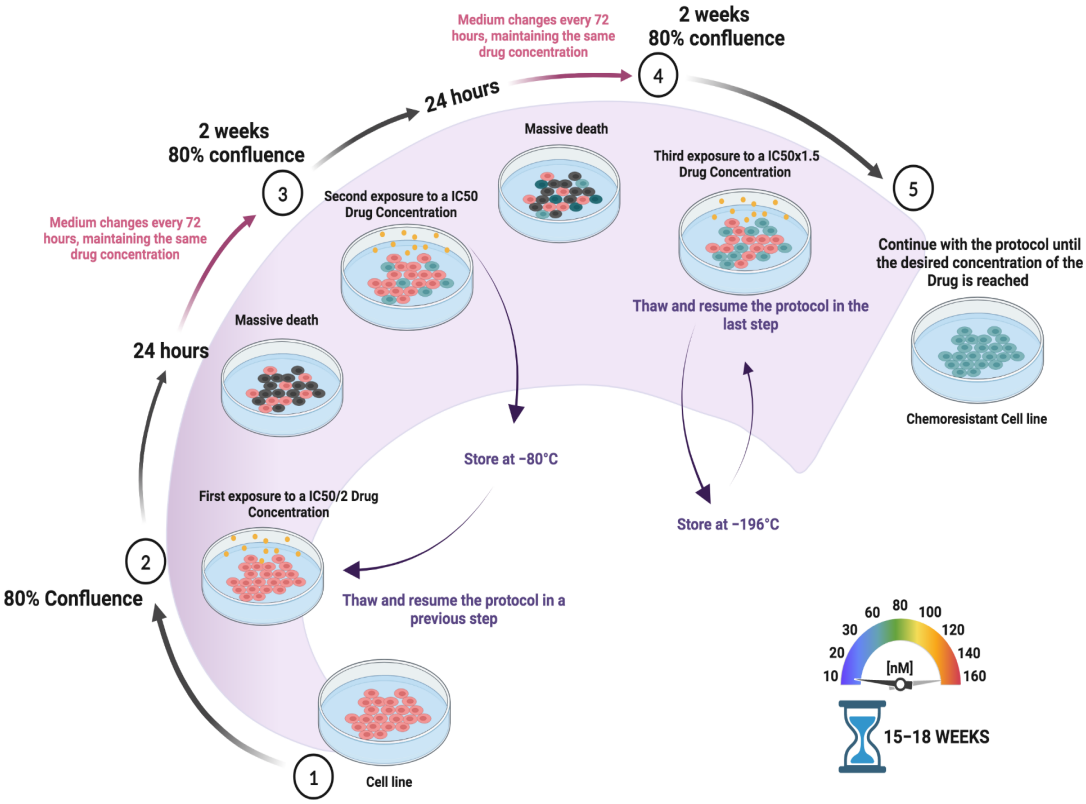


Figure 28. Schematic representation of the protocol used to develop drug-chemoresistant cell lines. To establish resistant tumour cell lines, increasing concentrations of the chemotherapeutic drug are periodically administered during a certain

period of time, between 15 and 18 weeks, depending on the cell type. Briefly, cells are incubated with increasing concentrations of the drug in the culture medium from $IC_{50}/2$ to the desired final concentration. The medium is changed every 72 h, while maintaining the drug concentration. Passage is done when cells acquire an 80% confluence (approx. every 2 weeks). It is recommended to freeze a cell stock in liquid nitrogen when acclimated to each drug concentration (the level of chemoresistance is not affected) as it is increased. (*Toledo, B et al., Int. J. Mol. Sci. 2023*).

Amongst the huge variety of anti-cancer agents, we have focused on DNA-damaging drugs (DDDs), Gemcitabine (GEM) (dFdC), 5-fluorouracil (5-FU), cisplatin, and doxorubicin as the main treatments for high-incidence tumours. Gemcitabine and 5-FU are "anti-metabolites", analogous to pyrimidine-based nucleotides, namely, cytosine and thymine/uracil, respectively, while cisplatin is an alkylating agent that generates DNA adducts and doxorubicin is a topoisomerase II inhibitor [505]. Tumour cells protect themselves from the aggression of cytotoxic agents through a series of mechanisms that range from preventing the entry of the drug to repairing the damage caused (Figure 29). Accordingly, in the face of aggression, tumour cells raise their first line of defense, preventing the entry of the drug and favoring its efflux through membrane pumps to compromise intracellular drug accumulation [506]. Further, DDDs are kept out of the nucleus where they perform their cytotoxic action by interfering mechanisms, for instance, lysosomal sequestration, as described for platinum-based treatment resistance [507]. In addition, the inactivation of chemotherapeutic agents, such as GEM or 5-FU, has also been shown to contribute to the clinical failure of chemotherapy [508] (Figure 29).

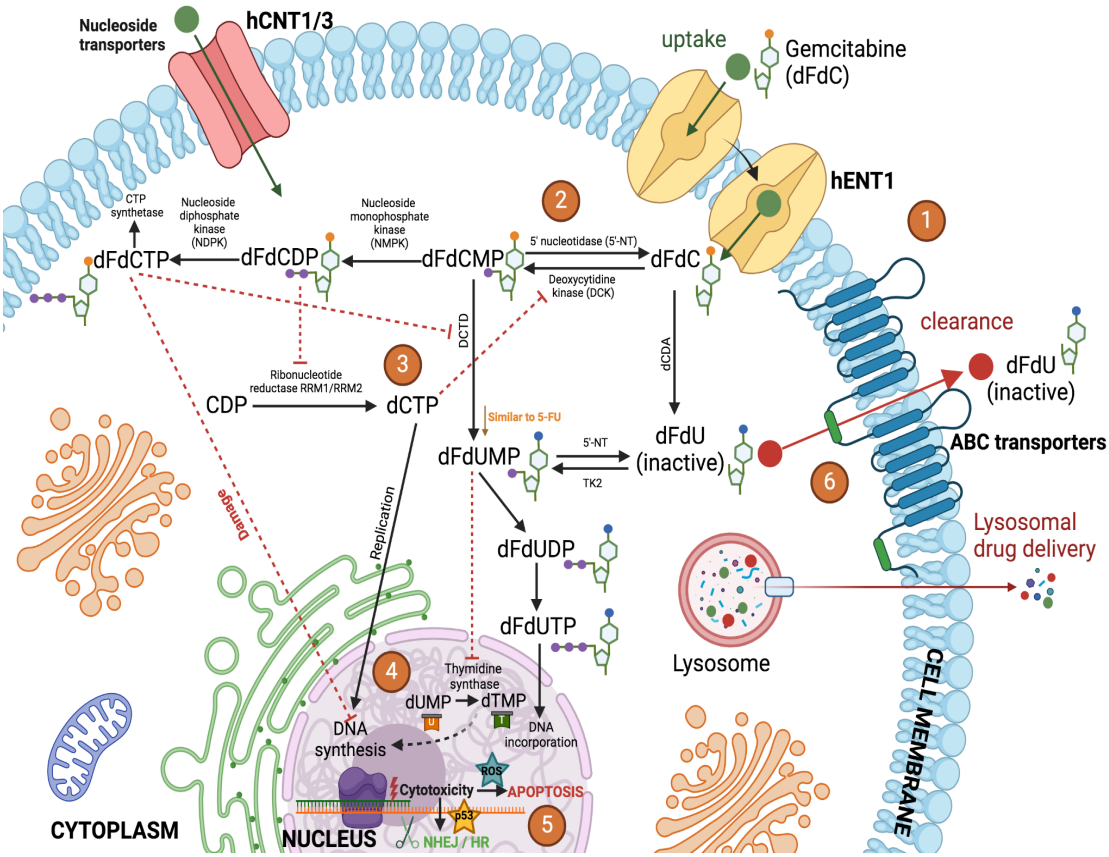


Figure 29. Mechanism of action of Gemcitabine via cellular metabolism. (1) Once administered, Gemcitabine (dFdC) is transported into cells by nucleoside transporters such as hENT1. (2) Gemcitabine is then phosphorylated into Gemcitabine monophosphate (dFdCMP) by deoxycytidine kinase (DCK), and dFdCMP is subsequently phosphorylated to Gemcitabine diphosphate (dFdCDP) and Gemcitabine triphosphate (dFdCTP) by nucleoside monophosphate kinase (NMPK) and nucleoside diphosphate kinase (NDPK). (3) dFdCDP potently inhibits ribonucleotide reductase (RR) RRM1/RRM2, resulting in a decrease in the competing deoxyribonucleotide pools necessary for DNA synthesis. RR transforms cytidine diphosphate (CDP) into deoxycytidine diphosphate (dCDP), and its inhibitory effect leads to the decreased concentration of competitive deoxycytidine triphosphate (dCTP) pool cells required for DNA synthesis, thus promoting the binding of dFdCTP to DNA. Gemcitabine exerts its cytotoxic effect mainly through the inhibition of DNA synthesis by being incorporated into the DNA strand as the active dFdCTP. dFdCTP suppresses activation of dFdCMP by inhibiting deoxycytidine monophosphate deaminase (DCTD). (4) dFdCTP in the nuclei inhibits Deoxyribonucleic Acid (DNA) and Ribonucleic Acid (RNA) synthesis. 2′,2′-difluorodeoxyuridine monophosphate (dFdUMP) inhibits thymidine synthase (TS), resulting in the depletion of the deoxythymidine monophosphate (dTMP) pool, but it can also be phosphorylated to 2′,2′-difluorodeoxyuridine triphosphate (dFdUTP) for its DNA incorporation or dephosphorylated to 2′,2′-difluorodeoxyuridine (dFdU) and then transported out of the cells. (5) Gemcitabine cytotoxicity activates DNA damage responses such as NHEJ, HR, and *p53*. Gemcitabine can also induce DNA damage by further promoting the accumulation of ROS, thereby enhancing apoptosis. On the other hand, *p53* mutations can

contribute to intracellular ROS accumulation and thus to genomic instability, which is characteristic of malignant cells. (6) The majority of dFdC is inactivated mainly by the deoxycytidine deaminase (dCDA)-mediated conversion to dFdU and then excreted through the ABC transporters. Deamination of dFdCMP to dFdUMP by DCTD and its subsequent dephosphorylation form, dFdU, is another inactivation pathway of dFdC. (*Toledo, B et al., Int. J. Mol. Sci. 2023*).

Moreover, the basal DNA repair mechanism of eukaryotic cells is enhanced in tumour cells after prolonged exposition to cytotoxic agents, implying the resistance to treatment in many cancer types including diffuse large B-cell lymphoma [509]. Finally, apoptosis evasion, mainly through *p53* mutations, has been described in a wide spectrum of cancers as a mechanism of cytotoxic drug tolerance [510].

While highly proliferative cancerous cells are more sensitive to DDDs, low-proliferative cancer cells tend to be more resistant. In this sense, cancer stem-like cells have been defined as low-proliferative/quiescent cells, with high invasive, metastatic, and chemoresistant potentials, which undergo the epithelial-to-mesenchymal transition (EMT) process [511]. Thus, the acquisition of a CSC-like phenotype upon exposure to chemotherapy can be considered as a pro-survival tumour cells' mechanism [512].

CSCs chemo and radio resistance are both supported by the intrinsic characteristics of their quiescent nature: (i) increased DNA repair mechanism, (ii) the ability to escape cell death [513], and (iii) the flexibility of their metabolism [514]. In addition, CSCs chemoresistance is also due to an increased drug efflux through ABC transporters [515]. In fact, a wide variety of studies have shown the strong relationship between stemness developed through upregulation of stemness markers, such as Nanog, OCT4, SOX2, and CD44, and drug resistance via increased drug efflux [516]. Similarly, it has been demonstrated that the dedifferentiation of melanoma cells toward a CSC-like status was accompanied by an increased xenobiotic efflux capacity and thus, an alteration of the therapeutic agent uptake [517]. Other studies have correlated EPCAMhigh/CD44+ colorectal CSCs with oxaliplatin tolerance through increased DNA repair capacity, altering

the cell cycle checkpoints or ROS scavenging [518]. Notably, the authors also mentioned some molecular pathways, i.e., Notch, WNT/β-Catenin, and the Janus kinase/signal transducer and activator transcription (JAK/STAT), which are significantly involved in the CSCs maintenance and thus, in the acquisition of a chemoresistant phenotype. In the same context, Matou-Nasri and colleagues identified the key role of the p38/MAPK and NFKβ signaling pathways in the survival of acute myeloid leukemia CSCs and their chemoresistance to 5-fluorouridine through the inhibition of the therapy-related induction of apoptosis [519]. Specifically, JAK/STAT3 pathway has been shown to play an important role in CSCs. This pathway is activated by IL-6 and the EGF, among other factors [520]. Activated JAK/STAT3 triggers JAK activation, which phosphorylates STAT3. P-STAT3 dimerizes and enters the nucleus inducing the expression of genes related to cancer progression and EMT and stemness, resulting in an increase in chemoresistance [520,521]. In addition, the JAK/STAT3 pathway in breast cancer has been shown to increase chemoresistance via the regulation of the lipid metabolism-activating fatty acid oxidation [522]. Other studies have shown that an overexpression of STAT3 in colorectal CSCs increased chemoresistance while STAT3 degradation enhanced cell chemosensitivity and decreased stem cell markers expression [523].

Here, we compare tumour cells' chemoresistance mechanisms against common treatments in high-incidence tumours, introducing novel approaches to reduce this cell population and therefore, chemoresistance.

4.4 Boarding and landing gates

To exert their anti-cancer effects, DDDs have to reach their molecular targets inside the nucleus, mainly DNA and/or DNA synthesis-related enzymes. Undoubtedly, the trafficking across the plasma membrane and the drug influx/efflux ratio determine the cytotoxic agent intracellular concentration. Thus, dysfunctions in drug uptake pumps lead to an increase in chemoresistance. For instance, Zeng and co-workers (2021) described the volume-regulated anion channel (VRAC) as a mediator of cisplatin uptake. Moreover, it has been documented that the cisplatin influx promoted by the organic cation transporter 1

(OCT1) in esophageal squamous cell carcinoma shows a significant positive correlation between a low expression of OCT1 and a reduced sensitivity to cisplatin, along with a poor prognosis [524] (Figure 30).

In addition, the ion transporters OCT1, OCT2, OCT3, and OATP1A2 (organic anionic transporter 1A2) have been shown to promote the cellular uptake of doxorubicin under physiological conditions [525]. Furthermore, Wang and colleagues reported the participation of the OAT2 transporter in 5-FU uptake in hepatocellular carcinoma cells, which explained the correlation between OAT2 downregulation and acquired chemoresistance [526] (Figure 30).

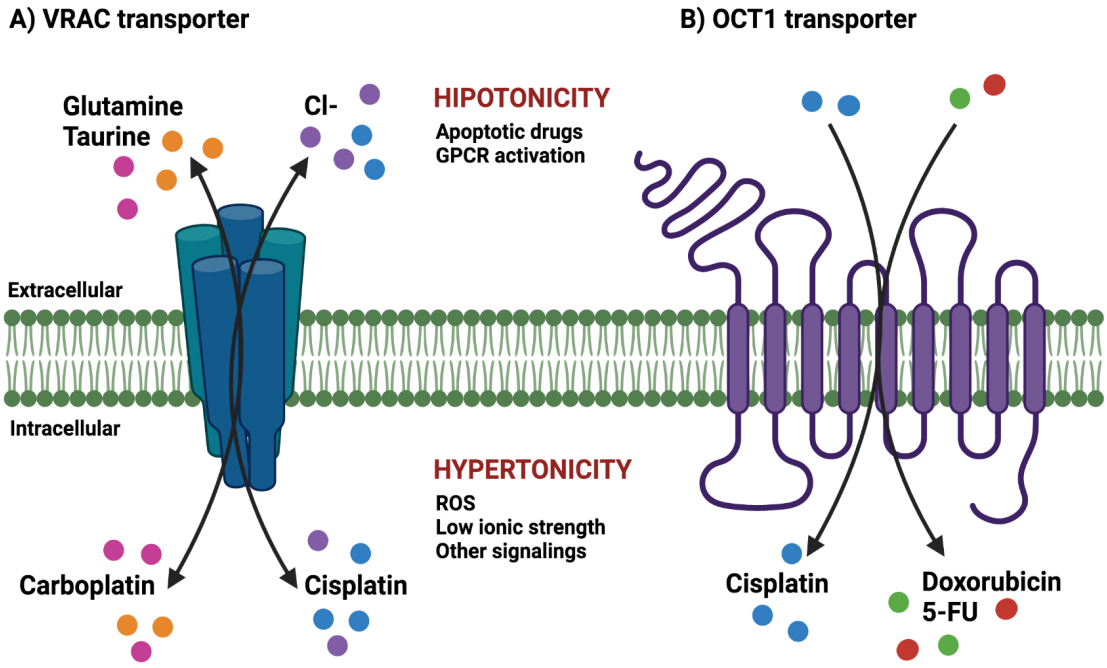


Figure 30. VRAC and OCT1 transporters mode of action. Image created with Biorender.com.

On the other hand, the increased efflux of therapeutic agents has been widely described as a central mechanism leading to multidrug resistance, with special emphasis on the upregulation of the ABC superfamily with up to 48 different subtypes [527]. The huge heterogeneity among efflux pumps has clinical relevance since it dictates the substrate-binding affinity. In fact, Mora Lagares and colleagues (2021) [528] have shown the implication of ABC transporters transmembrane domains, with a large proportion of non-conserved residues, in the

establishment of the substrate-binding pocket, suggesting different substrate specificities that are characteristic of each ABC member. According to this idea, a specific cytotoxic drug may be recognized and expelled out of the tumour cell by only certain ABC pumps. For instance, GEM is ejected from the cells by overexpressed ABCC4 [529] or ABCC5 [530] transporters, leading to chemoresistance in pancreatic cancer treatments (Figure 29). It has also been demonstrated that the downregulation of hENT1, a nucleotide transporter associated with GEM uptake, may also contribute to the acquisition of chemoresistance by pancreatic cancer cells [531] (Figure 29). In regard to cisplatin, some studies identified ABCB1 (MDR1), ABCG2 (BCRp), ABCC1, and ABCC4 as mediators of drug extrusion, thereby contributing to cisplatin tolerance [532]. Similarly, ABCA5, ABCC2, and ABCB5 [533] have also been reported to exert the same effect.

Additionally, 5-FU has been documented in colorectal cancer cells to be expelled from the intracellular space by ABCC11, but surprisingly, not by the widely recognized ABCB1 drug efflux pump [534]. In a recent study using non-small lung carcinoma cells, it was reported that 5-FU can also be a substrate of ABCA5 and ABCC1 transporters [533]. Moreover, in a hepatocellular carcinoma study, the overexpression of ABCB1, ABCB5, ABCC1, or ABCG2 had the potential to induce resistance to doxorubicin, and this fact was prevalent in hepatic CSCs rather than in non-stem cancerous cells [526] .

Furthermore, the subcellular distribution of chemotherapeutic agents is key to supporting their cytotoxic effect. In this regard, it has been shown that the major vault protein (MVP) can promote doxorubicin transference from the nucleus to the cytoplasm and then to the extracellular space, inducing drug tolerance [535,536]. Reinforcing the importance of subcellular drug location, the copper transporter ATP7B has been shown to induce platinum-derived compounds lysosomal sequestration and their subsequent exocytosis, thereby promoting cisplatin chemoresistance in ovarian cancer cells [507].

Collectively, these data reflect the importance of the bidirectional trafficking across the plasma membrane, which is mediated by multiple transporters. Nevertheless, it is relevant to note that different tissues, tumour types, and even distinct cell lines of the same tumour may exhibit specific expression patterns of

such transporters [525].

4.5 Drug Metabolism

Antimetabolites cytotoxic drugs exert their effect by incorporation into RNA and DNA molecules producing fatal errors, which lead to cell death. GEM and 5-FU are representative examples of antimetabolites that are analogous to pyrimidine-based nucleotides, namely cytosine and uracil/thymine, respectively [537] (Figure 29). Thus, the pyrimidine metabolic activity of target tumour cells may significantly influence drug availability and cytotoxic efficacy. Of note, dihydropyrimidine dehydrogenase (DPD) has been shown to be an important enzyme in the pyrimidine catabolic route which catalyzes a two-electron reduction in pyrimidine bases [538]. In fact, DPD deficiency in bladder cancer cells has been associated with GEM sensitivity, whereas the overexpression of *DPYD* (DPD-coding gene) was linked to GEM resistance via its catalytic inactivation [508] (Figure 29). Similarly, *DPD* upregulation in colorectal cancer has also been shown to promote 5-FU tolerance through intracellular 5-FU transformation into inactive metabolites [539].

Moreover, cytidine-metabolizing enzymes, such as cytidine deaminases (CDA), have been shown to interfere with Gemcitabine-based therapies. A representative example is CDA, whose upregulation has been related to GEM resistance in pancreatic cancer via metabolic neutralization [501]. Interestingly, other cytidine deaminases, such as the AID and APOBEC family, have also been related to key roles in cancer biology [540]. Hence, it seems reasonable to propose a relevant crosslink between GEM efficiency and these enzymes. In the case of CDA, its GEM-neutralizing capacity has also been detected outside tumour cells, for instance in systemic blood circulation [512]. Parallelly, the secretion of pyrimidine nucleosides, such as deoxypyrimidine dehydrogenase (*DPD*), by TAMs in pancreatic ductal adenocarcinoma has been observed. Deoxypyrimidine dehydrogenase promotes Gemcitabine resistance through the competitive attenuation of its uptake by tumour cells and by increasing its metabolic neutralization [541]. Furthermore, hypoxic TAMs have been proven to be the main contributors to *DPD* expression in colorectal cancer, implying the relevance

of surrounding stromal cells in promoting resistance to chemotherapeutic antimetabolites [542]. Importantly, Malier and colleagues also noted that mice-derived macrophages did not express significant levels of *DPD*, contrary to human TAMs.

Once inside the cells, GEM activated metabolites: difluorodeoxycytidine monophosphate (DFDCMP), the diphosphate form (DFDCDP), and subsequently, the triphosphate compound (DFDCTP) are phosphorylated by the enzyme deoxycytidine kinase (dCK) before achieving therapeutic effectiveness [543,544] (Figure 29). Consequently, it has been revealed that an increased expression of dCK in high-grade meningioma cells determined the intracellular activation of GEM leading to a significant increase in drug sensitivity [545]. Along with its misincorporation into synthesizing DNA strands (DFDCTP metabolite), GEM (DFDCDP form) can also hamper the synthesis and repair of DNA via the inhibition of RR. Indeed, the high expression of RR and the activation of the RR large subunit (RRM1) were associated with poorer patient outcomes and GEM resistance in pancreatic cancer [543] (Figure 29).

On the other hand, 5-FU has to be converted to the active metabolite: fluorodeoxyuri- dine monophosphate (FDUMP) by mediation of enzymes such as thymidine kinase 1 (TK1) in order to exert its cytotoxic effect [546]. 5-FU causes DNA damage by being mistakenly incorporated into DNA- and RNA-based molecules, and by the induction of TS inhibition, thus, hampering de novo thymidine synthesis. Moreover, the upregulation of TS has been shown to attenuate 5-FU cytotoxicity and to lead to drug resistance in a cholangiocarcinoma study [547].

Regarding CSCs, many studies have reported that the acquisition of a stem-like phenotype by cancerous cells may be accompanied by a deep metabolic reprogramming. To note, glycolysis is commonly enhanced in CSCs, as has been described in the case of glioblastoma CSCs [548]. The authors specified that this phenomenon along with the acquisition of a stem-like phenotype were induced by the long non-coding RNA HULC. In a similar way, another study has exposed that biomechanical forces derived from the extracellular matrix contributed to the dedifferentiation of colorectal cancer cells toward CSCs through the enhancement of glycolysis and HIF1 expression [549]. However, it has been highlighted that the

metabolic reprogramming of CSCs may be much more flexible/reversible according to their phenotypic plasticity. Indeed, CSCs are able to transit between a quiescent, low-metabolic phenotype with little energy needs and a proliferative behavior with high energy costs [550]. Interestingly, it has been revealed that the acquisition of cisplatin tolerance by non-small cell lung carcinoma cells was associated with their glycolysis/oxidative phosphorylation metabolic flexibility and with an increased mitochondrial function [551]. Moreover, the dedifferentiation of cancerous cells toward CSCs can also be accompanied by other metabolic alterations. In regard to this, the overexpression of OCT4 has been related to the CSC-like phenotype along with the enhancement of both glycolysis and the oxidative pentose phosphate pathway [552]. These events may be of special relevance considering the well-known role of the pentose phosphate pathway in the defense against reactive oxygen species (ROS), which can also be correlated with the acquisition of chemoresistance (explained in the following section). According to the many metabolic changes surrounding the CSC-like phenotype, it seems reasonable to hypothesize that nucleotide metabolism could also be altered in CSCs. In agreement, it has been confirmed that the TS enzyme may be essential for the maintenance of the CSC-like status of triple-negative breast cancer cells, which has also remarkably been associated with an increased activity of DPD enzyme [553]. In addition, others observed that the overexpression of RRM2 (related to nucleotide synthesis) was closely correlated with the stemness of squamous cells of oral carcinoma [554]. These facts may be relevant considering the participation of DPD, RRM2, and TS enzymes in the drug tolerance of cancerous cells, as mentioned above.

4.6 DNA Damage Repair and Cell Proliferation

Upon reaching the nucleus, DDDs cause a variety of DNA lesions. Depending on the level of DNA damage, several types of DNA-damage responses (DDRs) are triggered and these can be classified into two main groups (Figure 31): (i) pro-survival responses (i.e., DNA repair and/or cell cycle arrest/premature cell senescence) and (ii) cell death (i.e., pro-apoptotic signaling). Specifically, DDRs are characterized by complex and multifactorial phosphorylation cascades

comprising a wide variety of molecules such as DNA sensors (i.e., MRN complex, ATM, or ATR), DDR transducers such as checkpoint kinases (for example CHK1 or CHK2), and mediators/effectors such as *p53* and executioners (i.e., DNA repair-, cell cycle arrest-, or apoptosis-related factors) [555]. Considering that the final goal of cancerous cells relies on surviving at all costs, the reported association between the optimization of DDR through *ATM/ATR/p53* axis upregulation and the tolerance to chemotherapeutic drugs such as GEM in pancreatic cancer is not surprising at all [556]. Relevantly, some degree of specificity in the induction of certain DDR pathways according to the type of DNA damage has been shown. For instance, the activation of ATM/CHK2 signaling has been mainly correlated to double-stranded DNA breaks while the enhancement of the ATR/CHK1 axis has been mainly linked to single-stranded DNA breaks [557,558]. Similarly, different DNA repair mechanisms have been described according to the type of DNA damage. For instance, in the repair of double-strand DNA breaks, the high-fidelity homologous recombination (HR) or the error-prone non-homologous end-joining (NHEJ) are involved [559] (Figure 29). On the other hand, nucleotide excision repair (NER) is induced by DNA adducts [560] and single-stranded DNA breaks are repaired by base-excision repair (BER) [561]. Generally, DDDs can trigger several DNA repair responses, as has been revealed in the case of Gemcitabine-based treatment, which can induce either NHEJ [562] or HR [563].

Nonetheless, some trends with certain chemotherapeutic treatments have been identified, such as NER activation to overcome DNA adducts caused by alkylating platinum compounds [564].

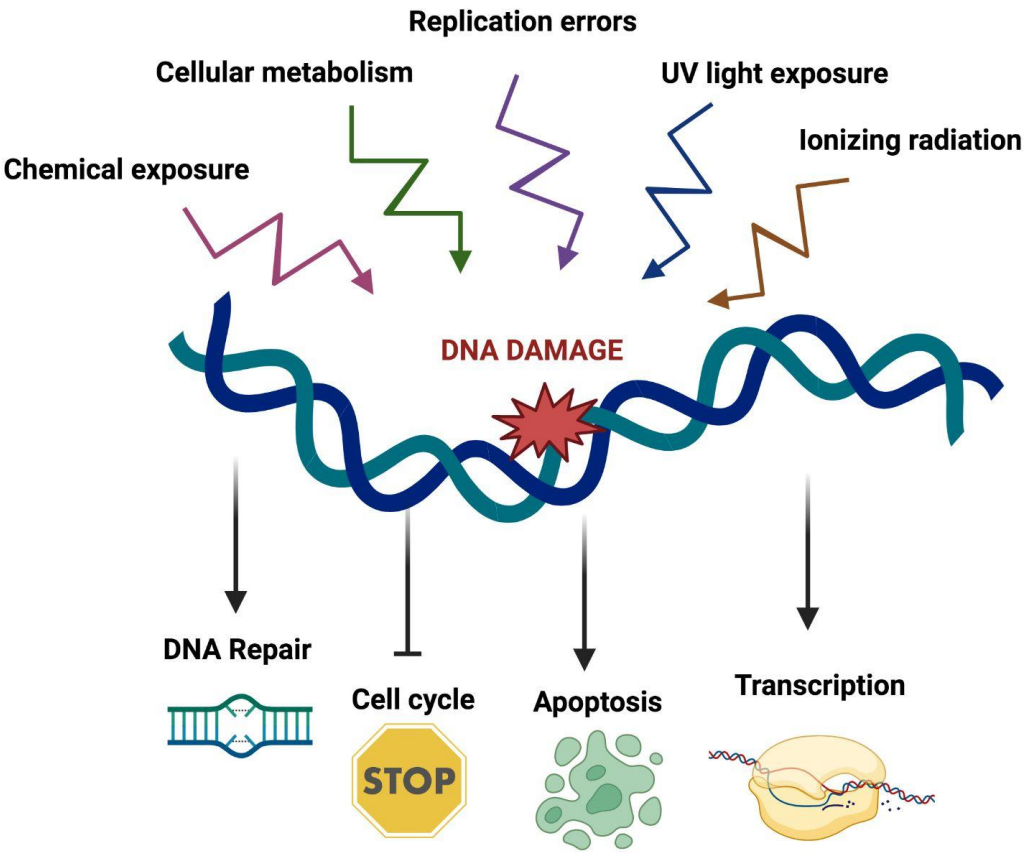


Figure 31. Principal DNA-damage responses or DDRs. Image created with Biorender.com.

However, HR repair machinery has also been shown to be involved in the resolution of DNA damage induced by a wide spectrum of cytotoxic agents, including GEM, 5-FU, cisplatin, and doxorubicin [505]. The development of suitable experimental protocols to simultaneously test a broad range of DNA repair responses may be of great interest. In regard to this, Ge and co-workers pointed out that the transcriptional profiling of DNA repair-associated genes does not always correlate with the real DNA repair capacity. Therefore, they developed a "cometchip platform" based on the previously established comet assay that is suitable for the parallel assessment of multiple repair pathways such as BER, NER, and NHEJ [561]. Furthermore, it may be relevant to note that DNA damage and, thus, the DNA repair capacity can also be enhanced in some cellular contexts that are different from the exposure to therapeutic compounds. For instance, a high-proliferative phenotype may elicit excessive proliferative stress, which has

been associated with the overactivation of DNA repair signaling [565]. On the other hand, a close correlation between chronic inflammation and the induction of oxidative stress and DNA damage has been established [566]. Considering the uncontrolled proliferation of tumour cells and the documented pro-inflammatory microenvironment surrounding solid tumours since the earliest stages of their growth [567], a remarkable DNA repair capacity even in the absence of antitumour drugs seems like a reasonable suggestion. Importantly, the reinforcement of DDRs by a pro-inflammatory microenvironment may be a significant difference between *in vitro* and *in vivo* chemoresistance-based studies.

Parallelly, cell cycle arrest promoted by DNA damage is a well-known cellular response in physiological conditions [568]. In fact, DNA-induced cell cycle arrest after chemotherapy might be a pro-survival cellular response, which may lead to chemoresistance by providing sufficient time to complete DNA repair. However, some studies have interpreted the inhibition of DNA damage-mediated cell cycle arrest by a cancerous cell as a drug-resistant mechanism [569]. With regard to this controversy, the authors also noted the importance of carefully considering the balance between the DNA repair potential and the cell cycle arrest/growth inhibition in chemoresistance-based research. Moreover, distinguishing between punctual/short-term cell cycle arrest and permanent/long-term cell cycle blockade (also known as “cellular senescence”), both promoted by DNA damage, may be relevant to clarify such a dichotomy. In this sense, the induction of cellular senescence in several types of cancers upon treatment with doxorubicin and etoposide has been shown [570]. On one hand, the induction of tumour senescence as an anti-cancer strategy to inhibit tumour proliferation and growth has been considered [571]. Nevertheless, the plasticity and reversibility of the senescent-like phenotype leading to a more aggressive and invasive behavior and even the promotion of disease relapse and metastasis have also been pointed out [570]. Interestingly, these are well-documented negative events that are closely related to the CSC population, the EMT process, and chemoresistance [572]. In agreement, the promotion of cellular senescence and a stem-like phenotype after sustained and long-term DNA-damaging conditions, i.e., radiotherapy, has been identified [573]. Additionally, it has been indicated that DDDs can also be associated with the EMT process and tumourigenesis [574].

Therefore, cellular senescence and the acquisition of a CSC-like phenotype seem to support chemotherapy through pro-survival tumour cell behavior [512].

4.7 p53 and Reactive Oxygen Species (ROS) Levels: Role in chemoresistance

Nuclear DNA damage triggers cell death mainly by the activation of *p53*, also known as "the guardian of the genome" [575]. In fact, *p53* phosphorylation leads to the phosphorylation cascade toward apoptosis-based cell death [555] (Figure 32). In healthy cells, the expression of *p53* is usually low, with a half-life of about 20 min [576]. However, after cellular stress, the *p53* half-life extends to several hours, promoting different responses such as cell-cycle arrest, senescence, apoptosis, regulation of cellular energy metabolism, antioxidant defense, DNA repair, and immune system regulation [577]. Accordingly, the mutant *p53* protein has been shown to interfere with a variety of processes such as the regulation of cell survival, DNA damage repair, and drug resistance [578]. *p53* versatility has been shown to be driven by different levels of phosphorylation and it has been shown that *p53* can act as a "cell cycle arrestor" permitting DNA repair and cell survival when it is phosphorylated on Serine15 (Ser15) and/or Serine20 (Ser20). Nevertheless, additional phosphorylation on Ser46 under severe DNA damage conditions may change its role to "killer", leading to apoptosis [555]. In summary, as a cellular "gatekeeper", *p53* recognizes whether DNA damage is irrevocable and acts accordingly by inducing apoptosis [577,579]. Therefore, *p53* plays a dual role by activating either a mechanism that leads to apoptosis or one that enhances DNA repair and cell survival (Figure 32).

The mutated *p53* gene (TP53) has been detected in approximately 50% of all human tumours, such as breast, brain, lung, or colorectal carcinomas, among others [580], which may deeply condition the prognosis and clinical outcomes of oncological patients. Particularly, germline *p53* mutants are mainly associated with Li–Fraumeni Syndrome, which is characterized by a high risk of oncogenesis [580,581]. Some mutations can make the *p53* protein unable to recognize and interact with *p53*-binding sites located in its target genes. In accordance with this, Donzelli and co-workers (2012) showed that mutant *p53* conferred tolerance to

doxorubicin, cisplatin and 5-FU by procaspase-3 downregulation, and apoptosis inhibition [582]. Furthermore, *p53* mutations promote the acquisition of new and distinct oncogenic properties by interacting with different genes, which is generally referred to as "gain of function" alterations (GOF) [583]. Specifically, mutant *p53* can reach the promoter of target genes through the interaction with several sequence-specific transcription factors including NF-Y, E2F1, NF-kB, and the Vitamin D receptor (VDR) [584]. GOF mutations may promote tumour progression and possibly lead to resistance to a variety of anticancer drugs. For instance, studies have shown that mutant *p53* may be associated with chemoresistance in an independent way of its pro-apoptotic role, increasing the expression of the MDR1 efflux pump (ABCB1) [585]. Additionally, a relationship between mutant *p53* and EFNB2 (ephrin-B2), a receptor tyrosine kinase involved in cell invasion, migration, angiogenesis, and tumour resistance, has been established [586]. Moreover, it was reported that mutant *p53* increases EFNB2 expression in colorectal carcinoma cell lines upon treatment with 5-FU [587].

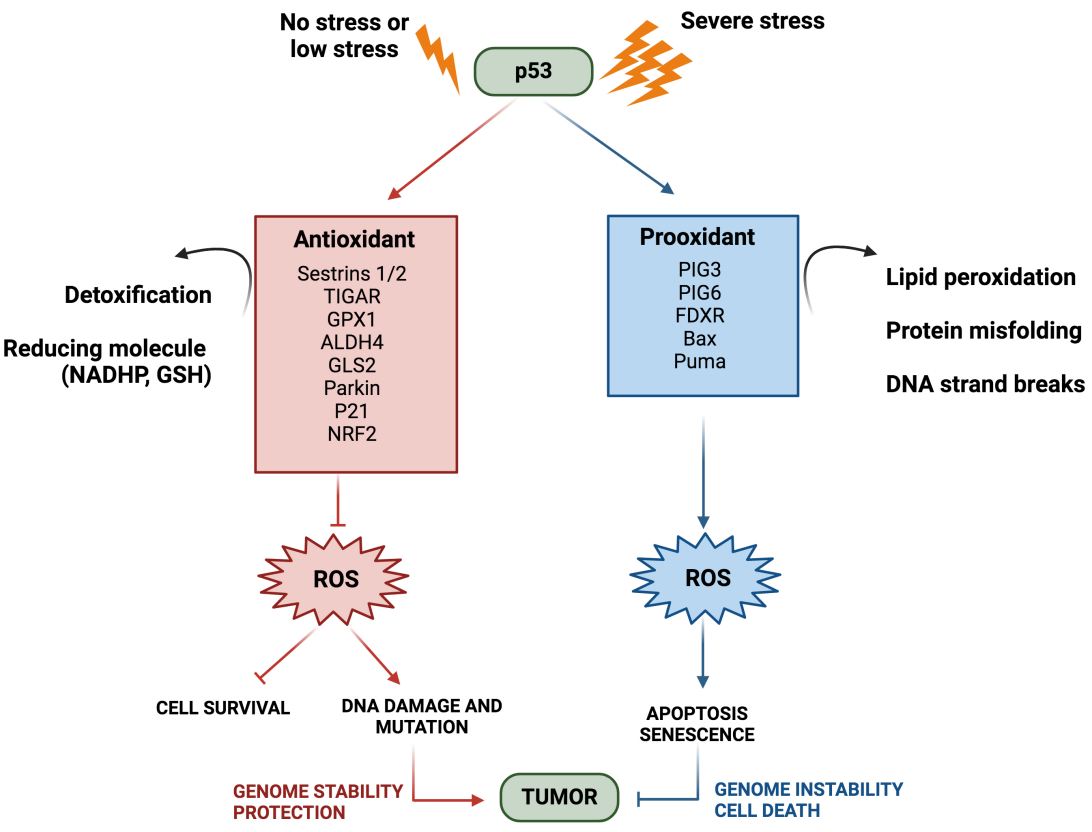


Figure 32. Role of p23 and ROS in tumour development. Image created with Biorender.com.

The authors also showed that EFNB2 induced 5-FU resistance through the upregulation of the ABCG2 drug efflux transporter, mediated by the activation of the c-Jun/JNK signaling pathway. Further, it has been observed that *p53* knockdown reduced cell proliferation and resistance to cisplatin, adriamycin, and etoposide in several cancer cell lines [588]. On the contrary, an overexpression of *p53* has been associated with Gemcitabine tolerance in pancreatic cancer [589]. Thus, *p53* phosphorylation status and not only its expression patterns should be taken into account to explain observed controversies regarding *p53* expression and chemoresistance.

Apart from the high rate of *TP53* mutations, the remaining 50% of cancers may exhibit *p53* dysregulation or alterations in *p53*-related pathways [590]. To note, it has recently been discovered that the overexpression of CD147 may promote the acquisition of GEM resistance by pancreatic tumour cells by interfering with the activation of *p53* upon *ATM/ATR/p53* complex formation, thus preventing cell apoptosis (REF: CD147). Similarly, the overexpression of MSIM2 may cause resistance to GEM and cisplatin in pancreatic cancer by negatively regulating *p53* (REF: MSIM2). Notably, *p53* has been documented to control its protein levels through a negative feedback loop involving MDM2 [591]. *P53* can act as a transcription factor activating MDM2, which can then enhance *p53* protein ubiquitination and degradation [592]. Upon cellular stress, mediated for example by DNA damage, MDM2 activity decreases, leading to an increase in *p53* levels through its protein stabilization (non-degradation) [593]. As a consequence, a re-increase in MDM2 is observed, which in turn, may promote *p53* protein degradation. In physiological conditions, nuclear concentrations of both *p53* and MDM2 may be mutually maintained at low levels [591]. Nonetheless, the dysregulation of *MDM2/p53* balance could be associated with severe disorders such as tumourigenesis and poor clinical prognosis [593], hence, interfering with such a feedback control may represent a promising therapeutic

strategy in oncology. In addition, *p53* dysregulation could also lead to deficient immune system responses [594], which may play a key role in cancer immune evasion. For instance, Major Histocompatibility Complex Class I (MHC-I) was described to be positively regulated by *p53*, promoting damaged cell recognition by T cells.

Parallelly, ROS levels have been reported to be increased in cancerous cells due to both environmental (smoking or UV) and internal mechanisms (ROS are considered as an inevitable by-product of cellular metabolism). Therefore, the increased metabolism of high-proliferative cancerous cells may result in elevated ROS production [595]. Furthermore, ROS generation can be related to well-known cancer features, for instance, the overactivation of oncogenes such as C-myc, Kras, or BRCA1, or the alteration of integrins during metastasis. In cancer cells, ROS could play a dual role: under basal conditions, ROS play a critical role in maintaining cellular proliferation and homeostasis [596], whereas high ROS concentrations may inhibit cell cycle progression and induce apoptosis [597] (Figure 32). Relevantly, many anticancer compounds, namely doxorubicin, cisplatin, or 5-FU, can induce DNA damage by further promoting the accumulation of ROS and thereby enhancing apoptosis in tumour cells [558]. Importantly, this study also indicated that ROS, like H2O2, can trigger the activation/phosphorylation of *p53* through a DDR-independent manner. Strikingly, mutant *p53* can also induce ROS accumulation due to the loss of its antioxidant potential, i.e., by causing antioxidant enzyme imbalance [598]. Hence, *p53* mutations can contribute, at least in part, to the intracellular ROS accumulation and thus, to the genomic instability characteristics of malignant cells [599] (Figure 32). As it has been revealed that cancer cells could enhance their antioxidant mechanisms to counterbalance excessive oxidative stress, this could be considered as chemoresistant behavior. For instance, the overexpression of antioxidant enzyme Isocitrate Dehydrogenase 1 (IDH1) reduced ROS levels and promoted GEM resistance in pancreatic ductal adenocarcinoma [600]. Similarly, cisplatin can interact with endogenous nucleophiles such as reduced glutathione (GSH), which makes the redox balance prone to oxidative stress [601]. Thus, the observed susceptibility of cisplatin-based treatments to cytoprotective antioxidant

molecules [602,603] may be determined in a double way: ROS direct neutralization and cisplatin activity mitigation by the overexpression of GSH. Several resistant routes can be triggered in response to elevated ROS levels, involving endoplasmic reticulum (ER) stress, autophagy, cell cycle perturbations, and the acquisition of a CSC-like phenotype through the EMT process [604]. Supporting the malignant cells' adaptation to high ROS accumulation, recent evidence suggests that prolonged chemotherapy can reduce the overall ROS concentration, thus leading to drug tolerance [605]. As an additional example, Gemcitabine-induced oxidative stress accompanied by an increase in antioxidant genes, i.e., *NRF2*, *SOD1*, *SOD2*, *CAT*, or *GPX1*, has been demonstrated [606]. Additionally, oxygen availability may significantly determine anticancer drug efficiency. While hypoxic conditions may limit ROS production, the recovery of normal oxygen levels can elevate the cytotoxicity of DDDs, such as doxorubicin, by increasing the generation of ROS and ultimately, alleviating the chemoresistance [607]. Therefore, the development of hypoxic environments in almost all solid malignancies may represent an important difference between *in vitro* and *in vivo* and/or clinical studies. However, the therapeutic feasibility of pro-oxidant drugs in cancer with mutant *p53* status remains to be well-defined [608].

4.8 Targeting chemoresistant cells as an Alternative Approach

As revised in previous sections, the acquisition of a quiescent, CSC-like phenotype by well-differentiated and chemoresistant cancerous cells provides them with sufficient time for successful DNA repair after the cytotoxic assault. Nevertheless, chemoresistance can rely on multiple additional mechanisms. Indeed, the correlation between breast, colorectal, and lung chemoresistant cells, the upregulation of ABC family members, such as ABCC1, and the cisplatin and doxorubicin tolerance have been demonstrated [609]. Moreover, it has been revealed in a colorectal cancer study using 5-FU as a chemotherapeutic agent that a high drug metabolic detoxification capacity is a typical property of dormant CSCs-like cells [610].

In addition, these cells usually exhibit enhanced DNA repair mechanisms in a similar way to healthy stem cells, which may lead to increased DDDs tolerance. What is more, the key importance of DNA repair-related pathways/proteins in the maintenance of a CSC-like phenotype, for instance, by regulating the EMT process, has been reported [611]. The chemoresistance capacity of them also relies on avoiding apoptosis mediated by the enhancement of BCL2 family proteins, such as BCL-XL, as has been determined by the Medema group in a colorectal cancer study [612]. Interestingly, they documented the reliance of different antiapoptotic proteins according to the tumour progression stage, which may suggest the existence of distinct antiapoptotic mechanisms between healthy cells, well-differentiated cancerous cells, and CSCs. Altogether, these data reinforce the crucial importance of developing therapeutic strategies with the aim of eliminating such a cancer cell subpopulation (Figure 33).

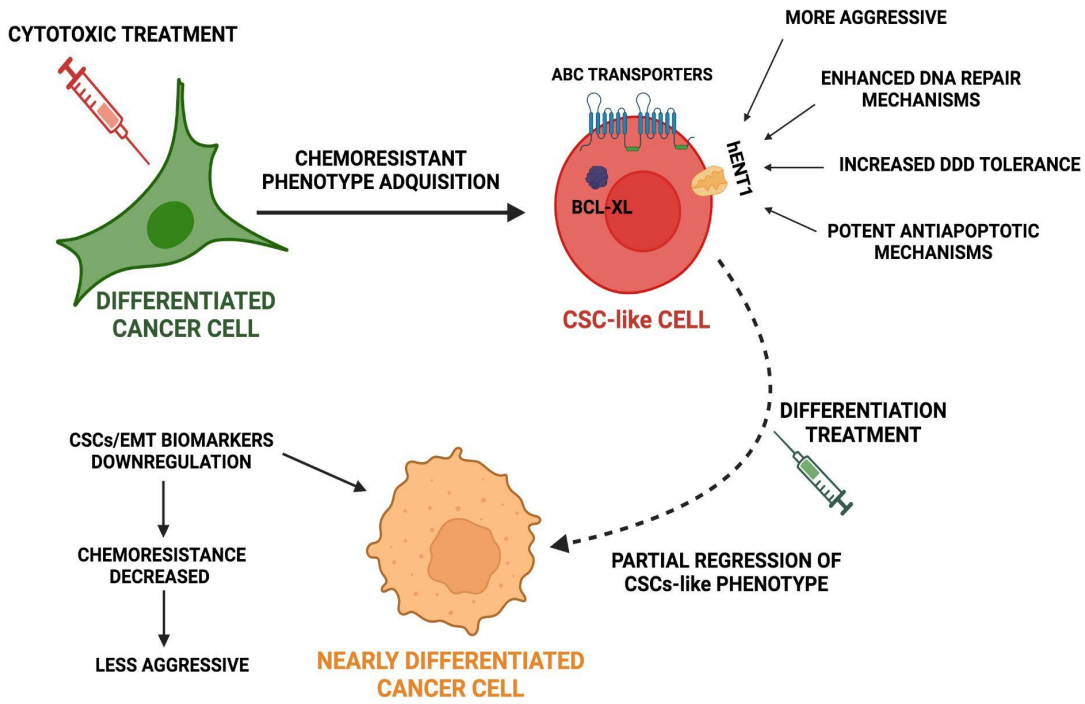


Figure 33. Cytotoxic treatments induce cancer stem cells-like phenotype acquisition together with increased chemoresistance. Approaches to re-differentiate these chemoresistant cells could improve current treatment. After cytotoxic treatment, differentiated cancer cells acquire a quiescent CSCs-like phenotype, through the successful repair of damaged DNA. CSCs, after cytotoxic assault trigger protective mechanisms, such as the expression of ABC transporters and mechanisms to repair DNA, to increase tolerance to DDDs and to avoid apoptosis. Novel strategies focusing on new

non-toxic anticancer compounds could induce regression of the CSCs-like phenotype toward a differentiated phenotype with less chemoresistant potential. (*Toledo, B et al., Int. J. Mol. Sci. 2023*

In a tumour mass and surroundings, the CSCs population is maintained by the dedifferentiation of tumours cells located in the borders (revised in Hernández-Camarero et al., 2018) [613]. Thus, CSCs are a population that subsists after chemotherapy, enhances drug resistance, and reappears constantly upon tumour cell dedifferentiation. All these facts make the development of long-term therapies to manage and control the revival of CSCs populations necessary, while avoiding chemotherapy that is associated with severe side effects (Figure 33).

In this regard, strategies that control and deregulate the CSCs population are interesting approaches.

For instance, targeting pathways that regulate CSCs-like cells has been proposed. One of the main candidates has been the Notch pathway and to this end, different strategies have been tested: (i) gamma-secretase inhibitors (GSIs) [614]; (ii) monoclonal antibodies targeting Notch signaling [615], and (iii) pan-Notch inhibition [616]. Another strong candidate has been the Wnt pathway, whose inhibition has been achieved by an IgG4 mAb (DKN-01) that targets Dkk1 and suppresses canonical Wnt signaling via negative feedback [617]. In addition, efforts have been undertaken to control the hedgehog signaling pathway, with the use of SMO inhibitors and GLI inhibitors [618]. Furthermore, CSC-directed immunotherapy has also received attention. CSC lysates are used to generate CSC-specific T cells that directly target the subpopulation of CSCs within tumours [619]. The relevance of these approaches is supported by their translation to the clinical arena. In fact, several clinical trials on therapies targeting CSCs are actually ongoing or have already been completed [620].

Therapeutic strategies against CSCs

Therapeutic targeting of CSC-like cells is a promising approach in cancer treatment, given their role in tumour initiation, progression, and treatment

resistance. Various strategies and techniques are being explored to specifically target and eradicate CSCs. Here are some current strategies and emerging techniques for targeting these cells, along with the challenges and prospects associated with developing therapies focused on CSCs (Figure 34) [621]:

1. **Current Strategies:**

- Differentiation Therapy: Inducing CSCs to differentiate into non-stem cancer cells, which are more susceptible to conventional therapies [622].
- Targeted Therapies: Developing drugs that specifically target CSC surface markers or signaling pathways essential for CSC survival and self-renewal [623].
- Immunotherapy: Stimulating the immune system to recognize and eliminate CSCs through tar geted immunotherapeutic approaches [477].
- Combination Therapies: Using combinations of drugs or treatment modalities to target both CSCs and bulk tumour cells, overcoming treatment resistance [624].
- Metabolic Targeting: Targeting metabolic pathways that are essential for CSC survival and gro wth, as CSCs often have distinct metabolic profiles [625].

2. **Emerging Techniques:**

- Gene Editing Technologies: Using gene editing tools like CRISPR-Cas9 to target specific genes involved in CSC maintenance and survival [626].
- Nanoparticle-Based Drug Delivery: Utilizing nanoparticles to deliver therapeutic agents specifically to CSCs, improving drug efficacy and reducing off-target effects [622].
- Organoid and 3D Culture Models: Using advanced cell culture models to study CSC behavior and screen for novel CSC-targeting compounds [627].
- Single-Cell Sequencing: Identifying and characterizing CSC subpopulations at the single-cell level to develop personalized treatments targeting CSC-specific vulnerabilities [628].

3. **Challenges and Prospects:**

- Heterogeneity: CSC heterogeneity within tumours presents a challenge in developing therapies that effectively target all CSC subpopulations.
- Resistance Mechanisms: CSCs exhibit mechanisms of treatment resistance that

can limit the efficacy of targeted therapies aimed at eradicating them.

- Validation of Targets: Identifying and validating specific CSC targets for therapeutic intervention is crucial but can be complex due to the plasticity and adaptability of CSCs.
- Clinical Translation: Translating preclinical CSC-targeting strategies into effective clinical therapies remains a significant challenge, requiring rigorous clinical testing and validation.

Nevertheless, the depletion of CSCs by itself has not been shown to be effective in reducing chemoresistance because other factors, such as TME [127], play a key role in CSCs regulation and stem maintenance via the transition of non-stem cells to stem cell states [629]. Thus, strategies should focus on directing CSCs differentiation (Figure 34) instead of CSCs eradication.

In conclusion, the development of novel therapeutic approaches directed against CSCs and the TME could reduce chemoresistance and the associated adverse effects.

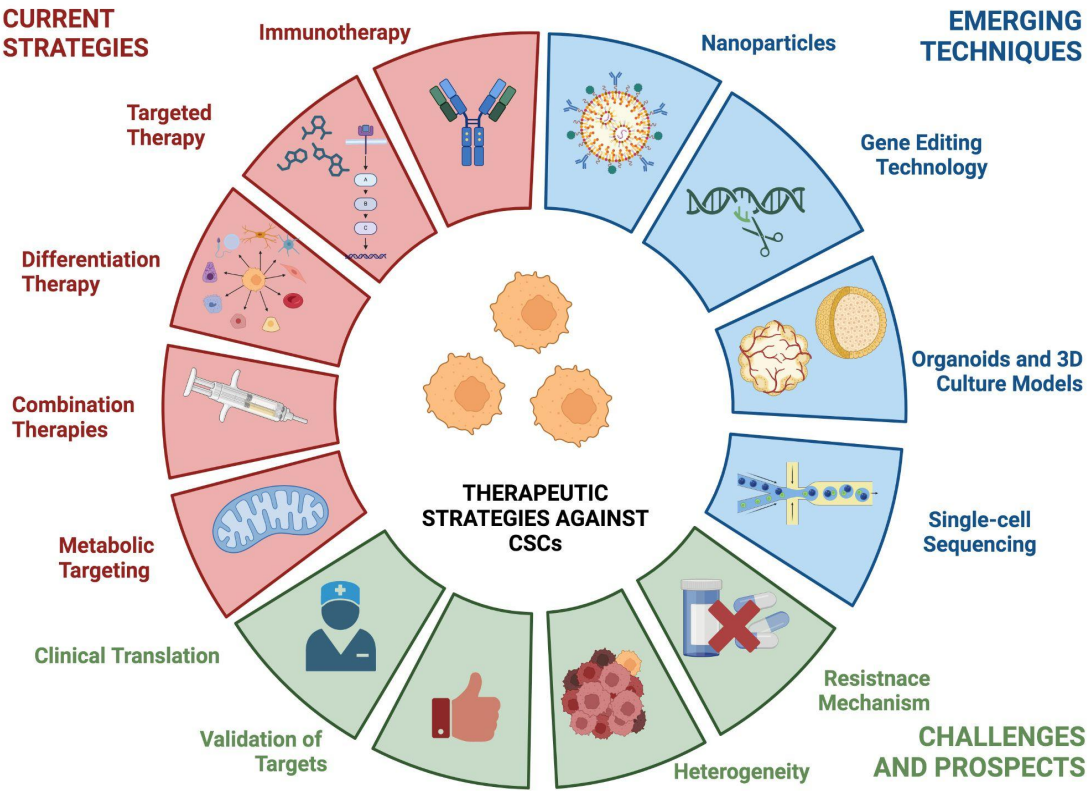


Figure 34. Different therapeutic strategies against CSCs. Image created with Biorender.com.

4.9 Molecular mechanisms of chemoresistance

In the field of medical research, gaining a deep understanding of the intricate molecular mechanisms underlying chemoresistance is of the utmost importance. Within the intricate web of cellular processes, various molecular players collaborate to orchestrate resistance, diminishing the efficacy of conventional treatments [630]. Thus, understanding these mechanisms holds great significance in the development of precision therapies that can overcome chemoresistance. While significant strides have been made in understanding the molecular underpinnings of this malignancy, therapeutic progress has been impeded by the common inherent and acquired chemoresistance [105]. This thesis seeks to provide an overview of the existing knowledge surrounding the molecular mechanisms implicated in chemoresistance in PC (Figure 35).

A) Genetic Alterations

Genetic alterations play a crucial role in the development of chemoresistance in PC. For instance, mutations in KRAS, which are detected in more than 90% of PC patients, have been associated with resistance to multiple chemotherapeutic agents (Figure 35). Certain studies have also suggested that targeting KRAS may lead to the attenuation of chemoresistance and improve the therapeutic response in PC patients. Recent preclinical studies have shown that the use of SHP2 inhibitors (SHP099), MEK inhibitors (cobimetinib), and KRASG12C inhibitors (sotorasib), can enhance the efficacy of chemotherapeutic agents in PC cells carrying KRAS mutations, as well as an acceptable safety profile in pretreated patients with KRAS-mutated advanced PC [631-633]. Alterations in TP53, have also been identified as key factors contributing to this resistance (Figure 35). Pan and colleagues demonstrated that the loss of p53 function has been associated with aggressive tumour phenotypes, chemoresistance, and poor prognosis in PC patients, involving several mechanisms, such as dysregulation of cell cycle arrest, DNA damage repair, apoptosis, and autophagy [634]. Mutations that impact genes responsible for

repairing DNA damage, such as BRCA1/2, ATM, and PALB2, have been extensively studied for their role in response to chemotherapy [635,636] (Figure 35). These genes encode important proteins involved in homology-directed repair (HDR), which is a system for accurately fixing DNA double-strand breaks (DSB) [637]. When there are mutations that disrupt the function of these genes, HDR is impaired, and cells rely more on error-prone repair mechanisms like non-homologous end joining and other DSB repair systems [638]. In 2018, Blair et al. found that a group of PC patients with BRCA1/2 mutations had better outcomes when treated with platinumbased adjuvant chemotherapy compared to non-platinumbased adjuvant chemotherapy or nothing [639]. Additionally, a retrospective study on 262 patients, of which 50 had HDR mutations, showed that HDR-mutated patients had better PFS when treated with first-line platinum therapy [640]. Furthermore, a recent phase II study demonstrated that GEM+cisplatin treatment is effective against PCs with both BRCA1/2 and/or PALB2 mutations [641.

B) Epigenetic Modifications

Several epigenetic modifications, such as DNA methylation, histone acetylation and deacetylation, as well as histone methylation have been implicated in chemoresistance in PC (Figure 35). Most of these studies have demonstrated that disrupting DNA methylation homeostasis is a key factor in the development of human cancer. Furthermore, these studies have contributed to the realization that alterations in methylation patterns play a crucial role in distinguishing tumour cells and rendering them resistant to chemotherapy [642]. The abnormal methylation patterns in PCs have been extensively described [643]. Aberrant expression of noncoding RNAs, such as microRNAs and long noncoding RNAs, can also impact the response to chemotherapy as they can act as oncogenes or tumour suppressor genes [644-646] (Figure 35). Hong and colleagues found that miR-21, miR-27a, miRNA-146a, miRNA-196a, and miRNA200a were the most upregulated miRNAs, while miR-20a, miR-96, and miR-217 were significantly downregulated in PC tissues [647,648]. This

suggests that some miRNAs could promote oncogenic processes while others have tumour suppressive effects. Several studies showed the role of miR-21 in chemoresistance and poor prognosis [649-651], and the involvement of other miRNAs has been recently reviewed by Vahabi and collaborators [652].

C) Epithelial to Mesenchymal Transition (EMT)

EMT is a critical process involved in the aggressive nature of cancers, leading to invasion, metastasis, and drug resistance [653] (Figures 25 and 35). El Amrani et al. conducted a study revealing that GEM induces EMT-like changes in PC cells [654]. These changes are mediated by the extracellular regulated kinase (ERK)-zinc finger E-box binding homeobox 1 (ZEB1) pathway. Inhibition of ERK1/2 phosphorylation or ZEB1 expression leads to a reduction in chemoresistance and invasion of GEM resistant (GR) PANC-1 and MIAPaCa-2 cells. Additionally, GEM induced overexpression of CD44, CD24, and CD326 in GR cells compared to sensitive cells. Further studies have identified that AMPK-related kinase 5 and upregulation of glycolysis contribute to GR through EMT mechanisms. Kuwada et al. demonstrated that the induction of EMT in PANC-1 and BxPC-3 cells by TAMs render them more migratory and thereby promote growth and chemoresistance [655]. Lastly, EMT is closely linked to the modulation of the TGF-β pathway which is a key regulator of autophagy and apoptosis [656]. The TGFβ-miR200-MIG6 pathway orchestrates a kinase switch linked to EMT, resulting in resistance to EGFR inhibitors [657]. Noteworthy the antipsychotic drug brexpiprazole is able to overcome osimertinib resistance in both lung cancer and PC by suppressing survivin protein. This action subsequently inhibits TGF-β/SMAD signaling, preventing EMT [658,659]. The regulation of hMENA isoforms by TGF-β1 is pivotal in TGF-β1-induced EMT, presenting potential targets for the development of innovative prognostic and therapeutic strategies in PC [660].

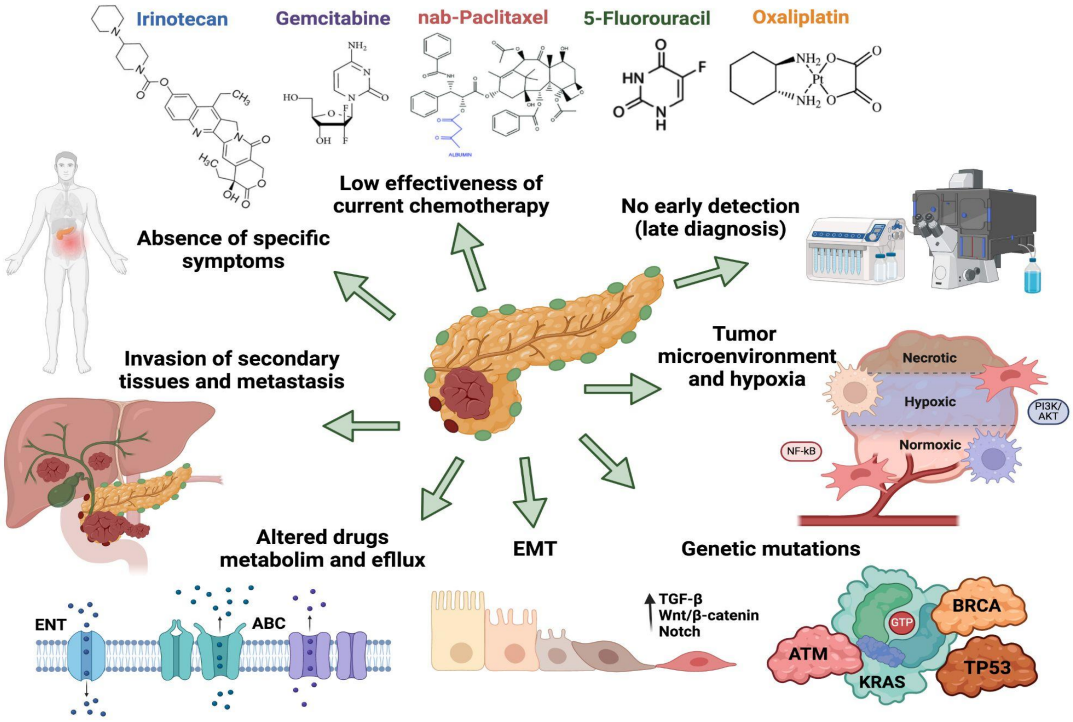


Figure 35. Molecular mechanisms of chemoresistance in PC. Chemoresistance in pancreatic cancer is mainly contributed by the EMT phenotype, genetic modifications, the hypoxic TME the great metastatic capacity of tumour cells and the deregulation of metabolic pathways induced by chemotherapeutic agents, including changes in drug entry and exit transporters and changes in enzymes involved in drug effects. This is supported by the factors of an absence of symptoms that allow early detection and diagnosis, as well as the effective reduction of current cytotoxic drugs. (*Toledo, B et al., Exp. Rev. Clin. Pharmacol. 2024*).

D) Tumour Microenvironment

Approximately 80% of PC tumour mass is constituted by stroma comprising cellular components (stellate cells, fibroblasts, endothelial and immune cells) and an extracellular matrix (ECM). Tumour stroma contributes to tumourigenesis by promoting invasion and enhancing tumoural angiogenesis, however the new vessels are often defective, explaining the hypoxic environment often observed in TME [661]. In addition, the stroma creates physical barriers that limit drug penetration and promotes drug resistance, by activating signaling pathways that are involved in cell proliferation [662,663] (Figure 35). Various growth factors (such as TGF-β, VEGF, CTGF, HGF, FGF), matricellular proteins, metalloproteinases, tissue inhibitors of metalloproteinases, and cytokines have been documented to impact drug effectiveness.

Sonic hedgehog (Shh), a soluble ligand, over-expressed by neoplastic cells in PC, drives formation of a fibroblast-rich desmoplastic stroma. Surprisingly, Shh-deficient tumours are more aggressive and exhibit undifferentiated histology, increased vascularity, and heightened proliferation features [383]. Together, these data demonstrate that some components of the tumour stroma can also act to restrain tumour growth and should not be inhibited.

Some stromal cells, including CAFs and immune cells, can play an active role in promoting chemoresistance. They provide cancer cells with survival signals, activate signaling pathways that facilitate drug efflux, and secrete cytokines that hinder apoptosis [9,67]. A therapeutic option may involve reversing the protumourigenic signals of CAFs or transforming them into a non-CAFs phenotype 're-educating' CAFs to adopt a non-tumour-associated state [440,441]. One such approach involves the use of epigenetic modulating agents like the demethylating agent 5-AZA. The administration of 5-AZA can transform an aggressive PC model into a less aggressive and more drug-sensitive phenotype. This transformation has been observed *in vivo*, with reduced tumour growth following engraftment of treated transformed cells. Additionally, when combined with GEM, 5-AZA exhibits a noticeable inhibitory effect on the growth of GR-PC cells [442]. A phase II trial with azacitidine and anti PD-1 pembrolizumab as second-line treatment in PC did not show improved PFS, however one patient had a notably long survival and maintained response, indicating the need for predictive biomarkers [664].

Lastly, the ECM, composed of proteoglycans, HA, and collagen, plays a crucial role in influencing interstitial fluid pressure and blood vessel distribution, contributing to hypoperfusion, hypoxia, and altered cancer cell metabolism. This, in turn, reduces drug delivery and activity [476,665] (Figure 35).

In summary, PC presents one of the most formidable challenges in oncology, marked by its aggressive nature and limited treatment success, largely attributed to the pervasive issue of chemoresistance. This comprehensive analysis delves into the intricate molecular mechanisms underlying chemoresistance in PC, highlighting genetic alterations, epigenetic modifications, EMT, altered drug influx and efflux, and the TME as key contributors.

E) Altered drug influx and efflux

Human equilibrative nucleoside transporter-1 (hENT-1 or SLC29A1) has been shown to play a crucial role in the intracellular accumulation of nucleoside-based chemotherapeutic agents in PC cells, especially GEM [130,666] (Figure 35). Previous studies reported that the downregulation/inhibition of hENT-1 resulted in cell chemoresistance to GEM and a retrospective analysis of the phase III clinical trials RTOG-9704 and ESPAC-1/3 supported the role of hENT1 as a predictive biomarker of GEM efficacy, with patients with high hENT-1 expression having a significantly longer OS [667-669]. Of note, this association was missing in patients treated with 5-FU, suggesting a predictive and not prognostic role for hENT-1.

Similarly, another transport called dENT-2 (SLC29A2) has been identified in various PC cell lines, although with decreased expression compared to hENT-1 [670]. It has been shown that h-ENT2 expression and its mRNA levels decrease after treatment with GEM in resistant cell lines [671]. Expanding on these findings, deactivation of both hENT-2 and hENT-1 results in diminished GEM uptake and sensitivity.

Efflux is the active process through which cells pump drugs out of their cytoplasm to avoid drug buildup. Thus, the expression of efflux pumps like ABC multidrug transporters like ABCB1 have been found to play a role in drug resistance, drug distribution, and toxicity [672] (Figure 35). For instance, in the context of irinotecan, ABCB1 plays a role in the cellular uptake of both the prodrug and its active metabolite SN-38. However, the ABCB1 1236C>T variant markedly reduces irinotecan clearance, the ABCC2 3972T>C variant is linked to toxicity, and cells over-expressing ABCG2 are recognized as resistant to both irinotecan and SN-38 [673].

Chen and collaborators reported a significantly lower promoter methylation level of the ABC transporter family (ABCB1, ABCC, and ABCG2) in a GR cell line (SW1990/GZ) compared to primary cells [674]. Another study indicated a gradual decrease in the promoter methylation level of ABCB1, ABCC, and ABCG2 during the establishment of GR in PANC-1 and BxPC-3 cells [675]. This provides evidence of the theoretical feasibility of predicting

MDR of PC through independent indicators such as the promoter methylation level of ABCB1, ABCC and ABCG2. However, clinical trials evaluating the predictive role of these transporters did not translate into successful clinical application.

Other interesting studies evaluated the expression and activity of drug-metabolizing enzymes. Recently, a study conducted by Yada et al. investigated the role of cytochrome P450 enzymes in chemoresistance of PC, showing that mRNA expression of cytochrome-P450 was upregulated in a concentration-dependent manner following GEM treatment. Moreover, the sensitivity to GEM increased with the use of a cytochrome-P450 inhibitor, indicating that this enzyme may be related to GR in PC [676].

The combination of nab-paclitaxel and GEM is another primary treatment option for patients diagnosed with advanced or metastatic PC [94]. Notably, the efflux of paclitaxel is facilitated by phosphorylated glycoprotein [677] and a recent study showed its key role in the resistance of SUIT2, PANC-1, and Patu-T cells [678]. The glycoprotein known as secreted protein acidic and rich in cysteine (SPARC) has been extensively studied as a potential biomarker for assessing the activity of nabpaclitaxel, correlating it with PC cell proliferation and metastasis [316]. An initial immunohistochemical study conducted on 36 patients revealed a dose-dependent correlation between higher levels of SPARC expression and improved OS, however this was not confirmed in following studies [679]. Furthermore, nab-paclitaxel has exhibited the ability to inactivate cytidine deaminase, the enzyme which mediates the conversion of GEM into its inactive form, thus resulting in increased levels of GEM and a higher response rate in KPC models [680].

Other intracellular mechanisms of drug resistance are represented in Figure 36:

- Cell Signaling Pathways: Dysregulated cell signaling pathways in cancer cells can contribute to drug resistance. For example, activation of pathways like PI3K/Akt and Wnt can promote their survival and proliferation while conferring resistance to chemotherapy-induced cell death [392].

- DNA Repair Mechanisms: cancer cells often have enhanced DNA repair mechanisms, which help them repair DNA damage caused by chemotherapy or radiation. This efficient DNA repair reduces the effectiveness of these treatments in eradicating chemoresistant cells [130].
- Metabolism plays a crucial role in drug resistance mechanisms within tumour cells since they often exhibit altered metabolic pathways that enable them to survive and thrive even under therapeutic pressure. For instance, these cells can shift between glycolysis and oxidative phosphorylation to adapt to different environments, a type of metabolic flexibility that enhances their survival and resistance to chemotherapeutic agents. This metabolic adaptation supports the energy and biosynthetic demands of chemoresistant cells, aiding in the repair of drug-induced damage and evasion of apoptosis [502].
- Drug compartmentalization is a key mechanism of drug resistance in CSC-like cells. This process involves the sequestration of chemotherapeutic drugs into specific intracellular compartments, such as lysosomes or vacuoles, thereby reducing the drug's effective concentration at its target site. By compartmentalizing the drugs away from critical cellular machinery and DNA, CSCs can minimize the cytotoxic effects of these treatments. This strategy not only protects the cells from the intended therapeutic damage but also allows them to survive and proliferate despite the presence of high drug concentrations in the surrounding microenvironment [130].

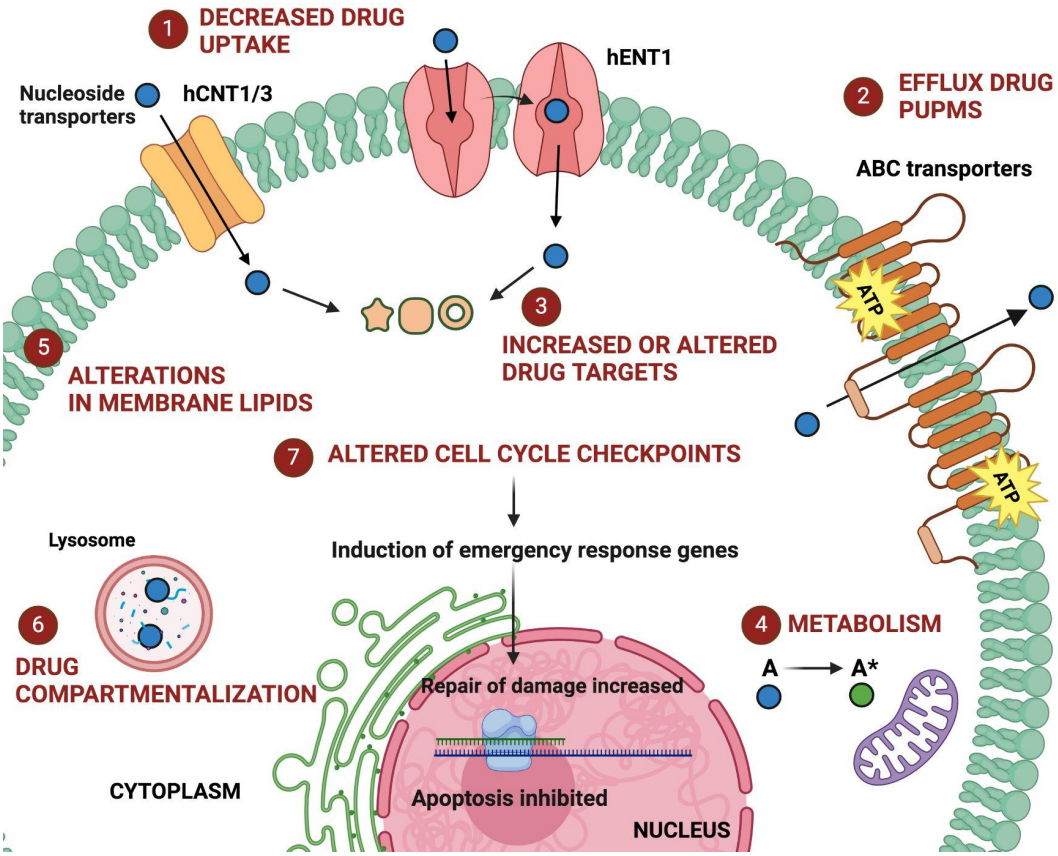


Figure 36. Different chemoresistant mechanisms in cancer cells. Image created with Biorender.com.

4.10 Overcoming Chemoresistance

As seen before, the mechanisms underlying chemoresistance are complex, numerous, and often intertwined. Despite these difficulties, several attempts have been made to try to overcome chemoresistance in PC (Table 3).

Given the genetic alterations associated with chemoresistance, one approach is to combine standard chemotherapy with drugs directed against genes involved in chemoresistance, and this subject is explored in the dedicated chapter on target therapy (Figure 37). Regarding epigenetic changes that can lead to chemoresistance, Kurdistani et al. analyzed the role of histone modifications in GR PC [644]. The researchers evaluated the effects of histone deacetylase inhibitors (HDACi) on PC cell lines and patient-derived xenograft models. They found that treatment with the HDACi vorinostat sensitized cells to GEM, resulting in decreased cell viability and increased apoptosis. Analysis of histone modifications revealed that treatment with vorinostat led to increased acetylation

of histones H3 and H4, as well as decreased methylation of histone H3 lysine 27, which is associated with gene silencing. Furthermore, combination treatment with vorinostat and GEM resulted in improved survival in PC xenografts compared to either treatment alone. A neoadjuvant phase I trial with the combination of vorinostat plus multi TKI sorafenib and chemoradiotherapy demonstrated good tolerability and encouraging antitumoural activity [681]. This suggests that modulation of histone modifications through HDACi holds potential for overcoming GR in PC. Furthermore, DNA methyltransferase inhibitors have also being explored as epigenetic therapy: a phase I trial including PC of guadecitabine and durvalumab was associated with a good tolerability profile and although the efficacy was limited, a group of patients had prolonged benefit, indicating the need for better biomarkers of response for this class of drugs [682].

Epigenetic changes can also take the form of alternation in miRNA, thus several studies have explored the idea of miRNA silencing or the use of miRNA mimics [683]. Overall treatment with miRNA has been widely explored in the preclinical setting, however a phase I trial on a drug targeting miR-34a in solid tumours including PC [684], showed that the treatment was associated with high infusion related adverse events.

Efforts to address phenotypic changes associated with chemoresistance due to EMT have been undertaken by targeting crucial pathways, albeit with limited success. For instance, drugs aimed at Notch, a pathway implicated in the emergence of CD44-positive PC stem cells and EMT in preclinical models, yielded unsatisfactory outcomes [685]. In a phase I/II clinical trial, the monoclonal antibody against Notch2/Notch3 (tarextumab) failed to enhance OS in combination with GEM and nab-paclitaxel. Moreover, the gamma-secretase inhibitor MK0752, when combined with GEM, resulted in severe adverse effects, causing a high rate of patient withdrawal [686,687] (Table 3).

Table 3. Overcoming chemoresistance in PC. (*Toledo, B et al., Exp. Rev. Clin. Pharmacol. 2024*).

Target	Regimen	Results	Authors	Year
Histone modifications	HDACi Vorinostat	- Viability decreased and apoptosis increased in cell lines	Kurdistani S.K et al. [644]	2004
		- Combinated treatment improved survival in PC xenografts		
Oncogenic pathways	MRX34 (liposomal miR-34a mimic)	- Acceptable safety	Beg M.S et al. [684]	2017
		- Evidence of antitumour activity in some patients		
Notch pathway	Tarextumab (Notch antagonist)	- tumour growth inhibited	Yen W. C et al. [685]	2017
		- tumour initiating cells frequenty decreased		
Notch pathway	Tarextumab + GEM + Nab-paclitaxel	- Similar OS in patients	O'Reilly E. M et al. [686]	2017
Notch pathway	MK0752 + GEM	- Severe adverse effecs	Cook N et al. [687]	2014
EMT	Metformin	- Cancer cells proliferation inhibited	Gulla A et al. [688]	2022
		- Morbidity and mortility rates reduced		
		- Short term suvival rate slightly increased		
		- No differences in long term survival rate		
EMT	5-AZA	- Tumour growth reduced	Gailhouste L et al. [689]	2018

		- Less aggressive tumour model		
		- More drug/sensitive tumour model		
Hedgehog pathway	IPI-926	- No remarkable results	Jimeno A et al. [690]	2013
Hyaluronic acid	PEGPH20 + GEM + Nab-paclitaxel	- PFS and OS was slightly increased	Hingorani S R et al. [289]	2018
Hyaluronic acid	PEGPH20 + FOLFIRINOX	- Toxicity and secondary effects	Ramanathan R K. et al. [691]	2019
JAK2/STAT3 pathway	Ruxolitinib + Capecitabine	- Well tolerated by patients	Hurwitz H. I et al. [692]	2015
		- OS lightly increased		
JAK2/STAT3 pathway	Ruxolitinib + Capecitabine	- Clinical results do not improve in second-line treatment	Hurwitz H. I et al. [693]	2017

Abbreviations: CCA: cholangiocarcinoma, PC: pancreatic cancer, EMT: epithelial-mesenchymal transition, miR: microRNA, GEM: Gemcitabine, 5-AZA: 5-azacytidine, OS: overall survival, PFS: progression-free survival.

Recent studies have indicated that metformin, a medication used for diabetes, enhances the antiproliferative effects of PC cells by inhibiting EMT. This effect might potentially improve the overall prognosis of PC patients. In fact, therapeutic exposure to metformin was associated with reduced morbidity and mortality rates, but modestly increased short-term survival rates without affecting long-term survival rates in PC patients [688].

The stromal components in PC have been targeted using Hedgehog pathway inhibitors, such as IPI-926 or recombinant pegylated HA enzyme PEGPH20, however with unsatisfactory clinical outcomes [289,690]. A randomized phase II trial combining PEGPH20 with GEM and nab-paclitaxel in patients with high levels of HA only met the secondary endpoint of PFS and had to be temporarily halted due to increased thrombosis [289]. Similarly, a randomized phase II trial investigating frontline PEGPH20 combined with FOLFIRINOX in a non-biomarker-selected population was terminated early due to lack of effectiveness in the interim analysis [691]. Disappointing results have also been observed with inhibitors targeting the JAK2/STAT3 pathway, such as ruxolitinib, which did not show promising results in a phase III study when combined with capecitabine [692,693] (Table 3).

It is important to note that while these strategies show promise, overcoming chemoresistance remains a complex and ongoing research area. Clinical trials and translational studies are warranted to validate the effectiveness of these approaches in PC.

4.11 Molecular mechanisms of resistance to target therapy

In the *era* of precision medicine, targeted therapies have emerged as a promising frontier in the treatment of various malignancies. These therapies, designed to specifically target aberrant molecular pathways driving cancer growth, have demonstrated remarkable efficacy in numerous tumours [694,695]. However, a formidable challenge persists: the development of resistance to these targeted interventions. As with conventional chemotherapies, cancer cells exhibit a remarkable capacity to adapt and evolve, rendering once-effective

targeted therapies progressively less potent [696]. Broadly, there are three main mechanisms underlying resistance to target therapies [696] (Figure 37).

(1) On-target alterations: these include changes in the target receptor that cause the drugs to not be able to inhibit the pathway. A striking example of this mechanism can be found in EGFR mutated NSCLC: the use of adenosine triphosphate (ATP)-competitive inhibitors such as gefitinib and erlotinib is often associated with the development of a T790M point mutation in the EGFR gene, causing an increase in affinity for the physiologic substrate ATP and thus decreasing the inhibition on the downstream pathway by the target drugs [697]. Other than single amino acid changes, on-target alteration can also include RNA alternative splicing, as has been seen in melanoma BRAF V600E patients treated with TKI, or gene amplification, as seen in imatinib-treated patients with chronic myeloid leukemia [698,699] (Figure 37).

(2) Off-target alterations: to bypass a drug's inhibition on a specific survival pathway, cancer cells often exhibit upregulation of different proliferation pathways. For example, BRAF V600E melanoma patients have to be treated with both BRAF and MEK inhibitors because the use of BRAF monotherapy leads to an upregulation in NRAS and a consequent mitogen-activated protein kinase (MAPK) reactivation [700]. Similarly, NSCLC treated with EGFR, anaplastic lymphoma kinase (ALK), RET, or ROS-1 inhibitors, often display a secondary MET amplification driving resistance [701] (Figure 37).

(3) Phenotypic transformation: histological changes have been described in several cancers treated with TKI [702]. EGFR mutated and ALK fusion-positive NSCLC treated with TKI can exhibit a transformation to small cell lung cancer, sometimes retaining the original mutation [703]. More commonly the phenotypic trans- formation takes the shape of an EMT, in which alterations in gene expression and transcriptional mechanisms give rise to cells with mesenchymal properties such as changes in cells' polarity, weakened cellular adhesion, increased migratory capacity, and cytoskeletal remodeling [704] (Figure 37).

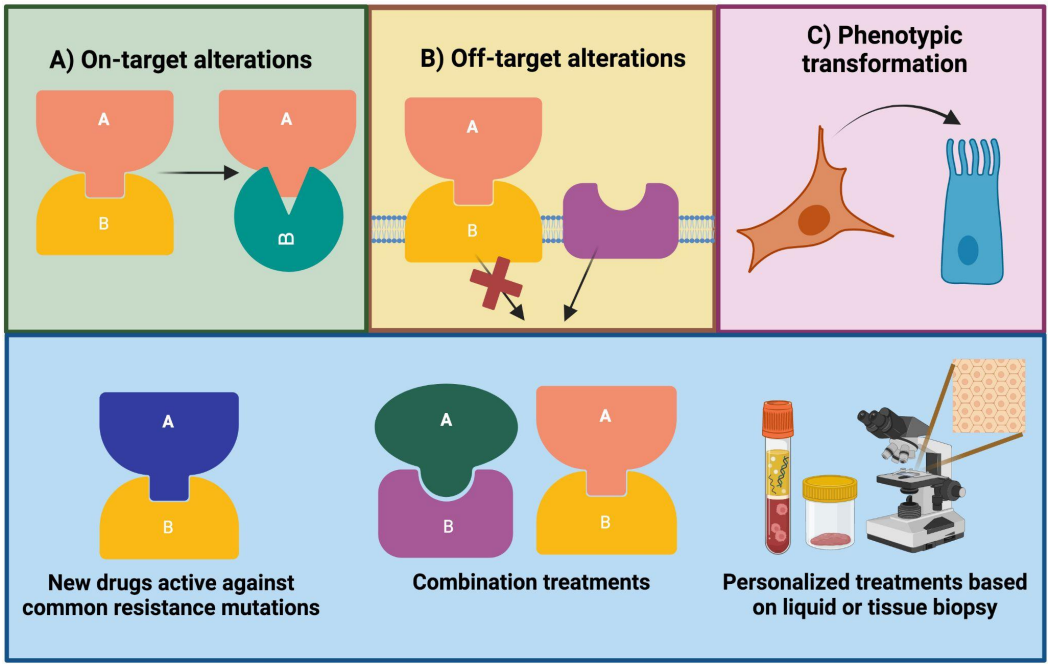


Figure 37. Mechanisms of resistance to target therapy and strategies to overcome them. There are three main mechanisms underlying resistance to targeted therapies: phenotypic transformations, on-target alterations and off-target alterations. Currently, attempts are being made to overcome this barrier by developing new drugs against common resistance mutations, combination of different treatments and personalized treatments based on liquid or tissue biopsy. (*Toledo, B et al., Exp. Rev. Clin. Pharmacol. 2024*).

To summarize, targeted therapies have managed to demonstrate remarkable efficacy by specifically attacking the aberrant molecular pathways that drive cancer growth. However, on-target, off-target and phenotypic changes can allow cancer cells to bypass the inhibition from target therapies. A comprehensive understanding of the underlying resistance mechanisms is essential to develop strategies to overcome them or to preemptively address them, thereby maximizing the long-term effectiveness of targeted therapies in the changing landscape of precision medicine.

A) Resistance to targeted therapy in PC

The genetic landscape of PC has been well analyzed in search of targetable mutations, but monotherapy treatment with target drugs did not have

much success in PC, possibly due to many dysregulated pathways with complex and multiple cross talks and the fact that the most commonly mutated genes do not have a corresponding target drug [105]. Thus, information on resistance mechanisms pertaining to PC specifically is scarce and limited to the few drugs that have been used in more advanced clinical trials. For example, although KRAS alterations are present in around 90% of PC cases (Figures 10 and 35), only a very small subset exhibits the G12C alteration that makes it susceptible to the target inhibitor sotorasib [705]. However, even in these selected patients, the recorded ORR to second or further-line target therapy was only 21% [633]. Preclinical data point to the activation of alternative signaling pathways and the development of new mutations in the KRAS gene as possible secondary resistance mechanisms to KRAs inhibitor, ongoing trials are evaluating allele-specific inhibitors and pan-(K)RAS inhibitors against the more common allele variants G12D, G12V, and G12R 290 [706].

Furthermore, the presence of KRAS alteration has been linked to resistance to another type of target treatment, EGFR inhibitor. For example, the combination of anti-EGFR antibody nimotuzumab plus GEM had an OS advantage only in KRAS wild-type patients [707]. Many other trials with EGFR inhibitors (both TKI and monoclonal antibody) failed to prove a survival benefit in unselected pancreatic patients, again, probably due to alternative active pathways [708]. It should also be noted that, although the combination of erlotinib in association with GEM is associated with an OS gain [90], this regimen is not often used in clinical practice as the benefit in the trial was minimal.

One of the few target therapies that has been approved in PC is the PARP inhibitor, olaparib, in the setting of BRCA1/2 germline-mutated patients that had not progressed after platinum-based first-line therapy. This is quite a rare scenario in which two mechanisms of DNA repair are blocked, one related to the BRCA gene and one to PARP enzymes, making the cancer cells extremely susceptible to DNA damage mechanisms. Despite this encouraging premise, maintenance therapy with olaparib after platinum-based first-line therapy was not associated with a gain in OS (18.9 vs 18.1 months), but only a PFS gain (7.4

vs 3.8 months in the placebo arm) [114]. Although clinical data on PC patients are not available, resistance mechanisms to olaparib have been studied in pancreatic cell lines, pointing to the development of multiple genetic alterations including upregulation of MDR genes and phenotype changes such as EMT, furthermore, in other cancer, resistance to olaparib was also associated with 53BP1 down-regulation and BRCA reversion mutations [709].

B) Overcoming target therapy resistance

Understanding the molecular mechanisms of resistance to targeted therapy is crucial for developing strategies to overcome or prevent resistance (Table 4). The first issue that needs to be addressed is how to recognize which resistance mechanisms are at play in each patient. Given the wide spectrum of resistance mechanisms and the high intratumoural heterogeneity of PC, it is unlikely that a single lesion biopsy will unravel the full complexity of the resistance mechanisms that can arise in each patient. Thus, it is crucial to implement tools that will give a more comprehensive picture of the resistance mechanisms during cancer progression. In this context, the implementation of liquid biopsy is emerging as a very valuable tool.

In PC, a meta-analysis on the use of liquid biopsy found an overall sensitivity of 70% and a specificity of 86% when compared to molecular analysis of tissue specimen; when looking at studies on *KRAS* mutations only, the sensitivity of liquid biopsy was a bit lower, around 65%, but with a high 91% specificity [710]. Of note, 78% of mutations detected in circulating free DNA were not found in the lesion biopsy, again confirming the likely inadequacy of single lesion biopsy [710]. Detecting the type or types of ongoing resistance mechanisms is only part of the problem, as then we need viable strategies to combat them (Table 4).

Multiple new drugs are currently being tested. For example, the novel KRAS inhibitor (MRTX1133), targeting the G12D mutation, has already demonstrated preclinical efficacy, showing tumour regression in the majority of tested cell line xenografts (8 out 11) and patient-derived xenografts [711], and is

now being tested in a clinical trials (NCT05737706) [712]. Another pan-KRAS inhibitor called BI-3406, currently in phase I trials, disrupts the binding of KRAS to its activator SOS1 [713], and similarly another KRAS G12X inhibitor called RMC-6236 is being tested in at least two phase I trials (NCT05379985, NCT06128551) [714,715]. Additionally, the SHP2 inhibitor TNO155 has shown promising safety and tolerability, and multiple clinical trials are underway to test its efficacy in combination with other therapies [716,717].

Another emerging therapy for PC involves targeting the Fanconi Anemia (FA) pathway, which is involved in DNA crosslink repair: ATR inhibitors, which inhibit the downstream signaling of the FA pathway, have been shown to be effective in preclinical studies when combined with chemotherapy agents such as GEM [718,719] (Table 4).

Table 4. Overcoming target therapy resistance in pancreatic cancer. (*Toledo, B et al., Exp. Rev. Clin. Pharmacol. 2024*).

Target	Regimen	Results	Authors	Year
KRAS	MRTX1133	- Tumour regression in xenograft models	Wang X et al. [711]	2022
KRAS	BI-3406	- Tumour growth suppression in xenograft models	Hofmann M. H et al. [713]	2021
Fanconi Anemia pathway	AZD6738 + GEM	- Tumour growth inhibition *in vitro*	Wallez Y et al. [718]	2018
		- Anti-tumour efficacy *in vivo*		
		- Drug sensitivity increased		
Fanconi Anemia pathway	VE-821	- Sensitization of normoxic and hypoxic PC cells to radiation/chemotherapy	Prevo R et al. [719]	2012
mTORC2 and PARP	PP242 + Olaparib	- Cell growth and invasion suppressed	Bu C et al. [720]	2023
		- Tumour cells more sensitive to drugs		
		- Tumour size reduced *in vivo*		
Multi-targets	Nab-paclitaxel + GEM + Cisplatin + Irinotecan + Capecitabine + Pembrolizumab + Olaparib	- Study ongoing	NCT04753879 [721]	2023
BRCA1/2 and PALB2	Niraparib + Dostarlimab	- Study ongoing	NCT04493060 [722]	2023

Abbreviations: ICC: intrahepatic cholangiocarcinoma, BTC: biliary tract cancer, EGFR: epidermal growth factor receptor, FGFR: fibroblast growth factor receptor, KRAS: kirsten rat sarcoma virus, PARP: poli-ADP ribose polymerase, GEM: Gemcitabine.

A different strategy consists in combining multiple target drugs to block multiple pathways, similar to what has already been done in other cancers like melanoma [723] (Figure 38). Such combination therapies can help overcome and prevent resistance by blocking alternative signaling pathways or compensatory mechanisms. In PC, the use of KRAS and MEK inhibitor has been associated with a compensatory activation of the AKT/mTOR pathway through cross talks proteins, thus the combination of either KRAS or MEK inhibitor along with an mTORC1/2 inhibitor has been used in preclinical models to prevent the activation of alternative pathways as a mechanism of resistance [724]. Several trials investigating combination therapies are underway, mostly in basket trials on solid tumours including PC, and involving different targets such as SHP2, ERK, MEK, KRAS, EGFR, VEGF, and CD4/6 [725-727]. Furthermore, given the use of olaparib in *BRCA1/2* mutated patients, one study explored the use of mTOR inhibitors in combination with PARP inhibitors, pointing to a synergic effect of this combination even in BRCA wild-type cell lines [720]. The combination of PARPi with immune checkpoint inhibitors has also been specifically used because BRCA insufficiency can trigger the production of type I interferon and pro-inflammatory cytokines, which can lead to an innate immune response that is dependent on the stimulator of interferon genes [728]. Furthermore, in a dose-dependent manner, clinical models have shown that PARP inhibition up-regulates PD-L1 and inactivates glycogen synthase kinase-3 (GSK3). As a result, T-cell activation is suppressed, which increases the death of cancer cells [729]. A phase I/II trial comparing PARPi niraparib plus nivolumab or ipilimumab as maintenance therapy after chemotherapy showed promising results in PFS for the niraparib–ipilimumab combination [730] and more trials are ongoing with olaparib plus pembrolizumab (NCT05093231, NCT04548752, NCT04753879) [731-733].

The combination of target therapy and immunotherapy is also being investigated (Figure 38). Currently, a phase II study (NCT04753879) is actively enrolling participants to evaluate the effectiveness of maintenance olaparib in combination with pembrolizumab after low-dose GEM, nab-paclitaxel, capecitabine, cisplatin, and irinotecan in previously untreated metastatic PC

patients [733]. Additionally, the National Cancer Institute is conducting a phase II trial (NCT04548752) comparing the use of olaparib alone versus the combination of olaparib and pembrolizumab in patients with metastatic germline BRCA1/2-mutated PC. Another trial (NCT04493060) is currently investigating the use of niraparib and dostarlimab, an anti-PD-1 antibody, in metastatic PC patients with BRCA1/2 and PALB2 mutations [734].

In conclusion, resistance to targeted therapy is a complex phenomenon driven by various molecular mechanisms. By unraveling these mechanisms and developing innovative strategies, we can enhance the effectiveness of targeted therapies and improve patient outcomes.

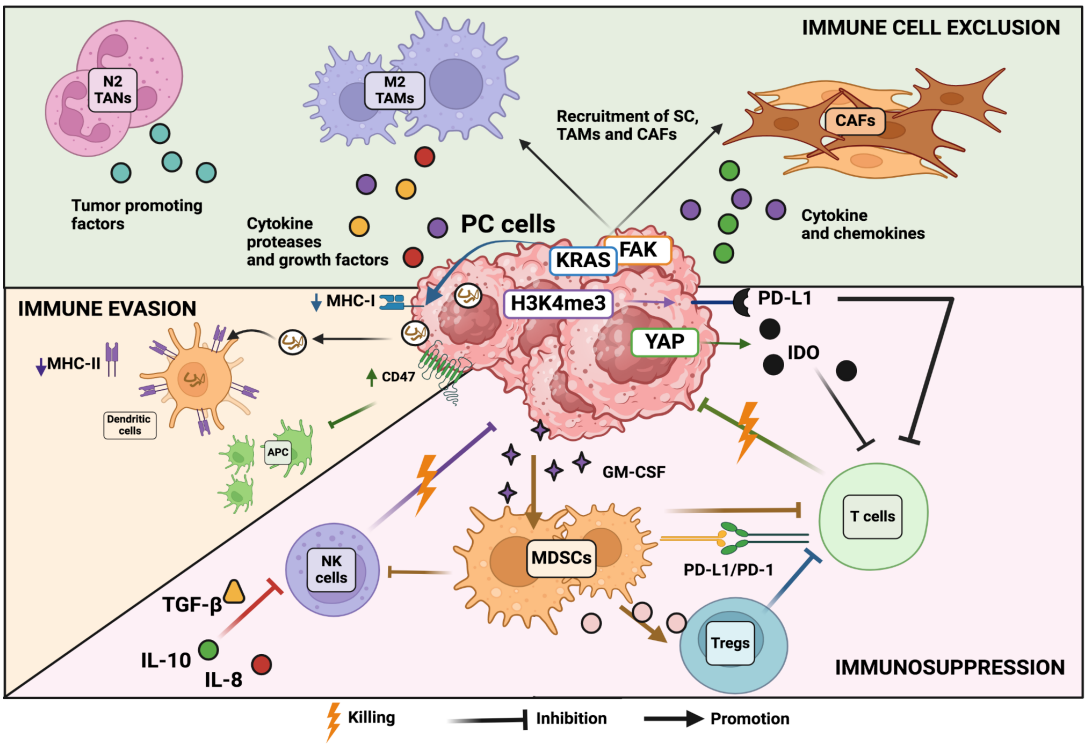


Figure 38. Immunoresistance mechanism in pancreatic cancer. (a) The tumour cells recruit and malignant the fibroblasts and macrophages of the tumour environment, thus the extracellular matrix becomes highly fibrotic and creates a physical barrier that prevents the infiltration of T cells into the tumour. (b) Immune evasion tactics in pancreatic cancer include: KRAS produced by the tumour cell reduces the expression of MHC I; just as miRNA-containing exosomes from tumour cells silence the expression of MHC II molecules in dendritic cells; and increased expression of antiphagocytic molecules such as CD47 prevents APC processing and tumour clearance. (c) The tumour suppresses immune responses through the expression of PD-L1, the recruitment of MDSC that activates regulatory T cells and inhibits T cells and NK cells, as well as the high expression of IDO

that also allows them to block the T cells. (*Toledo, B et al., J. Hematol. Oncol. 2024*).

4.12 Mechanisms of Resistance to Immunotherapy

Immunotherapy, heralded as a revolutionary paradigm in cancer treatment, has redefined the landscape of oncology by harnessing the intricate interplay between the immune system and malignant cells [735]. By empowering the body's immune defenses to recognize and combat cancer, immunotherapeutic agents have yielded unprecedented responses across a spectrum of malignancies. However, in PC, this class of drugs had to contend with high levels of primary resistance and the quick emergence of secondary resistance. The phenomenon of resistance to immunotherapy represents a complex interplay of biological mechanisms, whereby tumours employ a variety of strategies to evade or subvert the immune system's potent antitumour effects. Understanding the diverse molecular and cellular mechanisms that confer resistance to immunotherapy is paramount for advancing the effectiveness of these groundbreaking treatments. Broadly speaking there are two main components associated with immune evasion [736-738]. First, cancer cells possess intrinsic factors that play a crucial role in antigen presentation, a process vital for the immune system to recognize and eliminate cancer cells. However, loss of tumour neoantigen, changes in antigen presentation and processing, and hyperexpression of immune checkpoint proteins, can all help in the immune evasion. Furthermore, alteration of intracellular signaling due to genetic mutation, phenotypic transformation (such as EMT or a reversion to stem cell phenotype), and the secretion of metabolites to modify the tumour microenvironment are all possible causes of immune resistance as well. Second, the TME significantly shapes the presence, composition, and function of tumour infiltrating lymphocytes and other immune cells. The expression of immunosuppressive factors by the cancer cells, the presence of dysfunctional blood vessels, and a hostile, often hypoxic, environment are some of the factors contributing to immune evasion.

By illuminating the dynamic adaptations that underlie resistance, we aim

to catalyze the development of strategies capable of overcoming these barriers and extending the transformative potential of immunotherapeutic approaches in the fight against PC.

Resistance to immunotherapy in pancreatic cancer

Immunotherapy is not effective for PC, despite the increased knowledge of the genomic landscape and of the complex TME (Figure 38). Although it can be considered in patients with MSI or high tumour mutational burden, in this small subset of patients (around 2%), ORR to pembrolizumab has been reported between 62% and 18%, likely thanks to the high number of neoantigens being produced, capable of stimulating the immune system [118,739].

One culprit of immunotherapy's discouraging results is certainly the TME: PCs have a dense and prominent stroma, rich with immunosuppressive cells such as Treg cells, suppressive myeloid cells [740], CAFs and TAMs, but lack of effector T cells [741] (Figure 38). However, different compositions of TME have been described, with more mature-intermediate-immature characteristics, that correlate with prognosis, so it is possible that different subtypes may have different responses to immunotherapy [742]. Furthermore, PC cells often exhibit low expression of tumour antigens, limiting the ability of the immune system to mount an effective response, and they can upregulate immunosuppressive proteins such as PD-L1 and Cytotoxic T-Lymphocyte Antigen 4 [742] (Figure 38).

One of the main immune cells presented in the TME and which play a primary role in the fight against PC are tumour-associated macrophages or TAMs. The recruitment of TAMs to the TME is a complex interplay involving various cellular components and surrounding conditions (Figure 39). This process primarily relies on juxtacrine and paracrine signalling mechanisms, with cytokines and exosome secretion playing significant roles. A chemokine released by tumours that plays a key role in TAMs recruitment is granulocyte-macrophage colony-stimulating factor (GM-CSF). Studies have demonstrated that at low circulating levels, this factor exhibits anti-tumourigenic effects by

activating dendritic cells (DCs) within tumours. However, in advanced stages of cancer, characterized by high levels of GM-CSF, it switches to recruit TAMs and promotes oncogenesis. In fact, overexpression of this cytokine, as well as CSF, has been observed in various cancer types, including breast cancer, colon cancer, pancreatic cancer and cholangiocarcinoma, resulting in enhanced recruitment and infiltration of TAMs to the tumour site [94,108,743]. Additionally, CSF has been found to induce the production of IL-8 from TAMs in colon cancer cells. Subsequently, IL-8 activates the protein kinase C signalling pathway in colon cancer cells, leading to further release of CSF by the tumour, thereby attracting more TAMs [108]. This illustrates how the crosstalk between TAMs and cancer cells forms a vicious cycle (Figure 39). Apart from cytokines, certain tumour-released chemokines also play a significant role in TAMs recruitment. Major examples include C-C motif chemokine ligand 2 (CCL2), CCL5, CCL20, C-X-C motif chemokine ligand 4 (CXCL4) and CXCL12 [112-115]. Ultimately, the dependence on these tumour-released factors underscores the critical role of an inflammatory TME in facilitating the essential recruitment of TAMs, which become accomplices in tumourigenesis.

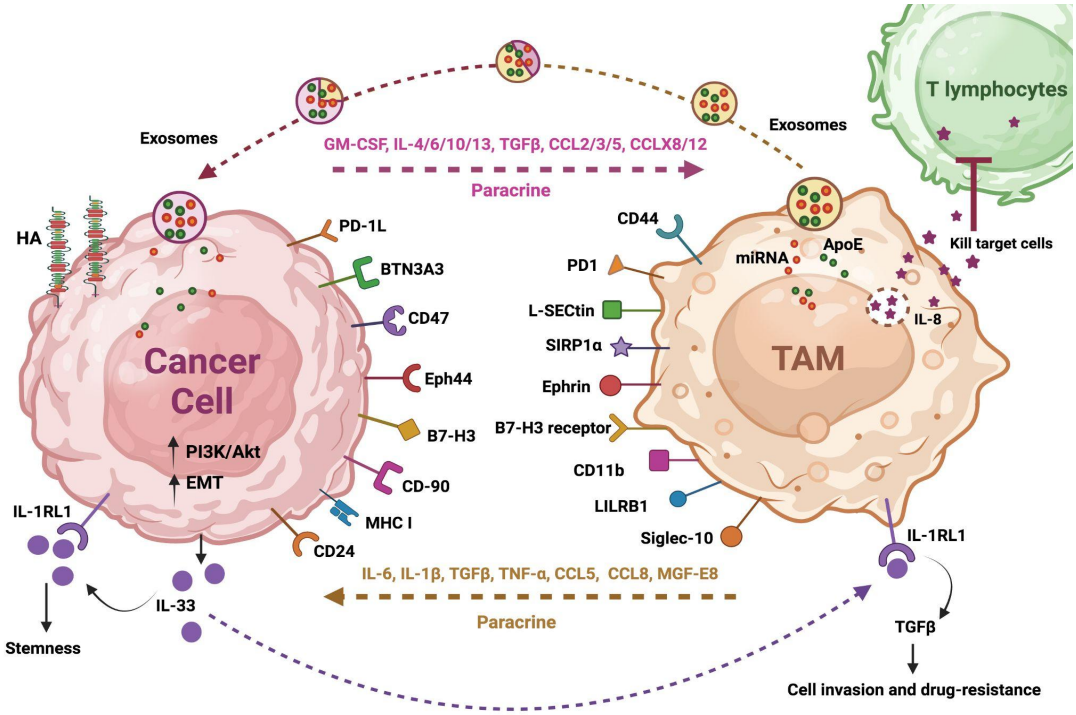


Figure 39. Crosstalk between tumour cells and tumour-associated macrophages (TAMs). TAMs secrete chemokines and cytokines such as IL-6, IL-8, and IL-10, which actively contribute to cancer advancement. Notably, IL-8 released by TAMs exerts cytotoxic effects on T lymphocytes. Additionally, various juxtacrine interactions between tumour cells and TAMs play a pivotal role in inducing immunosuppression. The PD-1/L1 signalling pathway further exacerbates tumour immune evasion by impeding the normal functioning of macrophages and other immune effector cells. Furthermore, the interaction of B7-H3 with its receptor has been implicated in the inhibition of T lymphocytes, thus facilitating tumour immune evasion. The SIRPα/CD47 and CD24/ Siglec-10 pathways serve as the "do-not-eat-me"signal, wherein tumour cells over-expressing CD47 and CD24 are recognized as self-normal cells, thereby evading phagocytosis by macrophages. Another significant mechanism of tumour evasion involves LILRB1/MHC class I component β2-microglobulin, which inhibits the phagocytosis of tumour cells by macrophages. Exosomes facilitate intercellular communication by transporting various molecules, including exosomal mRNA, circRNA, lncRNA, miRNA, lipids, and proteins. Interestingly, exosomes exhibit dynamic alterations in their cargo during transit from the origin to the destination cell. ApoE is highly expressed in TAMs and is transferred, along with other molecules, via exosomes to cancer cells, activating the PI3K-Akt signalling pathway and promoting cytoskeletal remodeling, EMT, and cancer cell migration. Other juxtacrine mechanisms, such as Eph44-ephrin interaction, regulate immune cell processes, including proliferation, survival, apoptosis, activation, and migration. CD44, a transmembrane adhesion molecule, plays a crucial role in binding and metabolizing hyaluronic acid (HA) and serves as an e ective phagocytic receptor, influencing inammation, phagocytosis, and multi-drug resistance. Interactions such as CD44-HA, BTN3N3–L-SECtin, CD90-CD11b, and Eph44-ephrin also also trigger signals that support the maintenance of cancer stem cells. Furthermore, IL-33 released by tumour cells sustains stemness via autocrine interaction with IL-1RL1, while also promoting tumour cell invasion and drug resistance through TAM-mediated TGF-β release and TAM proliferation and differentiation in a paracrine manner. This intricate interplay results in the amplification of the aforementioned crosstalk. Abbreviations: Apolipoprotein E (ApoE), C-C motif chemokine Ligand, (CCL), circular RNA (circRNA), epithelial-mesenchymal transition (EMT), granulocyte-macrophage colony-stimulating factor (GM-CSF), hyaluronic acid (HA), interleukin (IL), Janus kinase (JAK), long non-coding RNA (lncRNA), macrophage colony-stimulating factor (M-CSF), milk fat globule- EGF factor 8 (MGF-E8), major histocompatibility complex (MHC), microRNA (miRNA), messenger RNA (mRNA), programmed death-ligand 1 (PD-L1), sialic acid binding Ig-like lectin (Siglec), signal transducer and activator of transcription 3 (STAT3), tumour-associated macrophages (TAMs), transforming growth factor beta (TGF-β), tumour necrosis factor alpha (TNFα). Created with Biorender.com. (*Toledo, B et al., J. Hematol. Oncol. 2024*).

Interestingly, TAMs' status has also been shown to be strongly linked to cancer stage and prognosis. In general, early-stage tumours are associated with a predominance of anti-tumour TAMs, while advanced-stage tumours are associated with a predominance of pro-tumour TAMs. Therefore, the ratio of pro-tumour to anti-tumour TAMs (also known as the TAM polarization index) has been proposed as a prognostic marker and therapeutic target in cancer [744,745]. Väyrynen and colleagues demonstrated that a high M1:M2 density

ratio in tumour stroma was associated with better cancer-specific survival after the evaluation of the polarization spectrum of TAMs in 931 colorectal carcinomas [746]. However, the localization of these cells has been shown to hold equal or even greater prognostic significance than simply assessing the presence of TAMs in the TME [747] (Figure 40).

Precise percentage figures can vary widely depending on the context and methodology used to study TAMs within the TME [748]. What is well-established is that their presence is linked with poor prognosis in many types of cancer, including breast cancer, ovarian cancer, pancreatic cancer, lung cancer, and melanoma, among others [749-752]. In PC, the density of TAMs, in particular those that are CD163+ and CD204+, is associated with advanced disease stage, increased vascularization, and reduced survival [753]. In breast cancer, high levels of TAM infiltration are associated with poor prognosis and increased risk of relapse [754]. Jeong and colleagues concluded that the infiltration of CD163+ M2-like macrophages in tumour nests was associated with larger tumour sizes and unfavourable prognosis for invasive breast cancer (IBC) patients, and served as an independent prognostic marker for reduced OS as well as DFS [755]. This is largely attributable to TAMs' roles in promoting tumour invasion and metastasis by secreting MMPs and other proteases as established in previous studies [756]. On the other hand, infiltration of CD11c+M1-like macrophages in tumour stroma was shown to be prognostic of increased OS and DFS in IBC patients [755]. Similarly, Mei and colleagues reported in their systematic review and meta-analyses that a high density of M1-like TAMs in the tumour islet was indicative of better OS in NSCLC patients, as compared to high density of M2-like TAMs in the tumour stroma which was associated with worse OS in NSCLC patients [750]. And interestingly, the density of total CD68+TAMs in either tumour islet or stroma was not correlated to patients' OS [750]. These findings once more reinforce the idea that the spatial distribution of M1- and M2-like TAMs is more reflective of disease prognosis than solely assessing the presence of different TAM phenotypes in the TME (Figure 40).

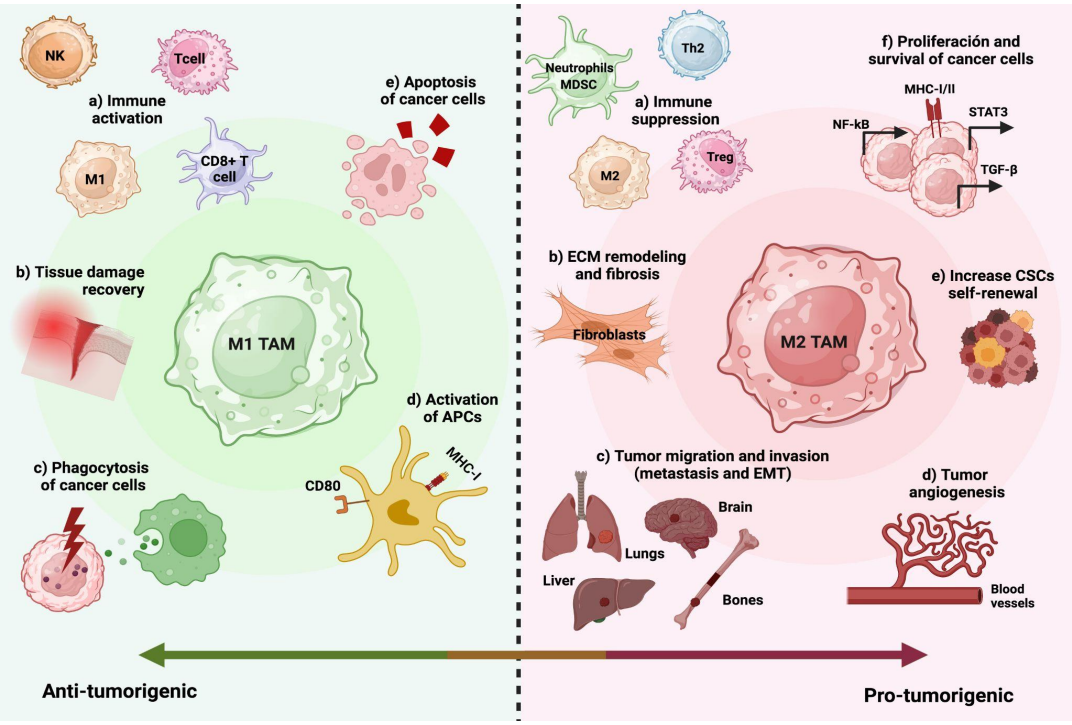


Figure 40. TAMs are involved in various anti- and pro-oncogenic processes. A key characteristic of macrophages is their intrinsic plasticity, the two extremes of which have been identified as M1-type and M2-type polarization. Depending on the paracrine signals they receive as well as the type of tissue, microenvironment and stage of the tumour, they may lead to one phenotype or another. On the left side of the figure, anti-tumoural M1-like macrophages are depicted. These macrophages contribute to T cell recruitment and immune activation, particularly by stimulating NK cells. They exhibit direct cytotoxic and phagocytic effects on tumour cells. Additionally, M1-like macrophages aid in tissue repair, promote the maturation of antigen-presenting cells (APCs) necessary for efficient antigen presentation, and actively induce apoptosis in cancer cells. On the right side of the figure, pro-tumoural M2-like macrophages are illustrated. These macrophages, conditioned by the hypoxic TME, release immunosuppressive mediators, support tumour proliferation, angiogenesis, invasion, and metastasis. They induce epithelial-mesenchymal-transition, facilitate tissue remodeling and in ammation, and enhance the self-renewal rate of CSCs. (*Toledo, B et al., J. Hematol. Oncol. 2024*).

The complex plasticity and context-dependent nature of TAM functions suggests that targeting TAMs for cancer therapy requires careful consideration of the specific TAM phenotype and its interactions with the TME [757,758] (Figures 39 and 40). Presently, several approaches are being explored to target TAMs as a target in cancer therapy, including reprogramming TAMs to an anti-tumourigenic phenotype, inhibiting their recruitment to the tumour site, and

enhancing the anti-tumour immune response [759-761] (Figure 41). However, further research is needed to unravel the complex interplay between macrophages and the TME [762-764].

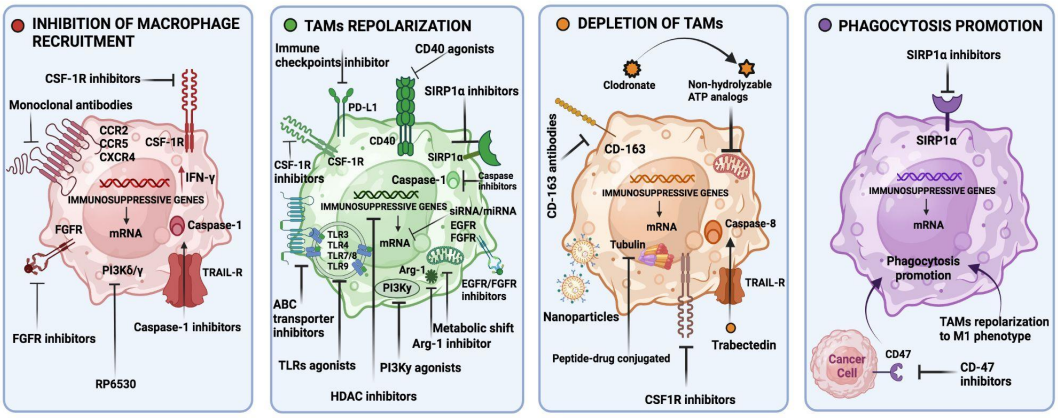


Figure 41. TAM-targeting approaches. An overview of the most promising strategies to combat tumour progression by targeting TAMs in cancer therapy. The approaches are categorized into inhibition of macrophage recruitment (red), repolarization of TAMs (green), depletion of TAMs (yellow), or promotion of phagocytosis (purple). (*Toledo, B et al., J. Hematol. Oncol. 2024*).

Overcoming immunotherapy resistance in pancreatic cancer

Overcoming immunotherapy resistance in cancer is a major focus of ongoing research and clinical trials and several strategies are being explored to enhance the effectiveness of immunotherapy. First of all, a better understanding of which patients will benefit from immunotherapy will certainly be helpful, but unfortunately, markers used in other cancers, such as PD-L1, did not prove to be useful. Currently, in PC, the use of immunotherapy alone is only recommended in MSI cases, however, there could be other subsets of patients that could benefit from it. For example, a small case series on 12 PC patients with homologous recombination deficiency showed that the use of combination immunotherapy with nivolumab and ipilimumab was associated with an ORR of 42%, with 4 patients reaching a complete response. Furthermore, the responders had a much higher level of TIL compared to non-responders, with a T cell inflamed signature on RNA expression analysis [765]. Furthermore, as stated before, several papers

have tried to subclassify PC according to the different levels of immune suppression, but so far these classifications were not correlated to response to immunotherapy [766-768] (Table 5).

Other than a better selection of patients, equally important is the development of better and more effective approaches to stimulate the immune system and combat immune resistance. Over the years, a viable strategy has been the use of combination therapies (Figure 37). The combination of different drugs that can target multiple immune checkpoints has had conflicting results: for example, the use of anti PD-L1 and anti CTLA4 was associated with ORR of around 20% when involving nivolumab and ipilimumab [769], while with the combination of durvalumab and tremelimumab the ORR was less than 5% [770]. Moreover, other combinations are being tested with drugs working on different mechanisms of action, such as immunotherapy plus targeted therapy or chemotherapy. As stated before, the combination of chemo-immunotherapy is now the standard first-line in some cancers, but many more are being evaluated such as anti VEGFR regorafenib + avelumab, or pembrolizumab + Lenvatinib, or chemotherapy plus lenvatinib and anti-PD-1 toripalimab [771-773]. Furthermore, given the immunosuppressive nature of some genetic alterations, the idea of combining target inhibitor and immunotherapy is being explored: anti IDH1 Ivosidenib + nivolumab, anti FGFR2 futibatinib + pembrolizumab, anti MEK cobimetinib + atezolizumab, or the implementation of chimeric antigen receptor-engineered autologous T (CAR-T) cell immunotherapy directed against EGFR positive cells, just cite a few [774,775] (Table 5). Furthermore, other than PD-L1 or CTLA4 inhibition, novel immune targets are being explored, alone or in combination with other immune-stimulating drugs, with the intent to modify the immunosuppressive effect of the tumour microenvironment and boost the effect of immune cells. Some of these targets are, for example: TIM-3, LAG-3, TIGIT, and VISTA plus in PC specifically CD40, CD11b, OX40, IDO, and B7/H3 [776-779].

Many other innovative immune strategies are in the early stages of testing. For example, the use of vaccines or oncolytic viruses, to enhance antigen presentation and improve the recognition of tumour cells by the immune system

[780]. Furthermore, the use of adoptive cell transfer therapy is being explored in different types of solid tumours, including PC [781]. It involves genetically modifying a patient's own immune cells, such as T cells or natural killer cells, to express chimeric antigen receptors or T cell receptors that specifically recognize tumour antigens; these modified cells are then infused back into the patient to target and kill cancer cells. For example, an early trial with the combination of allogeneic natural killer cells in combination with pembrolizumab showed good tolerability and some antitumour activity [782]. Finally, since epigenetic modifications can influence the expression of genes involved in immune response regulation, the modulation of these epigenetic changes through specific drugs has the potential to sensitize the tumour to immunotherapy and overcome resistance: for this reason, several new drugs such as DNA methyltransferase inhibitors, HDAi, BET inhibitors, and EZH2 inhibitors are being tested [783] (Table 5).

To conclude, the road to overcome resistance to immunotherapy is still long. The lack of predictive tools to implement a better selection of patients that will likely benefit from immunotherapy and the complex immunosuppressive environment of PC, are significant challenges that need to be overcome. Hopefully, an even better understanding of the resistance mechanisms at play and the ever-changing new ideas and technologies being studied will allow for a breakthrough soon.

Table 5. Overcoming immunoresistance in pancreatic cancer. (*Toledo, B et al., Exp. Rev. Clin. Pharmacol. 2024*).

Regimen	Phase	N	Median OS (months)	Authors	Year
Ipilimumab + Nivolumab	N.S	12	N.S	Terrero G et al. [765]	2022
Atezolizumab + Autogene Cevumeran + mFOLFIRINOX	I	34	18	Rojas L. A et al. [780]	2023
IMP321 + GEM	I	18	25	Wang-Gillam A et al. [777]	2013
GEM + Nab-paclitaxel + Indoximod	II	135	10.9	Bahary N et al. [778]	2018

Abbreviations: N: number of patients, BTC: biliary tract cancer, N.S: not said, OS: overall survival, GEM: Gemcitabine, mFOLFIRINOX: oxaliplatin, leucovorin, irinotecan and fluorouracil, NK: natural killer, EGFR: epidermal growth factor receptor.

5. TRYPSINOGEN AND CHYMOTRYPSINOGEN - AN OVERVIEW

There is an urgent requirement to create alternative therapeutic approaches that improve the clinical effectiveness of existing treatments for pancreatic ductal adenocarcinoma (PDAC), particularly in light of the significant side effects linked to traditional chemotherapy and radiotherapy. In this context, British embryologist John Beard introduced the "trophoblastic model of cancer" in the early 20th century, a concept that was later revisited by Kelley in the mid-20th century and by González and Isaac at the close of the 20th century and the start of the 21st. Drawing from his research on embryonic development, Beard proposed that pancreatic enzymes, including trypsin, played a role in regulating the trophoblast—which exhibits behavior akin to cancerous cells—and could therefore be utilized in cancer treatment [784]. Over the last few decades, a multitude of studies have highlighted the anticancer properties of pancreatic enzymes [revised in 785].

5.1 Trypsinogen and Chymotrypsinogen

By secreting a number of enzymes that are necessary for the breakdown of nutrients, the pancreas plays an important part in digestion. As zymogens—also known as (pro)enzymes—these enzymes are produced by acinar cells [786]. Zymogens are transported to the small intestine and activated there after being secreted. Trypsinogen and chymotrypsinogen are the zymogens that receive the most research. Enterokinase converts trypsinogen into trypsin in the small intestine. Trypsin can activate other pancreatic zymogens once it is activated, such as the transformation of chymotrypsinogen into chymotrypsin [787]. Poor nutrient absorption can result from a lack of these enzymes' production. Exocrine pancreatic insufficiency is most frequently caused by chronic pancreatitis and cystic fibrosis [788] (Figure 42).

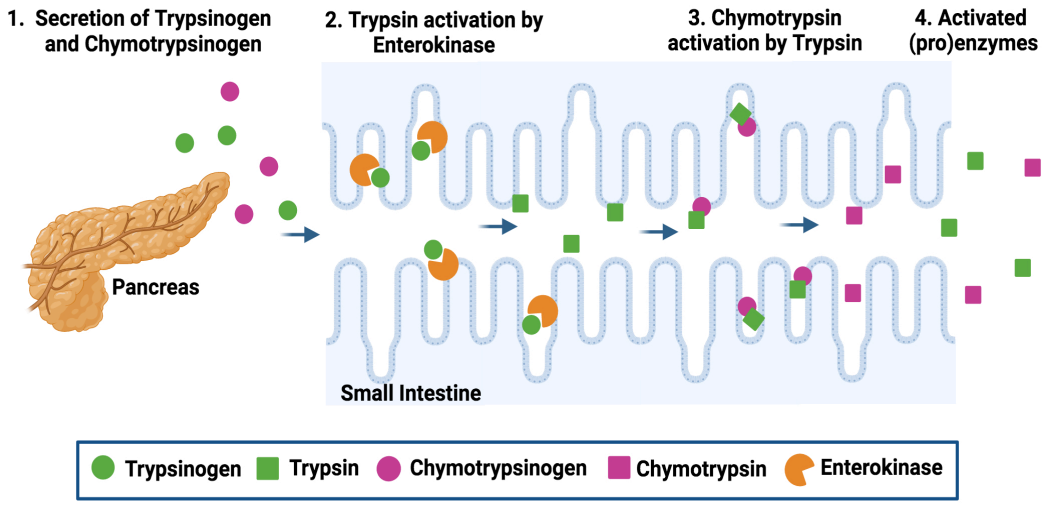


Figure 42. Activation mechanism of (pro)enzymes in the small intestine based on González-Titos et al.'s study (2021). Image created with Biorender.com.

Despite their structural similarity, both (pro)enzymes belong to the serine protease family and have important differences. The (pro)enzymes have distinct substrate affinities because of the amino acids that make up their active sites, one of the most significant distinctions [789]. In addition, a number of isoforms with various functions have been identified [790,791].

In addition, it has been demonstrated through research that the human pancreas produces a variety of isoforms of Trypsinogen and Chymotrypsinogen. In particular, Trypsinogen's three distinct isoforms have been identified: Mesotrypsinogen, an anionic isoform, and a cationic isoform The most common is the cationic isoform, followed by the anionic isoform and the Mesotrypsinogen, which makes up less than 5 percent [790,792]. The optimal pH, amino acid composition, and molecular weight of the cationic and anionic Trypsinogens are all comparable [793]. When it comes to chymotrypsinogen, it includes chymotrypsinogen A, which is the most widespread isoform and, upon activation, changes into chymotrypsin A; Chymotrypsinogen B, the other primary isoform that transforms into chymotrypsin B upon activation; and Chymotrypsinogen C, which can be activated into chymotrypsin C, also known as elastase-2A, despite being less common than the other two.

5.2 The impact of pancreatic proteinases on tissue repair, inflammation and the immune system

Trypsin and chymotrypsin have been used to treat burns, fractures, and accidental and surgical injuries ever since the 1960s [794]. In addition, a number of studies have shown that this combination of pancreatic enzymes can be used as an analgesic as well as an anti-inflammatory, anti-edematous, fibrinolytic, antioxidant, and anti-infective agent [795] (Figure 43). Trypsin and chymotrypsin are especially effective when taken orally to treat the early stages of inflammation. Compared to plasmin, 1-antitrypsin has a higher affinity for trypsin and chymotrypsin, allowing plasmin to remain available for fibrinolysis for a shorter time. Blood clots are reduced, circulation is restored, inflammation and edema are reduced, and damaged tissue is encouraged to heal through this process [796]. Patients undergoing orthopedic surgery or suffering from sciatica treated with the trypsin-chymotrypsin combination had shorter recovery times and reported experiencing no adverse effects [794]. In addition, this enzyme combination reduced inflammation and helped burn patients recover quickly [797] (Figure 43).

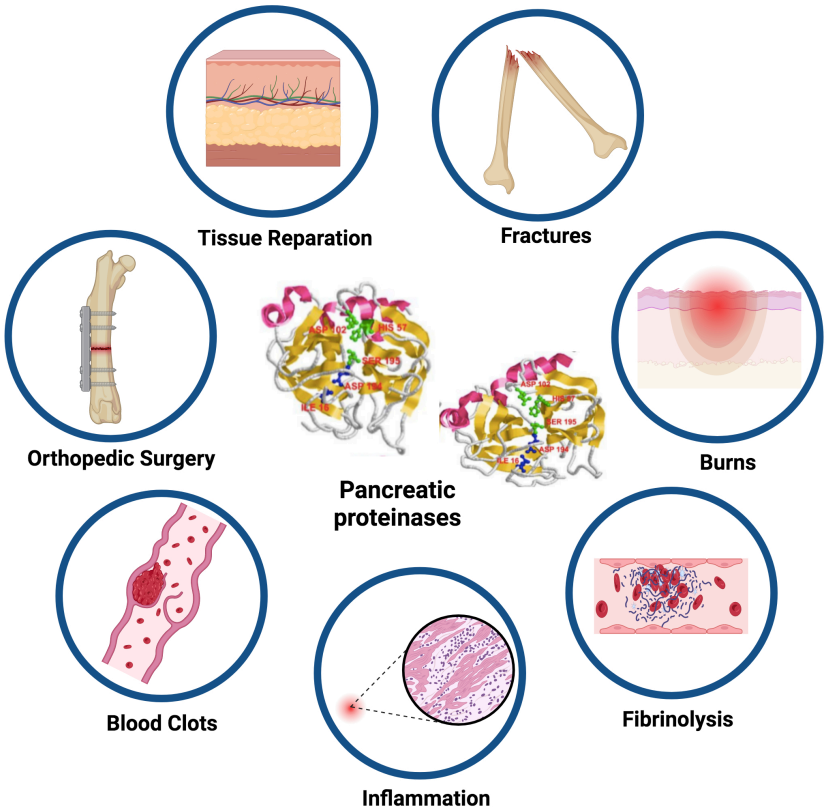


Figure 43. Applications of pancreatic proteinases related to tissue repair, inflammation and immune system. Image created with Biorender.com.

5.3 Anti-tumour effects of pancreatic proteinases

In conclusion, pancreatic enzymes actively aid in the reduction of inflammation and enhancement of the immune system, both of which are essential components in the formation of tumours.

As a cancer treatment, enzyme therapy has been used in a particular order. Patients served as the subject of the initial trials, which were followed by animal models and *in vitro* testing. We will present a chronology-based summary of these advancements here.

The safety and efficacy of pancreatic enzyme therapy have been evaluated in numerous cancer research studies. A summary of these studies, which included over 1600 patients treated with pancreatic enzymes for a maximum of seven years, can be found in Table 6. In a variety of cancers, including pancreatic adenocarcinoma [798], cervical cancer [799], head and neck cancer [800], colorectal cancer [801], pancreatic cancer [802], multiple myeloma [803], and breast cancer [804, 805], treatment with pancreatic enzymes led to improved survival rates, reduced disease recurrence, and minimized the occurrence of metastasis. Additionally, the enzymes were reported to be tolerable and to have no side effects.

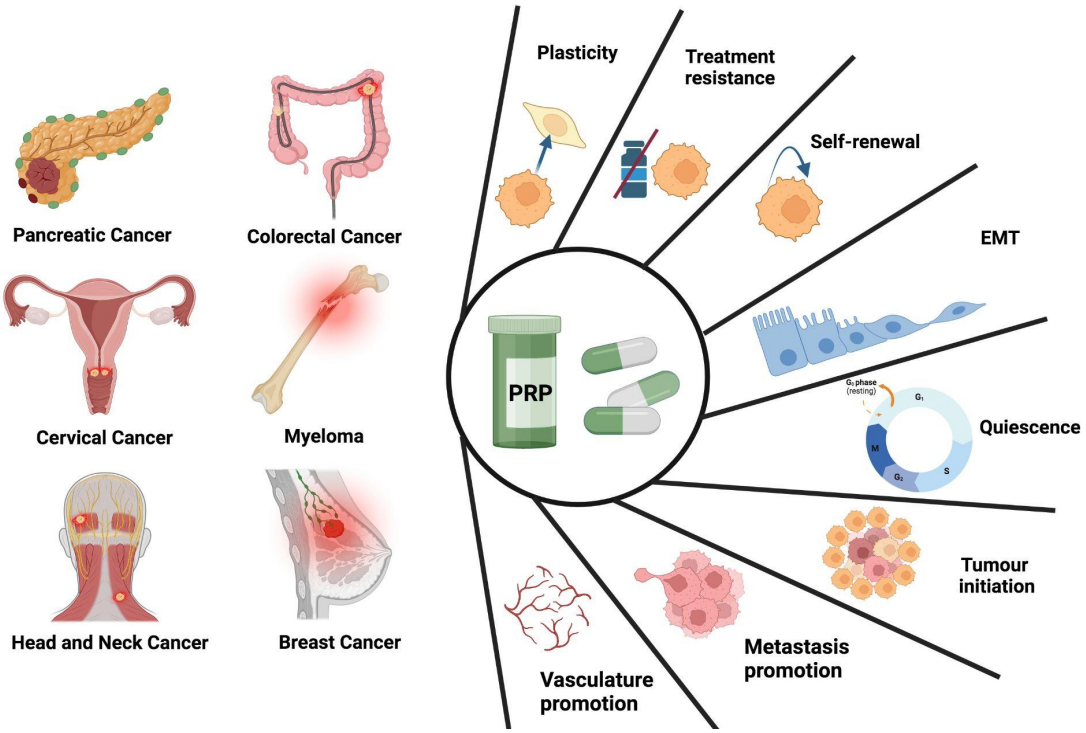


Figure 44. Different cancer models where the effectiveness of PRP has been evaluated are shown on the left. The fundamental processes during cancer that have been inhibited by PRP treatment are detailed on the right. Image created with Biorender.com.

However, it is essential to acknowledge these studies' limitations. Some studies were poorly designed, and the absence of double-blinding in some instances may have reduced the significance of the clinical outcomes, despite the fact that the overall results indicated a positive impact on cancer treatment. For instance, Gonzalez and Isaacs [798] discovered that patients who received pancreatic enzyme therapy had longer periods of survival. However, this study lacked a control group for comparison and had a small number of patients. Likewise, Dale et al. [799] demonstrated that when used as an adjuvant therapy, the treatment aids in the reduction of radiotherapy-related symptoms; however, the study lacked double-blinding and did not examine the effect of the enzyme treatment alone on patient survival. Other studies have shown that pancreatic enzymes can lessen the side effects of chemotherapy and radiation therapy [800-802], but the short duration of these trials may make the results less significant.

On the other hand, Sakalová and colleagues' better-designed study [803] found that patients with stage III multiple myeloma who received enzyme treatment outlived those who did not, with survival times of 83 months and 47 months, respectively. Beuth and co. [804] as well as Peran et al. [806] also found that tumour patients treated with pancreatic enzymes had longer survival times.

Last but not least, it is important to mention the conclusion of a controlled, observational study by Chabot and colleagues that compared Gemcitabine-based chemotherapy and pancreatic proteolytic enzyme therapy for PC treatment. "Among patients with pancreatic cancer, those who chose Gemcitabine-based chemotherapy survived more than three times as long and had a better quality of life than those who chose proteolytic enzyme treatment" was the study's conclusion [802].

Proteolytic enzyme treatment's anti-metastatic and anti-tumour effects were demonstrated in animal models in studies. A wide range of tumours induced in mice, including melanoma [807,808], Lewis lung carcinoma [809], mammary tumour [810], ovarian cancer [806], and pancreatic cancer [806,811,812], all produced positive results. The main findings of these studies are summarized in Table 7, which suggests that these digestive proteases have a lot of potential as an alternative treatment for cancer. PRP's efficacy in advanced solid tumours was designed to be evaluated, and a variety of studies and assays were used to confirm it. After Caco-2 cells were treated with PRP, which induced enterocytic differentiation, promising results were obtained [813]. The cells developed cell-specific differentiated structures like microvilli and tight junctions, giving them a polarized structure and an enterocyte-like phenotype. This finding is consistent with other research [814] that shows that some colon cancer cell lines (HT-29, Caco-2) are induced to form tight junctions or domes by pancreatic trypsin.

In addition, the pancreatic tumour cell line Panc-1 also demonstrated that the formulation of (pro)enzymes induces cell differentiation. Pancreatic cell differentiation is characterized by the formation of islet-like clusters by treated Panc-1 cells [815]. Other studies indicate that trypsinogen and chymotrypsinogen

treatment results in the formation of cellular aggregates similar to multicellular spheroids. They also talked about the benefits of using (pro)enzymes rather than active enzymes because (pro)enzymes can be activated specifically in cancer cells but not in healthy ones [816]. An increase in lamellar bodies and enhanced expression of markers associated with pancreatic differentiation further supported the differentiation of Panc-1 cells [813]. As a result, lineage-specific cellular differentiation and pancreatic enzymes may facilitate the re-entry of tumour cells into normal pathways, resulting in a less malignant phenotype and reduced proliferative capacity [813]. In addition, matrigel-based tube formation and fibrous capsule formation assays were used to evaluate PRP's antiangiogenic potential, and the results showed that the treatment reduced the number of capillary-like structures [806] (Figure 44).

Pancreatic CSCs, which are tumour-initiating cells thought to be responsible for metastasis due to their ability to remain dormant, were also used to test the PRP formulation's anti-cancer effects [817]. The expression of CSC-related markers such as ALDH [818], CD44, CD326 and CXCR4 [819] was found to decrease as a result of the treatment with PRP. Additionally, PRP treatment increased cell adhesion, differentiation, and expression of tumour suppressor genes while also promoting downregulation of essential genes associated with EMT. As a result, the treatment with (pro)enzymes prevented the expression of genes associated with the CSC phenotype, resulting in these cancerous cells becoming more differentiated and less harmful (Figure 44).

Table 6. Adjuvant proteolytic enzyme therapy in cancer patients' trials.

Trial Design/Cancer Type/No of patients/Reference	**Adjuvant Proteolityc/Enzyme therapy/Treatment Duration**	**Main Results**
Unblinded-single-arm/Adenocarcinoma/Treated group: 11 products / 2 years [798]	25-40 g of porcine lyophilised pancreas	Increased survival rate in the treated patients (17 months)
Retrospective cohort, with parallel groups/ Non-metastatic Breast cancer/ Control group: 1056- Treated group: 1283/ [804]	Mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe Mugos E)/ 485 days	Reduction of radiation therapy/chemotherapy side effects. Reduction of the disease-associated symptoms. Metastasis and recurrence decreased in treated patients.
Retrolective cohort analysis with parallel groups/ Colorrectal cancer/ Control group: 626 - Treated group: 616/ [801]	Mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe Mugos E)/ 9.2 months	Increased survival of treated patients (34.1 months). Reduction of the disease- associated symptoms
Retrolective cohort analysis, with parallel groups/ Multiple myeloma/ Control group: 99 - Treated group: 166/ [803]	Mixed proteolytic enzymes papain (100 mg), trypsin (40 mg) and chymotrypsin (40 mg) (Wobe Mugos E)/ 61 months	Increased survival of treated patients (3 years). Good proteolytic enzyme therapy tolerability
Prospective randomized open study/ Uterine cervix cancer/ Control group: 60 - Treated group: 60/ [799]	Radiation therapy + mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe Mugos E)/ Less than 2.5 months	Reduction of radiation therapy side effect
Non randomized study/ Breast cancer/ Treated group: 57/ [805]	Chemotherapy + mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe Mugos E)/ One year	Good proteolytic enzyme therapy tolerability
Non randomized controlled study/ Pancreatic cancer/ Control group: 23 - Treated group: 32/ [802]	Pancreatic enzymes, nutritional supplements, detoxification and organic diet/ 40 months	No effect was seen in the group of those treated with enzymes

Non randomized study/ Several types of cancer/ Treated group: 46/ [806]	Suppository formulation containing 8.92 mg of each bovine pancreatic (pro)enzymes (Trypsinogen and Chymotrypsinogen A) and 1.78 mg of α-amylase/ 9 months	Increased survival of treated patients

Table 7. Summary of *in vivo* assays with proteolytic enzyme treatment.

Animal model/ Tumour cells/ Duration of treatment/ Reference	**Treatment**	**Main results**
Female mice C57Bl6/ Melanoma cells (B16)/ 100 days/ [807]	45 mg/kg of the mixed proteolytic enzymes papain, trypsin and chymotrypsin (Wobe-Mugos E) rectally administered, twice daily.	Survival rate increased in the treated group (58.3%). Metastasis decreased in treated mice.
Female C57Bl6 mice/ Lewis lung carcinoma cells/ 100 days / [808]	0.1 mL saline solution containing 0.5 mg papain, 0.5 mg trypsin and 0.2 mg chymotrypsin, rectally administered, twice daily. Treatment commenced: (A): after extirpation; (B): 6 days before extirpation; (C): inmediately following transplantation	Survival rate increased in the treated groups (A: 60%; B: 90% and C: 100%). Metastasis decreased in treated mice.
Female F-344 rat/ Rat mammary tumour (R13762) cells/ 50 days/ [810]	20 g or 2 g porcine pancreatic extract (2 groups for each condition) + 10 mg Mg++ daily (only one group for each condition). Orally administered, daily.	No differences in the tumour growth were observed between the treated groups and control group. Also, no differences in the incidence of rats with pulmonary metastasis.
Female C56Bl6 mice/ Melanoma cells (B16)/ 100 days/ [820]	0.1 ml of 0.2 mg trypsin, 0.5 mg papain and 0.2 mg chymotrypsin rectally administered, twice daily. Treatment commenced: (E1): 24 h after intracutaneous transplantation; (E2) after extirpation of the primary Bl6 melanoma	Treated E1 group showed no tumour formation (36%) and small size of the tumour. Survival increased in treated groups (E1: 46% and E2: 30%). Metastasis and recurrence decreased in treated mice.
Male beige XID nude mice/ Pancreatic cancer cells (AsPC1)/ 60 days/ [812]	400 mg/kg of porcine pancreatic extracts, Orally administered, daily.	Survival rate increased in the treated group (79%). Tumour size decreased in the treated mice. No reported side effects

Female athymic Nude- Fox1nu mice and female C57BL/6 mice/ Human ovarian cancer cells (A2780) and mouse pancreatic tumour cells (Pan02)/ One month/ [806]	Ovarian tumour: 27.5/165 mg/kg (Group 2); 83.3/500 mg/kg (Group 3). Pancreatic tumour: 9.1/54 mg/kg (Group 2); 27.5/165 mg/kg (group 3). Intravenously injected, 3 days per week.	Tumour weight in the ovarian cancer mice treated with 83.3/500 mg/kg decreased. Tumour weight in the pancreatic cancer mice treated with both doses decreased.
NSG immunodeficient mice/ Pancreatic cancer (BxPc3 cancer stem cells)/ 3 months/ [811]	83.3/500 mg/kg Trypsinogen/Chymotrypsinogen A Intravenously injected, 3 days per week. Pre-treatment group: injections started 3 weeks before tumour induction. Pre-treatment+treatment group: injections started 3 weeks before tumour induction and followed for 9.5 weeks after tumour induction.	Tumour incidence decreased in treated groups. (41% in the pre-treated mice and 50% in the pre-treated+treatment group). Tumour growth decreased in treated mice.

5.4 Development of a novel anticancer treatment based on pancreatic (pro)enzymes formulation

Our group focused on developing a novel therapeutic drug in light of the evidence for the potential use of pancreatic (pro)enzymes as a cancer treatment. To begin, we conducted a blind test with a variety of protease combinations to determine the IC50 for a wide variety of cancer cell types. a collection of (pro)enzymes from the pancreas: A selection of trypsinogen, chymotrypsinogen, and amylase were tested further on three distinct cancer cell lines (Caco-2; OE33 and Panc1) The findings demonstrated that the treatment caused tumour cells to re-differentiate into a "healthy phenotype" similar to normal cells. In point of fact, colon cancer Caco-2 cells developed a polarized structure with an enterocyte-like phenotype and the development of cell-specific differentiated structures like tight junctions and microvilli. The pancreatic tumour cell line Panc-1 also demonstrated that the formulation of (pro)enzymes induces cell differentiation. Increased lamellar bodies, islet-like clusters, and enhanced expression of markers related to pancreatic differentiation were seen in treated Panc-1 cells [813]. Additionally, the treatment with the pancreatic (pro)enzymes increased the expression of epithelial markers like E-cadherin and decreased the expression of specific EMT markers [813].

Next, our group indentified that the optimal ratio of action of a trypsinogen and chymotrypsinogen A formulation was 1:6. The study was conduted evaluating the antiproliferative effects of different (pro)enzyme combinations across 24 human cell lines representing various carcinomas. The optimal formulation was named PRP [806].

It was proved that PRP reduced tumour cell proliferation, cell migration and antiangiogenesis [806].

Pancreatic CSCs, which are tumour-initiating cells that are thought to be responsible for metastasis due to their quiescent capacities, were also used to test the PRP formulation's anti-cancer effects [811,817]. The expression of CSC-related markers such as ALDH [818], CD44, CD326 and CXCR4 [819] was found to decrease as a result of the treatment with PRP. Additionally, PRP treatment increased cell adhesion, differentiation, and expression of tumour suppressor

genes while also promoting downregulation of essential genes associated with EMT. As a result, the treatment with (pro)enzymes prevented the expression of genes associated with the CSC phenotype, making these cancerous cells more differentiated and less harmful.

Figure 44 summarizes the potential effects of PRP treatment on various cancer-related events, as tested in different tumour models.

The cascade of reactions induced by the (pro)enzymes treatment is summarized in Figure 45. Briefly, PRP treatment may lead to the upregulation of RAC1β, which could prevent the hyperactivation of the TGF-β pathway. This, in turn, may inhibit the TGF-β-induced p38 pathway, resulting in the phosphorylation of YAP, which can then inhibit the Notch pathway. Additionally, phosphorylated YAP sequesters β-catenin in the cytoplasm, potentially blocking the canonical Wnt pathway [811]. These events contribute to the inhibition of the EMT process, a phenomenon known to give rise to the CSC phenotype and facilitate metastatic dissemination. Indeed, the EMT process significantly boosts metastasis-causing migration and invasion of surrounding tissues due to the loss of differentiation-associated features and cell-cell contacts [821]. As a result, the effects of PRP on cell differentiation, angiogenesis, stem-like phenotype inhibition, and EMT inhibition may represent a significant step forward in cancer treatment (Figure 45).

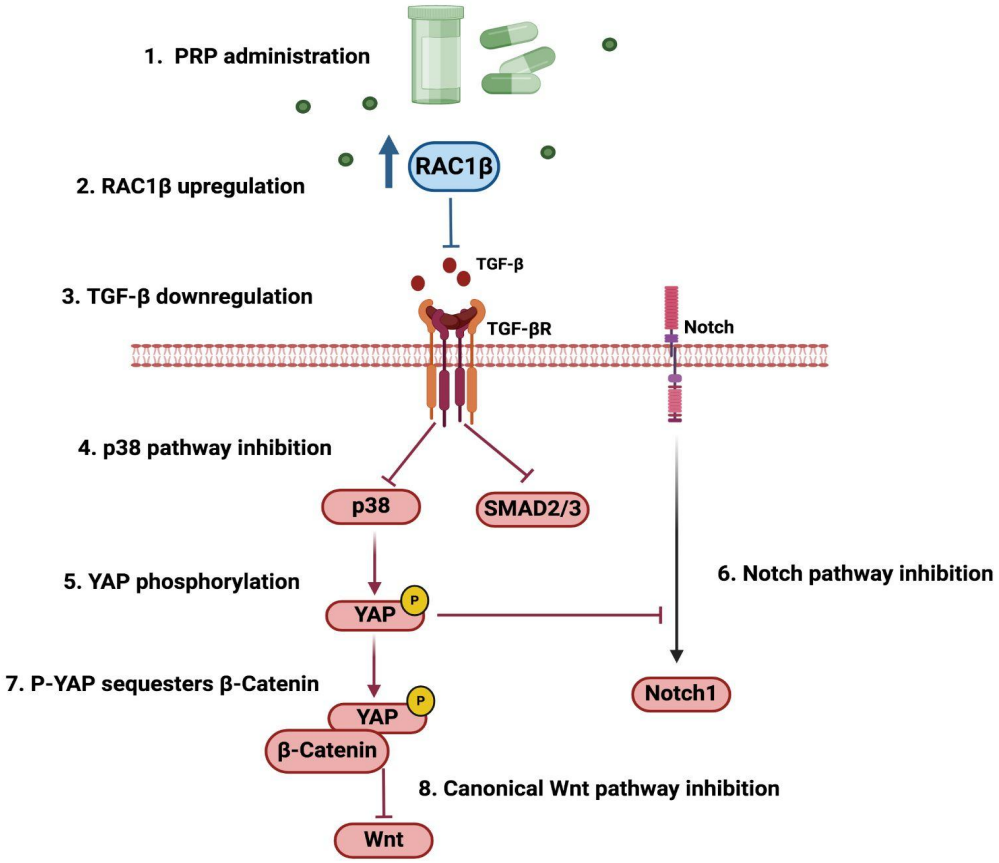


Figure 45. Possible effects of PRP treatment in different cancer related-pathways. Image created with Biorender.

HYPOTHESIS

HYPOTHESIS

The tumour microenvironment plays a crucial role in tumour development, invasion, metastasis, recurrence, and chemoresistance. PDAC is particularly notable for its highly fibrotic tissue, where CAFs are key components. These cells secrete various growth factors, cytokines, and ECM proteins, creating a supportive niche for tumour cells. This environment not only facilitates tumour growth and metastasis but also impairs the effectiveness of chemotherapeutic drugs. Most cancer cells in PDAC exhibit either intrinsic or acquired chemoresistance, leading to increased aggressiveness and the development of anti-apoptotic and DNA repair mechanisms. Consequently, the efficacy of standard chemotherapeutic agents is significantly reduced, resulting in poor therapeutic outcomes, rapid tumour progression, and high recurrence rates.

Recent studies have highlighted the antitumour potential of pancreatic (pro)enzymes, such as trypsinogen and chymotrypsinogen, particularly when used in combination as PRP. This formulation has been shown to induce re-differentiation of malignant cells toward a less malignant phenotype, reduce tumour cells and CSCs proliferation, and promote cell adhesion. Furthermore, treatment with these (pro)enzymes has been effective in inhibiting EMT, thereby reducing tumour aggressiveness, invasion, and metastasis.

The observed interference of PRP treatment with various cancer-related pathways suggests it could play a significant role in mitigating TME and chemoresistance. PRP could effectively reduce the CAF population within the TME by re-educating malignant cells toward a normal phenotype, disrupting the crosstalk between tumour cells and CAFs, and alleviating the fibrotic nature of the PDAC microenvironment. Additionally, the adjuvant use of PRP could enhance the anticancer effects of GEM and diminish the GEM-chemoresistant phenotype. Thus, PRP holds promise as a strategy to reduce chemoresistance and improve therapeutic outcomes in PDAC patients.

OBJECTIVES

OBJECTIVES

The primary objective of this doctoral thesis was to evaluate the impact of a synergistic combination of two pancreatic (pro)enzymes (trypsinogen and chymotrypsinogen A) on TME development, CAFs and tumour cell crosstalk, pre-metastatic niche formation, and GEM chemoresistance in a model of PDAC.

The secondary objectives are:

1. To deeply review recent studies on the development of the TME, macrophage interactions with cancer, and mechanisms of chemoresistance.
2. To induce the malignification of human fibroblasts through direct and indirect co-culture of pancreatic BxPC3 tumour cells and BxPC3-CSCs.
3. To evaluate the effect of the PRP formulation on the induction of malignification in fibroblasts caused by CSCs and over tumoural cells.
4. To evaluate the effect of the PRP formulation over tumoural cells.
5. To evaluate the modulation by PRP over the interaction between CAFs and non-tumoural cells within the TME.
6. To study the induction of a cancer-restraining CAFs like phenotype by the application of PRP.
7. To evaluate the effect of PRP over pathways involved in fibroblasts' malignification such as TGF-β pathway.
8. To determine whether PRP induces apoptosis in normal adult fibroblasts and increases apoptotic susceptibility in CAFs.
9. To evaluate *in vivo* the effect of PRP treatment on TME using a pancreatic cancer mouse model.
10. To obtain and characterize BxPC3 pancreatic cells resistant to GEM.
11. To evaluate whether PRP treatment can enhance GEM sensitivity in chemoresistant pancreatic cells.
12. To identify pathways that could be modulated by PRP in GEM-resistant cells.

MATERIALS AND METHODS

MATERIALS AND METHODS

Pancreatic (pro)enzymes

The bovine pancreatic (pro)enzymes Chymotrypsinogen and Trypsinogen were supplied by AppliChem (UK). A stock solution containing 6 mg/ml of Chymotrypsinogen A and 1 mg/ml of Trypsinogen was prepared by dissolving the pancreatic enzymes in phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO), and stored at −20°C. For each experiment, the stock solution was diluted in medium to achieve the desired concentrations. The final concentrations of the (pro)enzymes were selected based on literature data and previous clinical experiences [822].

Cell culture

Three PDAC cells lines were used: The human pancreatic cell line BxPC3 (ECACC 93120816) was sourced from the American Type Culture Collection (ATCC) (Rockville, MD, USA); Patu-T (mesenchymal phenotype) and Suit-2.007 (mesenchymal phenotype), kindly provided by Prof. Elisa Giovannetti (Vrije Universiteit Amsterdam, Amsterdam, The Netherlands). BxPC3 and Suit-2 were cultured in RPMI supplemented with 10% heat-inactivated bovine fetal serum and 1% penicillin/streptomycin. Patu-T was maintained in DMEM, supplemented the same way.

NAFs were isolated from human skin tissue obtained during abdominoplasty surgery and properly characterized (ethics committee reference: 0467-N-20); CAFs were obtained through various co-culture methods with tumour cells (explained below); CAF-educated fibroblasts (CEFs) were generated by culturing NAFs in CAFs conditioned medium for 14 days (explained below). Primary human umbilical vein endothelial cells (HUVECs) were also obtained from the ATCC (PCS-100-010) and cultured in EGM-2 growth medium (Lonza, Basel, Switzerland) supplemented with 2% fetal bovine serum (FBS) and endothelial growth supplement (BD Biosciences, San Diego, CA).

All cell lines were routinely maintained in T75 culture flasks at 37°C in a

humidified atmosphere containing 95% of air and 5% CO_2. The culture media used were: RPMI-1640 Medium for BxPC3 and Suit-2; Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA) for Patu-T, NAFs and CAFs; a mixture of 30% DMEM and 70% medium obtained from CAFs for the growth of CEFs. All media were supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 100 IU/ml penicillin (Invitrogen, Merelbeke, Belgium), and 100 µg/ml streptomycin (Invitrogen, Merelbeke, Belgium). HUVECs were cultured on collagen-coated Cytodex microcarrier beads (Sigma-Aldrich, St. Louis, MO). Cell dissociation for passaging was performed using the trypsin replacement TrypLE reagent (Life Technologies, Carlsbad, California, USA). Fibroblasts between passages 2-15 and HUVECs below passage 6 were used for all experiments.

Drugs administration

Throughout this Thesis, different types of treatments based on different drugs will be named, which are explained below:

Control or CTR: when no drug was administered to the cells.

+ GEM: cells received a concentration of 16 nM of GEM for each mL of medium. This administration was carried out every 72 h (the estimated number of times could be applied).

+ PRP: a volume of 12 mL of the final PRP mix was administered to the cells for each mL of medium. This amount was administered twice at an interval of 72 h.

+PRP+GEM: both drugs were administered together into the medium following the amount described above.

+PRP→GEM: once the two pulses of PRP were administered, we waited 72 h to administer in this case the corresponding amount of GEM (and could be applied the estimated times).

Isolation of healthy fibroblasts from human abdominal sample

Abdominal skin samples of 9 cm^2 in size are transported in DPBS from the operating room to the laboratory for the start of processing. In a laminar flow cabinet, they are kept in washing solution (penicillin 600,000 Ul and streptomycin

600,000 Ul in DPBS) for 30 minutes.

With the help of a scalpel and tweezers, the dermis is separated from the epidermis. The dermis is mechanically processed and deposited in a 50mL falcon. Subsequently, 2 mg/ml of collagenase type I (Gibco) is added and incubated for 18-24 hours at 37ºC while shaking. The solution is then neutralized with double the volume of specific culture medium for fibroblast culture (10% FBS). Finally, it is filtered through a 100 μm sterile filter and the resulting solution is centrifuged at 300g for 10 minutes.

Cells are seeded at a density of 100,000-140,000 cells/cm^2 in DMEM. Medium changes are made every 2-3 days, until a confluency of >80% is reached. Once confluence is reached, cell passage is carried out, which consists of cell recovery with TrypLE Select 1X at 37ºC and then seeding at a density of 2500-5000 viable cells/cm^2.

Primary and secondary sphere formation assay

An enriched subpopulation of cancer stem-like cells from the BxPC3 pancreatic cancer (adenocarcinoma) cell line was cultured in our specific sphere-forming medium (WO2016020572A1) [823] in ultralow attachment plates (Corning). Primary spheres were manually counted after 72 hours, disaggregated with TrypLE, and re-seeded to form secondary spheres after 72 hours (Supplementary 2).

Generation of resistant cell lines

To establish Gemcitabine-resistant (GR) cell lines, concentrations causing 50% reduction in cell growth (IC_{50}) were determined in parental cells. Cells were then exposed to the respective $IC_{50}/2$ of the drug and grown for at least 2 weeks with the drug until reaching 80% confluency. After acquiring resistance, the drug concentration was doubled (IC_{50}) and cells were cultured until they could grow to confluence. The process was repeated with stepwise increasing drug concentrations until the maximum tolerated concentration was reached after 15-18 weeks (Figure 28). Parental cells never exposed to the drug were cultured in

parallel with the resistant cells. To determine stable resistance, GR cells were grown in drug-free medium and baseline growth and resistance to the maximum tolerated concentration was analyzed by MTT assay at regular intervals for up to 2 months. The resistance factor was calculated as the ratio of the IC_{50} of resistant- versus IC_{50} of parental-cells.

2D Co-culture assay

To perform indirect co-culture, six-well plates (353502, Corning, Durham, USA) with polystyrene transwell inserts (353090, Corning, Durham, USA) with a pore size of 0.4 microns were utilized. 80,000 NAFs were seeded into each well of the plate. After 24 hours, two different co-cultures were established: i) 120,000 BxPC3 cells were seeded per transwell, or ii) CSCs derived from secondary spheres (originated from 300,000 BxPC3 cells) were added per transwell. 1.5 mL and 1 mL of medium were added to the well and transwell, respectively, with medium refreshed every three days.

For direct co-culture, 80,000 NAFs together with an enriched subpopulation of CSCs derived from secondary spheres (originated from 300,000 BxPC3 cells) were seeded per well of a 6-well plate with 2 mL of medium. Medium change was performed every 72 hours until reaching 90% confluence. After 14 days, fibroblasts were isolated using TrypLE reagent as they required a shorter detachment time compared to the tumour cells and were characterized with specific CAF markers.

For both types of cocultures, a prior evaluation was carried out to establish which medium was the most optimal that both cell types shared. Among the options (RPMI, DMEM and sphere medium), it was determined to apply sphere medium with 1% FBS (Supplementary 3)

Cells were treated twice with PRP (T/C 0.07/0.42 mg/mL), 24 hours after seeding and 72 hours after the first treatment, under three conditions: i) cancer cells were treated before co-culture with fibroblasts; ii) PRP was added directly into the co-culture; and iii) fibroblasts were treated after the co-culture, resulting in the formation of CAFs (Supplementary 4).

3D direct co-culture assay

An enriched subpopulation of CSCs derived from secondary spheres (originated from 300,000 BxPC3 cells) was seeded per well of a 6-well plate until full confluence was achieved. Simultaneously, 80,000 fibroblasts were also cultured to confluence in other wells. Confluent monolayer cultures of fibroblasts and BxPC3 cells were detached from the wells using a micropipette tip and introduced into 15 mL tubes with 5 mL of DMEM, aiming to maintain the BxPC3 monolayer inside and the fibroblasts outside, simulating a protective barrier. Tubes were centrifuged at 300g and 21°C for 5 minutes to form pellets, which were then incubated at 37°C with 5% CO_2 for 24 hours without agitation. The following day, tubes were gently tapped for pellet separation and medium change was performed while avoiding touching the pellet. Two PRP treatment models were implemented: maintaining the concentration used so far with established 72-hour breaks, or simulating a more frequent chemotherapy treatment with four doses instead of two, while halving the concentration (Figure 46).

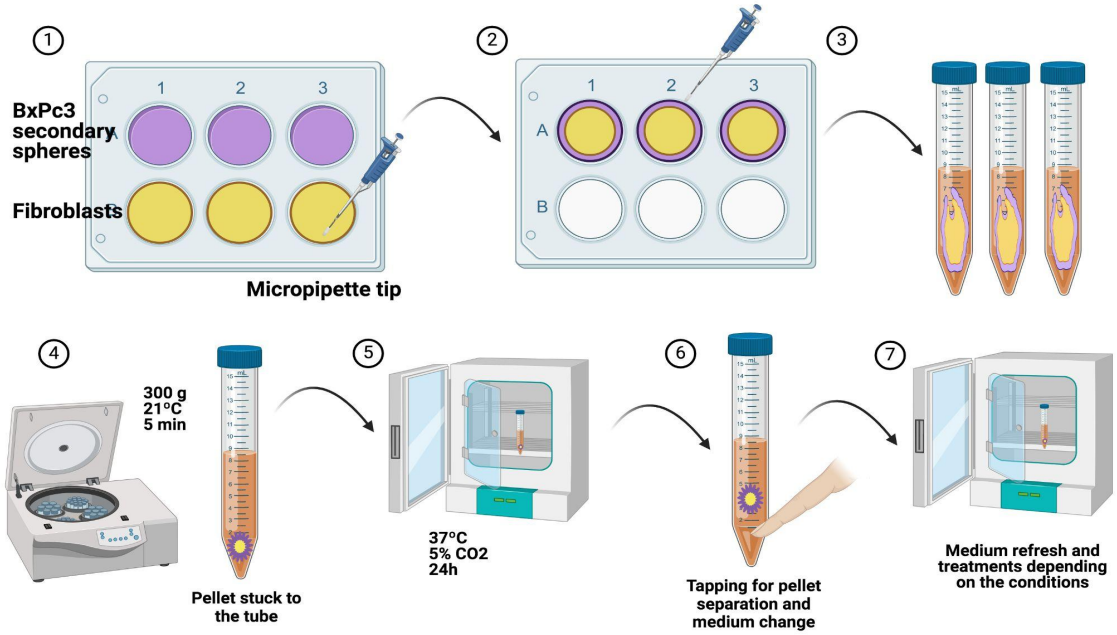


Figure 46. Protocol for the 3D direct co-culture assay. Image created with Biorender (pending publication).

Conditioned Medium (CM)

Culture medium was collected from different cell cultures under various conditions and labeled as follows (Figure 47):

i) Normal Medium (NM): Depending on the cells, it could be DMEM or RPMI.

ii) NAF CM: Medium collected from NAFs cultured in T75 flasks.

iii) NAF+PRP CM: NAFs were treated twice with PRP (T/C 0.07/0.42 mg/mL) on day 1 and day 4; on day 7, the media was changed, and media were collected from day 8 onwards.

iv) CAF CM: Medium collected from CAFs cultured in T75 flasks.

v) CAF+PRP CM: CAFs were treated twice with PRP (T/C 0.07/0.42 mg/mL) on day 1 and day 4; on day 7, the media was changed, and media were collected from day 8 onwards.

Collected media were frozen and stored at -20ºC for use within the following 3 months. For longer storage periods, CM was stored at -80ºC.

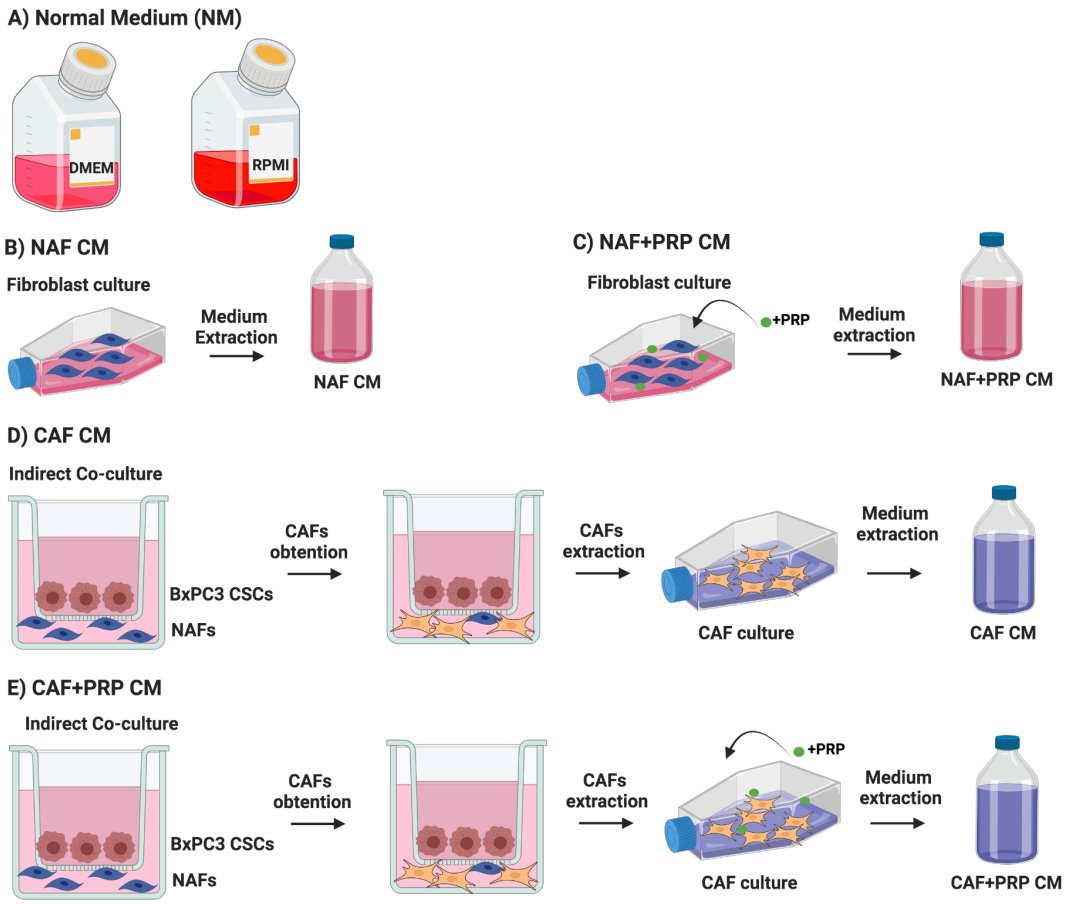


Figure 47. Conditioned Medium Protocol. Culture medium was collected from different cell cultures under various conditions and labeled as follows: A) NM. B) NAF CM. C) NAF+PRP CM. D) CAF CM. E) CAF+PRP CM. Conditioned media were frozen and stored at -20ºC for use within the following 3 months. For longer storage periods, conditioned media were stored at -80ºC. Image created with Biorender (pending publication).

CAF-educated fibroblasts (CEFs) obtention

100,000 NAFs were cultured in 7 mL of CAFs CM along with 3 mL of DMEM in a T75 flask, and the medium was changed every 72 hours. After 14 days of culture, CEFs were obtained and characterized (Figure 48).

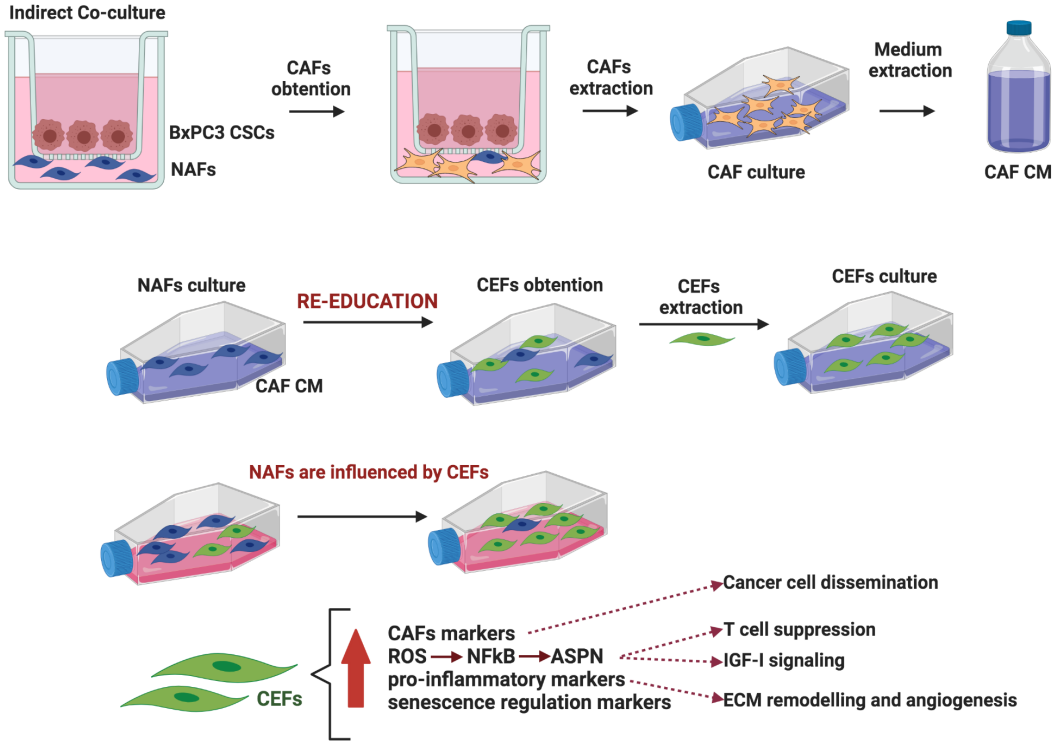


Figure 48. CEFs obtention and characterization. Following the protocol from Figure 47, NAFs (blue) were cultured in CAF CM. After 14 days, these fibroblasts were re-educated into a CEFs phenotype. CEFs (green) induce ROS production, which activates the NF-kB pathway; produce immune and inflammatory cytokines such as IL-1, IL-6 and CXCL8 which promotes ECM remodeling, angiogenesis and metastasis; and express CAFs markers which expands tumour and triggers cancer cell dissemination. CEFs sequentially educate resident NAFs to further amplify pro-tumour fibroblasts in the TME. Image created with Biorender (pending publication).

Angiogenic co-culture system

Human umbilical vein-derived endothelial cells (HUVECs) were cultured in complete EGM-2 growth medium (Lonza, Basel, Switzerland) under standard cell culture conditions at 37°C and 5% CO_2. Cells were used between passages 4 and 6. Multiwell dishes (12-well plates) were coated with 300 µL of Matrigel (Collaborative; BD PharMingen, San Diego, CA) at 4°C and incubated for 30 minutes at 37°C. HUVECs (1.2×10^5 cells/well) were added to the Matrigel-coated wells in complete culture medium, and 24 hours later, 80,000 NAFs and CAFs were seeded in chambers. The (pro)enzymes formulation was added after seeding the fibroblasts at a concentration of T/C: 0.07/0.42 mg/mL. After 14 days of co-culture, cells were photographed using a Leica DM 5500B fluorescent microscope (Solms, Germany).

Methyl thiazolyl tetrazolium (MTT) assay

The effect of PRP on cell viability and proliferation was assessed using the MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, St. Louis, MO). Briefly, cells (2×10^3 cells/well) were seeded onto 96-well plates and incubated for 24 h and then treated with different PRP concentrations (0.001x, 0.125x, 0.25x, 0.5x, 1x, 2.5x, 5x, 10x and 100x; where x= 6 mg/ml of Chymotrypsinogen A and 1 mg/ml of Trypsinogen) in a final volume of 200µL. 72 hours treatment was repeated and cells were maintained for 3 additional days. Thereafter, cells were processed as follows, 10 µL of 2 mM MTT reagent was added to each well and cells were incubated at 37 °C for 4 hours. 100 µL detergent reagent was then added and cells were left at room temperature in the dark for 3 hours. The medium was removed and 150 µL DMSO (D2650, Sigma-Aldrich, St. Louis, MO) was added to each well. It was shaken mechanically for 30 min and absorbance was recorded at 570 nm in Full-Automatic Microplane Reader MB-580 (Heales). The IC_{50} values were calculated from four parametric logistic curves by linear interpolation using Sigma Plot software. All of the experiments were plated in triplicate wells and were carried out at least twice.

SRB assay

For sulforhodamine B (SRB) assay, cells were seeded in 96-well flat bottom plates at a density of 3000-4000 cells/well. After 72h of drug exposure, plates were fixed with 50% TCA, and incubated with 0.4% SRB at 4ºC avoiding light for a minimum of 1 hour. Plates were washed with water 4 times and let them dry. Plates were marked with SRB for 15 min and then washed with 1% acetic acid to remove unbound SRB and air dried. 10 mM Tris was used to extract protein-bound dye and optical density was measured with a BioTek Synergy HT plate reader (SN 269140, BioTek Instruments Inc.) at 490 and 540 nm. IC_{50} was determined through interpolation in GraphpadPrism (version 9.0, Intuitive Software for Science, USA).

LIVE/DEAD Cell Viability assay

Cell viability staining was performed by the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes), according to the manufacturer's instructions. In brief, BxPC3 secondary spheres in different conditions were washed with PBS and labelled with the staining solution (1:4 calcein AM: EthD-1) (L3224, Invitrogen Kit). Images were taken by confocal microscopy (Nikon Eclipse Ti-E A1, USA) with a 10x magnification and analyzed using NIS-Elements software (Supplementary 5).

Proliferation Assay

Culture medium was aspirated and replaced with a mixture of 100uL of AlamarBlue® (DAL1025, Invitrogen) stain per 1mL of medium. Cells were incubated at 37 ºC for 1h in darkness and 100µL of medium was poured into a well of a 96 plate and proceeded with the fluorescence reading (560/590nm) (Synergy HT, BIO-TEK).

Colony formation assay in TME analysis

CSCs derived from secondary spheres, originating from 300,000 BxPC3 cells, were seeded in wells of a 6-well plate on day 0. Various culture conditions were tested: i) RPMI medium (NM); ii) RPMI medium + PRP (NM+PRP); iii) NAF CM; iv) NAF+PRP CM; v) CAF CM; and vi) CAF+PRP CM (Figure 47).

Cells were treated twice with PRP (T/C 0.07/0.42 mg/mL) on day 1 and day 4. Colony formation assays were conducted on day 7 and day 21 using Crystal Violet staining (C0775, Sigma-Aldrich, St. Louis, MO). Briefly, cells were washed twice in PBS and fixed with 4% paraformaldehyde (PFA) for 30 minutes. After three washes with PBS, cells were stained with 0.5% Crystal Violet in 5% methanol for 15 minutes at room temperature (RT) with gentle rocking. Individually stained colonies in each well were counted, and the colony formation fraction was calculated as follows: colony number/(number of cells seeded × plating efficiency), where plating efficiency was determined by the ratio of colony number to the number of cells seeded in the drug-free medium. Three independent experiments were conducted.

Colony formation assay in chemoresistance analysis

300,000 GR and WT from each cell line were seeded in wells of a 6-well plate on day 0. Various culture conditions were tested: i) Control; ii) +Gemcitabine; iii) +PRP; iv) +PRP first and then +Gemcitabine; v) +PRP+Gemcitabine at the same time.

Cells were treated twice with PRP (T/C 0.07/0.42 mg/mL) on day 1 and day 4. Colony formation assay was analyzed at day 19 using Crystal Violet staining (C0775, Sigma-Aldrich, St. Louis, MO). Briefly, cells were washed twice in PBS and fixed with 4% PFA for 30 minutes. After three washes with PBS, cells were stained with 0.5% Crystal Violet in 5% methanol for 15 minutes at RT with gentle rocking. Individually stained colonies in each well were counted, and the colony formation fraction was calculated as follows: colony number/(number of cells seeded × plating efficiency), where plating efficiency was determined by the ratio of colony number to the number of cells seeded in the drug-free medium. Three independent experiments were conducted.

***In vitro* wound-healing assay**

300,000 cells were seeded in wells of a 12-well plate with different conditions. Once the cells reached 100% confluence, wounds were created by scraping the

monolayer cells with a 200 µl pipette tip, and non-adherent cells were washed off with medium. At 0, 12, 24, 48, and 72 hours after the creation of wounds, treated and control non-treated cells were observed, and migration images were captured with a 4x objective using phase-contrast microscopy. Cell migration was determined by the rate of cells moving towards the scratched area, and the scratched area was quantified using ImageJ™ software. All experiments were plated in triplicate wells and were carried out at least three times.

Invasion assay

24-well plates with 8.0 µm pore polycarbonate membrane insert were used (CLS3422, Sigma-Aldrich, St. Louis, MO) and a mixture of medium was poured (with FBS) on them in a total volume of 500 uL. Once the transwells were positioned on each well, 100 uL of Matrigel + medium mixture (without FBS) was added in a 1:1 ratio and waited between 20-30 minutes until it was completely solidified. On top of the mixture, 80,000 cells were placed previously counted in medium (without FBS). After 5 days, the transwells were collected and the matrigel was gently removed.On the transwell filters 200 uL of Iodonitrotetrazolium chloride was added (I8377, Sigma-Aldrich, St. Louis, MO) previously diluted in distilled water (1mg / 1mL) for 1 hour. It could observe the staining under a 1x magnifying glass (Leica S9i) (Figure 49). Due to the FBS gradient generated in the culture, cells moved from the top of the transwell to the bottom (which is the well of the plate) through the matrigel. All cells that migrated were stained with Iodonitrotetrazolium chloride (red).

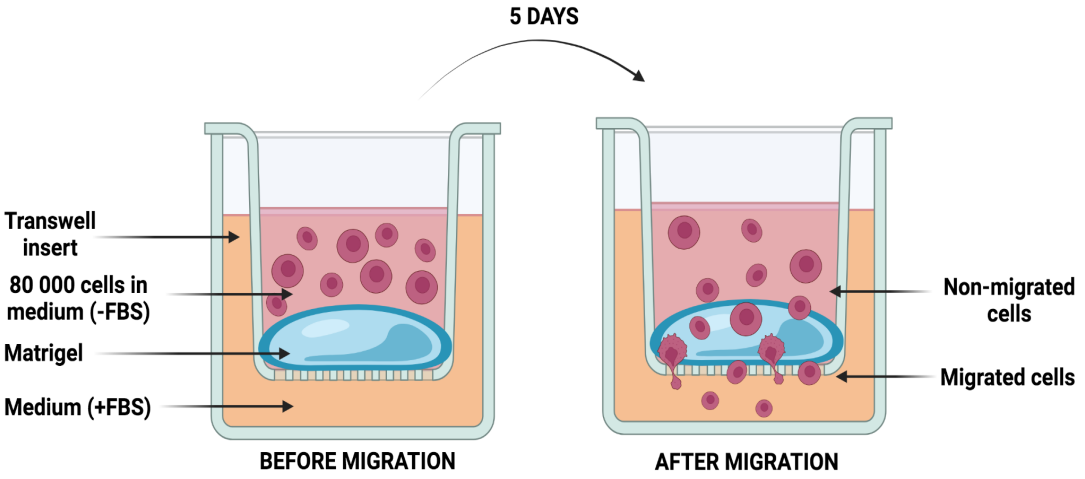


Figure 49. Diagram of the transwell-invasion assay. Cells in migration were added into a transwell insert with a porous membrane in different conditions. FBS was added into the bottom chamber to form a gradient so cells could migrate through the matrigel and the pores of the transwell membrane. Image create with Biorender.com (pending publication).

GSR calculation

For the calculation of Gemcitabine Sensitive Ratio (GSR), RNA was isolated after 24h Gemcitabine treatment and subjected to RT-qPCR. The four Gemcitabine metabolic genes quantified were hENT1, RRM1 and RMM2 (ribonucleotide reductase gene), and DCK. These four genes were selected based on a publication by Nakano et al. [824] who developed the GSR ratio. RNA was extracted using the protocol for the RNeasy Plus Mini kit (Qiagen). Total RNA concentration was measured and utilized for cDNA preparation using the SuperScript VILO cDNA Synthesis kit (Thermo Fisher). RT-qPCR samples were prepared using TaqMan Fast Advanced Master Mix (Thermo Fisher). Primers were acquired from Applied Biosystems and data were normalized to GAPDH. The gene expression (RQ) values for each gene were determined and used in the following formula to obtain the ratio:

$$GSR = [hENT1][DCK]/[RMM1][RMM2]$$

Because the genes required for metabolism (hENT1 and DCK) are in the

numerator and the genes responsible for reducing effectiveness of the drug (RRM1 and RRM2) are in the denominator, lower values of the ratio denote enhanced Gemcitabine resistance.

Treatment with TGF-βR1 Inhibitor

Cells were grown until reaching 80% confluence and then treated with Galunisertib (LY2157299, a TGF-βR1 inhibitor obtained from MedChemExpress, New Jersey, USA at a concentration of 5 μM. The treatment duration was 24 hours, 48 hours, and 72 hours post-malignification before subsequent assays were conducted [825]

Immunofluorescence analysis

For the Immunofluorescence analysis, the cells were fixed with 4% PFA in PBS for 30 minutes at RT and permeabilized with 0.5% Triton X-100 for another 30 minutes. Non-specific binding was blocked using a Blocking Reagent (11096176001, Roche, Basel, Switzerland) for 1 hour at RT. Subsequently, cells were incubated overnight at 4°C with primary antibodies: alphaSMA (ab7817, Abcam, Cambridge, UK), FAPalpha (ab28244, Abcam, Cambridge, UK), Smad2/3 (ab217553, Abcam, Cambridge, UK), Meflin (ab232986, Abcam, Cambridge, UK), VE-cadherin (ab33168, Abcam, Cambridge, UK), and CD31 (ab9498, Abcam, Cambridge, UK), ENT (ab181192, Abcam, Cambridge, UK) and ABCC5 (HPA044067, Atlas Antibodies, Stockholm, Sweden).

On the following day, cells were washed three times with PBS and then incubated with secondary antibodies (Alexa, Abcam, Cambridge, UK) for 2 hours at RT. After another three washes with PBS, cells were mounted with fluoroshield mounting medium with DAPI (ab104139, Abcam, Cambridge, UK). Photographs were taken using a Leica DM 5500B (Solms, Germany) fluorescent microscope equipped with Meta Systems Isis and NIH ImageJ software (National Institutes of Health, MA, USA). The integrated fluorescence intensity for each cell line was determined by computing the average fluorescence intensity of the image,

subtracting the background intensity, and normalizing the data.

RNA isolation and quantitative real time RT-qPCR assay

Total RNA was extracted using the TRIZOL reagent following the manufacturer's instructions (Life Technologies, Carlsbad, California, USA). cDNA was synthesized by reverse transcription of total RNA using the Reverse Transcription System kit (Promega, Madison, Wisconsin, USA) with oligo dT and random primers.

Real-time PCR (RT-qPCR) was performed using the SYBR-Green PCR master mix (Promega, Madison, Wisconsin, USA) according to the manufacturer's recommendations. Each reaction was performed in triplicate from cDNA dilutions. The comparative threshold cycle (Ct) method was used to calculate the amplification factor as specified by the manufacturer. RT-qPCR reactions were performed as follows: an initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 5s and 60°C for 30s, and a final cycle of dissociation from 60 to 95°C.

Human GAPDH was used as an internal standard to normalize variations in RNA quality in the quantities of input cDNA. Different controls were used for each experiment using the ΔΔCT method of relative quantification and assessment of reverse transcription performance, genomic DNA contamination, and RT-qPCR performance. All the samples were done in triplicate for each gene. A list of primers is summarized in Table 8.

Table 8. List of primers for RT-qPCR

Gene Name	Sequence (5'-3')	Size (bp)	Melting Temperature (ºC)
Meflin FRWD	TAGGTGGTATTGGATTCTGGCTGGG	25	71.10
Meflin REV	TTGAAGTAGTCGACGATGTCCTGG	24	68.20
FAPα FRWD	AACCTCATCTCCCACGGCTTC	21	68.60

FAPα REV	TTCTGTGCTTCTCCTCTTTGTCACT	25	66.30
FSP1 FRWD	CTTCCTGGGCTGCTTATCTGG	21	66.90
FSP1 REV	GTACTCGGGCAAAGAGGGTGA	21	67.40
SDF-1 FRWD	CCCGAAGCTAAAGTGGATTC	20	62.50
SDF-1 REV	TTCAGAGCTGGGCTCCTACT	20	63.00
SMAD2 FRWD	CCGGAATTCATGTCGTCCATCTTGCCATTCAC	32	80.7
SMAD2 REV	TCCGCTCGAGTTATGACATGCTTGAGCAACGCAC	34	82.7
SMAD3 FRWD	CGCGGATCCATGTCGTCCATCCTGCCTTTC	30	83.5
SMAD3 REV	TCCGCTCGAGTCAAGACACACTGGAACAGCGGA	33	84.3
IL-6 FRWD	GATTCAATGAGGAGACTTGCC	21	62.6
IL-6 REV	TGTTCTGGAGGTACTCTAGGT	21	56.9
IL-1 FRWD	CCACAGACCTTCCAGGAGAATG	22	60
IL-1 REV	GTGCAGTTCAGTGATCGTACAGG	23	57.2
CXCL8-FRWD	GAGAGTGATTGAGAGTGGACCAC	23	61.5
CXCL8-REV	CACAACCCTCTGCACCCAGTTT	22	59.8
EGR2 FRWD	CCTTTGACCAGATGAACGGAGTG	23	63.4
EGR2 REV	GAAGGTCTGGTTTCTAGGTGCAG	23	69.2
SNAIL FRWD	TGCCCTCAAGATGCACATCCGA	22	74.1
SNAIL REV	GGGACAGGAGAAGGGCTTCTC	21	67.4
SLUG FRWD	ATCTGCGGCAAGGCGTTTTCCA	22	75.1
SLUG REV	GAGCCCTCAGATTTGACCTGTC	22	66.2
E-CAD FRWD	GCCTCCTGAAAAGAGAGTGGAAG	23	66.3
E-CAD REV	TGGCAGTGTCTCTCCAAATCCG	22	70.6
N-CAD FRWD	CCTCCAGAGTTTACTGCCATGAC	23	65.8
N-CAD REV	GTAGGATCTCCGCCACTGATTC	22	65.9
SOX-2 FRWD	GCTACAGCATGATGCAGGACCA	22	69.5
SOX-2 REV	TCTGCGAGCTGGTCATGGAGTT	22	70.4

VIM FRWD	AGGCAAAGCAGGAGTCCACTGA	22	69.4
VIM REV	ATCTGGCGTTCCAGGGACTCAT	22	70
BAX FRWD	TCAGGATGCGTCCACCAAGAAG	22	70.6
BAX REV	TGTGTCCACGGCGGCAATCATC	22	76.3
Bcl2 FRWD	ATCGCCCTGTGGATGACTGAGT	22	67.7
Bcl2 REV	GCCAGGAGAAATCAAACAGAGGC	23	66.6
ABCB1 FRWD	TGGAGGAGCAAAGAAGAAGAAC	19	63.6
ABCB1 REV	GCAGCCAAAGTTCCCACCAC	18	69.0
ABCG2 FRWD	CAAAGGCAGATGCCTTCTTCG	21	68.0
ABCG2 REV	CATACTGAATTAAGGGGAAATTT	23	60.0
ABCC5 FRWD	GCTGTTCAGTGGCACTGTCAG	21	66.2
ABCC5 REV	TCAGCCCTTGACAGCGACCTT	21	70.4
hENT1 FRWD	TGTTTCCAGCCGTGACT	17	64.0
hENT1 REV	CAGGCCACATGAATACAG	18	64.3
RMM1 FRWD	CGCTAGAGCGGTCTTATTTGTT	22	61.6
RMM1 REV	TTGCTGCATCAATGTCTTCTTT	22	57.5
RMM2 FRWD	CCCGCTGTTTCTATGGCTTC	20	55.7
RMM2 REV	CCCAGTCTGCCTTCTTCTTG	20	58.0
DCK FRWD	TGCAGGGAAGTCAACATT	18	59.2
DCK REV	TCCCACCATTTTTCTGAG	18	55.4
DPD FRWD	CAACGTAGAGCAGATGTTGCAC	22	64.7
DPD REV	GAGCTGTCATGCAGAAATGGTTT	23	66.3
P53 FRWD	CCTCAGCATCTTATCCGAGTGG	22	66.9
P53 REV	TGGATGGTGGTACAGTCAGAGC	22	66.3
SNAIL FRWD	TGCCCTCAAGATGCACATCCGA	22	74.1
SNAIL REV	GGGACAGGAGAAGGGCTTCTC	21	67.4
SLUG FRWD	ATCTGCGGCAAGGCGTTTTCCA	22	75.1
SLUG REV	GAGCCCTCAGATTTGACCTGTC	22	66.2

E-CAD FRWD	GCCTCCTGAAAAGAGAGTGGAAG	23	66.3
E-CAD REV	TGGCAGTGTCTCTCCAAATCCG	22	70.6
N-CAD FRWD	CCTCCAGAGTTTACTGCCATGAC	23	65.8
N-CAD REV	GTAGGATCTCCGCCACTGATTC	22	65.9
SOX-2 FRWD	GCTACAGCATGATGCAGGACCA	22	69.5
SOX-2 REV	TCTGCGAGCTGGTCATGGAGTT	22	70.4
VIM FRWD	AGGCAAAGCAGGAGTCCACTGA	22	69.4
VIM REV	ATCTGGCGTTCCAGGGACTCAT	22	70

Western blotting

Whole cell lysates were obtained using RIPA lysis buffer (sc-24948, Santa Cruz Biotechnology, Texas, USA). Cytosolic and nuclear proteins were extracted using a nuclear protein extraction kit (Beyotime Biotechnology Co., Jiangsu, China). Protein concentrations were determined using Bio-Rad detergent-compatible protein assays (Bio-Rad Laboratories, Hercules, California, USA). Equal amounts of protein (20 μg) were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) gels. After electrophoresis, proteins were transferred onto nitrocellulose membranes (162-0115, Bio-Rad Laboratories, Hercules, California, USA) for 30 min at 25 V and blocked in 5% non-fat milk for 1 h followed by overnight incubation with primary antibodies (1:1000 dilution) at 4 ºC with shaking. After washing in Tris-buffered saline with Tween 20 (TBST), membranes were incubated with secondary antibodies (1:2000 dilution) for 2 h at RT, washed again in TBST, and detected using an enhanced chemiluminescence reagent kit (Beyotime Biotechnology Co., Jiangsu, China) on a LAS 3000 Imaging System (Fujifilm). Band intensities were determined using a gel image analysis system (Bio-Rad Laboratories, Hercules, California, USA) and normalized to β-actin, tubulin and GAPDH. Protein band intensities were quantitated using Image-J software (NIH). Primary antibodies used were anti-Human/Mouse Smad2/3 (AF3797, R&D Systems Bio-Techne, Minneapolis, Minnesota, USA), anti-Human Phospho-Smad2/3 (S465/S467, R&D Systems

Bio-Techne, Minneapolis, Minnesota, USA), anti-RMM2 (ab57653, Abcam, Cambridge, UK), anti DCK (ab2100536, Sigma-Aldrich, St. Louis, MO), anti-β-Actin (ab8227, Abcam, Cambridge, UK) anti-tubulin (ab4074, Abcam, Cambridge, UK) and anti-GAPDH (ab9485, Abcam, Cambridge, UK).

Analysis of Apoptosis by Propidium Iodide and Annexin V-FITC Staining

Apoptosis was detected using the apoptosis detection kit for annexin V-FITC (88-8005-74, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Both reagents annexin V-FITC and IP non-vital dye allowed the differentiation among: viable cells (no staining for both dyes), cells undergoing early apoptosis (positive for annexin V-FITC and negative for PI), cells undergoing late apoptosis (positive for both dyes), and necrotic cells (only showed IP staining). The results were representative of three independent experiments.

Animal care compliance

During the study, the care and use of animals were conducted in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals, 8th Edition, 2010 (National Research Council).

***In vivo* anti-tumour xenograft studies**

All procedures were approved and performed in accordance with the guidelines of the Research Ethics and Animal Care Committee of the University of Granada (approval number 12/07/2019/127). Mice were housed and maintained at temperatures ranging from 20 °C to 24 °C, with a relative humidity of 50%, and a light-dark cycle of 14 to 10 hours, with food and water provided ad libitum. Animals (n=5 per group) were randomly assigned to control (1st group), pretreatment group (2nd group), and pretreatment + treatment group (3rd group). The treated groups received Trypsinogen and Chymotrypsinogen A at a dose of 83.3/500 mg/kg in combination in a single injection administered 3 days per week by intravenous injection into the tail vein at a dosing volume of 10 mL/kg. The volume of dosing solution administered to each animal was calculated and adjusted based on individual body weight measured immediately before dosing.

Treatments were administered for 3 weeks before tumour induction (2nd group); and for 3 weeks before tumour induction and continued for 9.5 weeks after tumour induction (3rd group); the control group (1st group) was inoculated with physiological saline solution.

Tumours were generated by subcutaneous injections of BxPC3-CSCs, for which CSC spheres were disaggregated, and 10,000 counted viable cells per mouse were injected using 26-gauge needles. During the experiment, tumour volume was measured, and after 88 days all mice were sacrificed when showing clear signs of health decline in control mice (e.g., weight loss, ascites, or lethargy), and tumours were extracted and measured using a caliper.

Paraffin-embedded blocks of all tumours were sectioned at 5 μm and subjected to immunofluorescence. After deparaffinization and rehydration, tumour sections were labeled with primary antibodies: alphaSMA (ab7817, Abcam, Cambridge, UK), FAPalpha (ab28244, Abcam, Cambridge, UK), Smad2/3 (ab217553, Abcam, Cambridge, UK), and Meflin (ab232986, Abcam, Cambridge, UK), at 4°C overnight. Following washing with PBS, tumour sections were subsequently incubated with fluorescence-conjugated secondary antibodies (Alexa, Abcam, Cambridge, UK) for 2 h at RT. The sections were then mounted with fluoroshield mounting medium with DAPI (ab104139, Abcam, Cambridge, UK). Images were captured using a confocal fluorescence microscope (Leica DM 5500B microscope).

Statistical analysis

All analyses were performed at least in triplicate in each of at least three independent experiments and exhibited mean ± standard deviations. Statistical significance was calculated using Student's t-tests (*P < 0.05, **P <0.01, ***P <0.001). P < 0.05 was considered statistically significant. The Statistica 6.0 Program ws used to analyze the results.

CHAPTER 1

EFFICACY OF PRP AGAINST PANCREATIC TUMOUR MICROENVIRONMENT CELLS

CHAPTER 1

To investigate the effect of the PRP formulation on various cellular components of the TME, a series of cell co-culture experiments were conducted, including treatment at different stages of the process (Supplementary 7). The results indicated that PRP induced a: (i) reduced expression of fibroblast malignancy-related genes, (ii) decreased expression of EMT markers and increased expression of epithelial markers in cancer cells, (iii) inhibition of tumour cell proliferation and migration, (iv) dose-dependent suppression of malignant cell growth, (v) decreased endothelial cell proliferation (vi) downregulation of the TGFβ signaling pathway in CAFs, (vii) induction of pro-apoptotic biomarkers in CAFs, and stimulation of apoptosis and necrosis stages. Additionally, utilizing an *in vivo* model, it was demonstrated that PRP treatment reduced fibrotic markers within the TME and enhanced cancer-restraining CAFs markers such as Meflin.

1. RESULTS

1.1 Monolayer of BxPC3 and BxPC3-CSCs induce the transition from NAFs phenotype to CAFs phenotype

The first objective of our study was to obtain CAFs. To achieve this, NAFs were co-cultured with a monolayer of BxPC3 cells or with BxPC3-derived cancer stem cells (BxPC3-CSCs) using a transwell system (Fig. 50A). Phase microscopy images revealed that both co-culture conditions induced an activation of NAFs towards a malignant-like phenotype. Specifically, a change in the size and shape of NAFs co-cultured with tumour cells was observed under phase-contrast microscopy. NAFs co-cultured with a BxPC3 monolayer exhibited a larger and more star-shaped morphology (Fig. 50A, white arrows) compared to control NAFs, which appeared more elongated and thinner. Moreover, NAFs co-cultured with BxPC3-CSCs displayed an even larger size and a more stellate shape (Fig. 50A, white arrows).

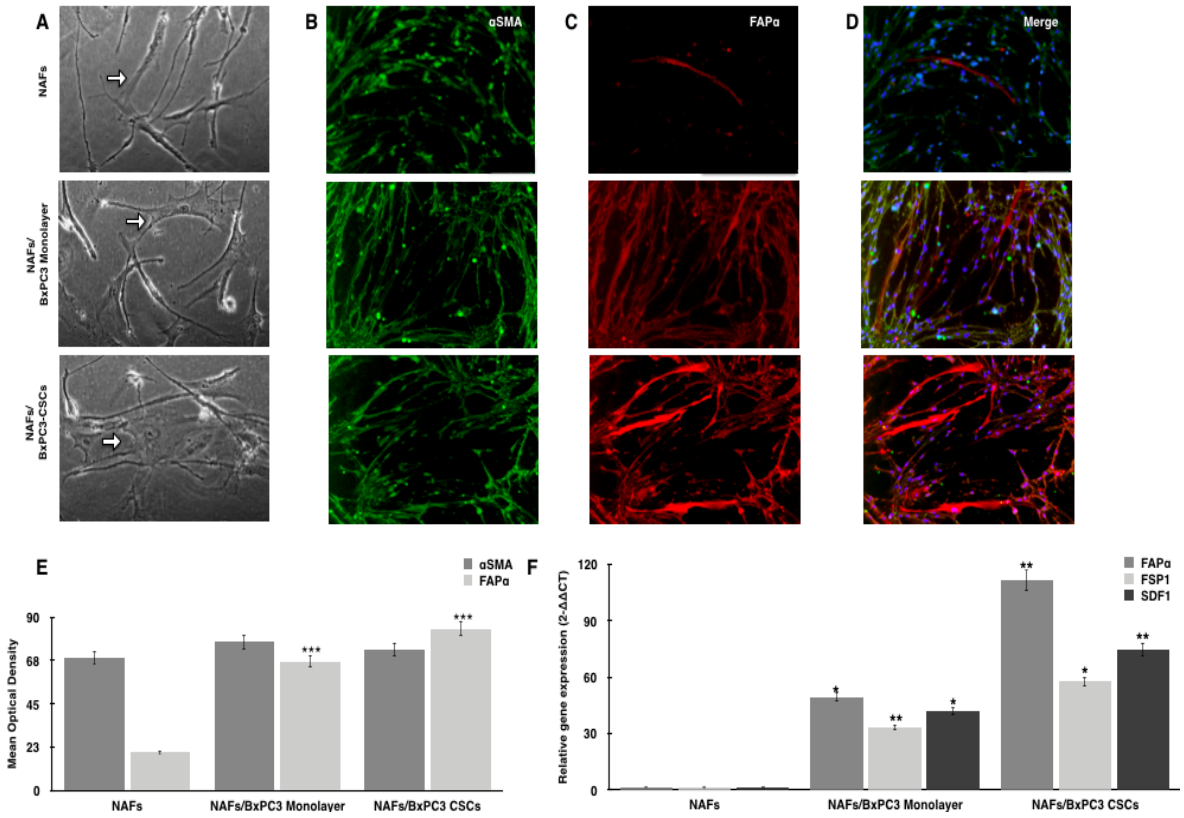


Figure 50. Indirect Co-culture of Monolayer BxPC3 or BxPC3-CSCs with Fibroblasts generates Cancer-Associated Fibroblasts. A) Representative images of phase-contrast micrograph of the bottom of the dish after removing the chamber containing BxPC3 grown in monolayer or BxPC3-derived CSCs. CAFs displayed a star-like morphology (white arrow), contrasting with the spindle shape of NAFs. (Original magnification 40x). B-D) Representative images of immunofluorescence staining with αSMA (B, green) and FAPα (C, red) antibodies. Nuclei were counterstained with DAPI (D, blue). (Original magnification 10x). E) Quantification of fluorescence intensity normalized by cell nuclei. F) RT-qPCR analysis of CAFs-related genes. The assays were conducted in at least three independent experiments. Error bars represent ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

An immunofluorescence assay targeting CAFs markers, alpha smooth muscle actin (αSMA) and fibroblast activation protein (FAPα), was conducted (Fig. 50B-D). αSMA staining was detected in both NAFs and CAFs, with minimal differences in expression between these cellular states. However, NAFs co-cultured with tumour cells showed an increased expression of the FAPα marker, which was more pronounced in fibroblasts co-cultured with BxPC3-CSCs, as can

be appreciated in the graphs showing fluorescence intensity of the labeled cells (Fig. 50E).

Subsequently, a RT-qPCR analysis was performed (Fig. 50F), revealing a significant increase in the expression of CAFs markers in NAFs cultured with a monolayer of BxPC3 (49-fold for FAPα; 33-fold for FSP; and 42-fold for SDF1), which was more pronounced when fibroblasts were cultured with BxPC3-derived CSCs (111-fold for FAPα; 57-fold for FSP; and 74-fold for SDF1). The significant increase in the expression of these genes after NAFs co-culture with BxPC3-derived CSCs indicated that this approach leads to a more pronounced pro-tumoural CAF phenotype.

1.2 Pancreatic (pro)enzymes formulation (PRP) inhibited CSCs induction of CAFs

After demonstrating that CSCs induced a more pronounced phenotypic change in normal fibroblasts, it was investigated whether the pancreatic (pro)enzymes formulation PRP could interfere with the malignification potential of CSCs and reverse the CAF-like state of fibroblasts cultured with CSCs (Fig. 51). To do so, co-cultures of NAFs and BxPC3-CSCs were established. Three different sets of experiments were performed: i) indirect co-culture of NAFs with BxPC3-CSCs that had been treated with PRP before the co-culture (represented in the figure as "before"); ii) NAFs and BxPC3-CSCs co-culture with PRP added during the co-culture process (represented in the figure as "during"); and iii) CAFs, obtained from the co-culture of NAFs and BxPC3-CSCs, that were treated with PRP after the co-culture (represented in the figure as "after"). Changes in the expression of CAF-related markers were studied (Figures 51A and 51B).

The representative immunofluorescence images of NAFs co-cultured with BxPC3-CSCs indicated the acquisition of a CAF-like phenotype with increased expression of the protein FAPα when compared with NAFs. Interestingly, when the co-culture was performed with CSCs previously treated with PRP (before), the expression of FAPα dramatically decreased, indicating an inhibition of the malignification potential of CSCs by PRP. On the other hand, the expression of

FAPα also decreased in CAFs treated with PRP (after), but it was less marked when the treatment was administered during the co-culture (during).

Furthermore, RT-qPCR analysis performed on NAFs and CAFs samples indicated that PRP treatment clearly induced a down-regulation of genes related to CAFs (FAPα, FSP1, and SDF1) when the treatment was administered "before" or "after" co-culture (Fig. 51C). A significant decrease in expression of 68, 39, and 48-fold for FAPα, FSP1, and SDF1 respectively could be seen when the treatment was applied prior to co-culture, and 76, 32, and 43-fold for those CAFs that were treated after malignification. The addition of PRP during co-culture also significantly down-regulated the expression of CAF markers: 94, 13, and 21-fold (Fig. 51C).

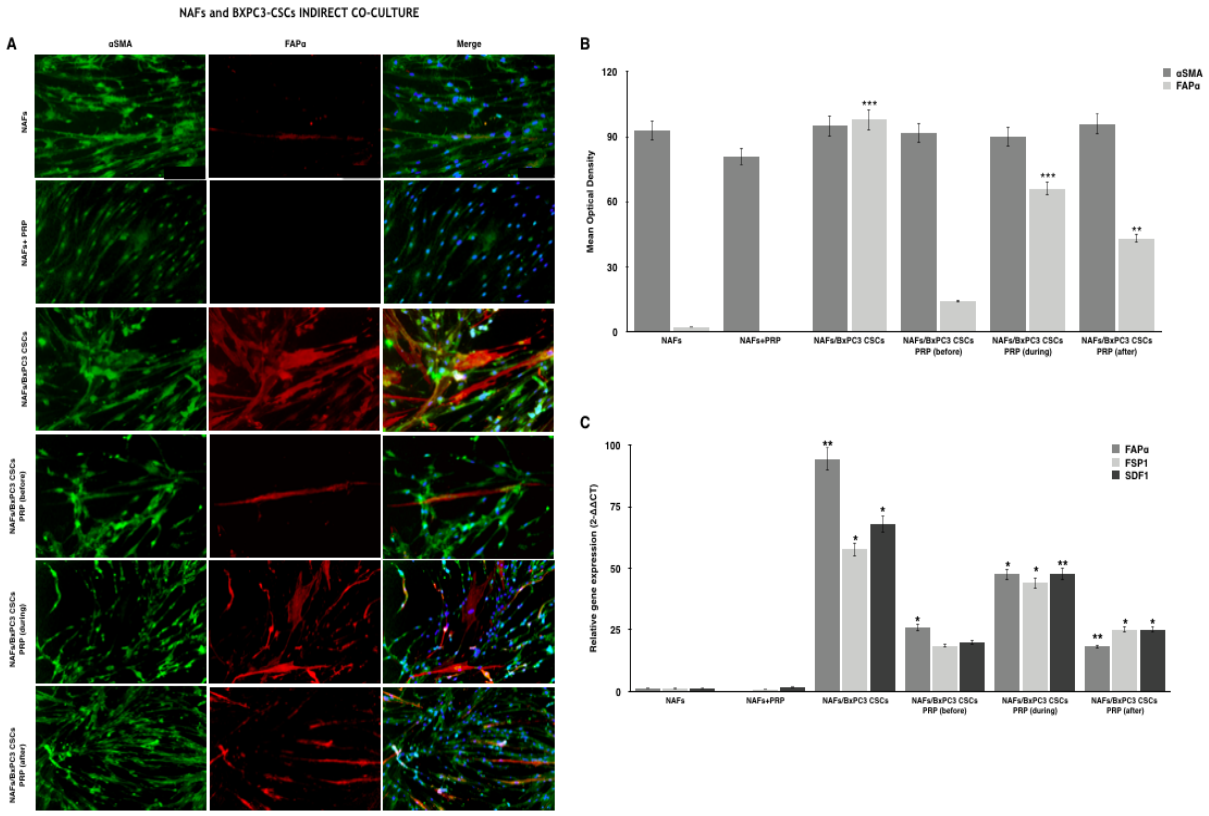


Figure 51. PRP interferes with the malignancy process of BxPC3-CSCs cells over fibroblasts. A) Indirect co-culture (transwell-system) of NAFs and BxPC3-CSCs cells. Representative immunofluorescence images of αSMA (green) and FAPα (red) expression in normal and malignant fibroblasts without and with PRP treatment before, during, and after the indirect co-culture. Nuclei were counterstained with DAPI (blue). (Original magnification: 20x). B) Quantification of fluorescence intensity normalized by cell nuclei.

C) RT-qPCR analysis of FAPα, FSP1, and SDF1 genes expression in NAFs and CAFs without and with PRP treatment before, during, and after the indirect co-culture.

To try to replicate the interactions observed in the tumour TME, BxPC3-CSCs were directly co-cultured with NAFs, both in a 2D model (without using a transwell system) and in a 3D model (explained in Figure 46). Figures 52A-B demonstrate how the direct co-culture strategy mirrored the results obtained with the transwell system (described earlier in Figure 50), with a decrease in the expression of FAPα observed when NAFs were co-cultured with previously PRP-treated CSCs. Notably, treatment during co-culture led to a higher level of inhibition of the CSCs' malignification potential, indicating that the treatment could affect direct cell-cell interactions.

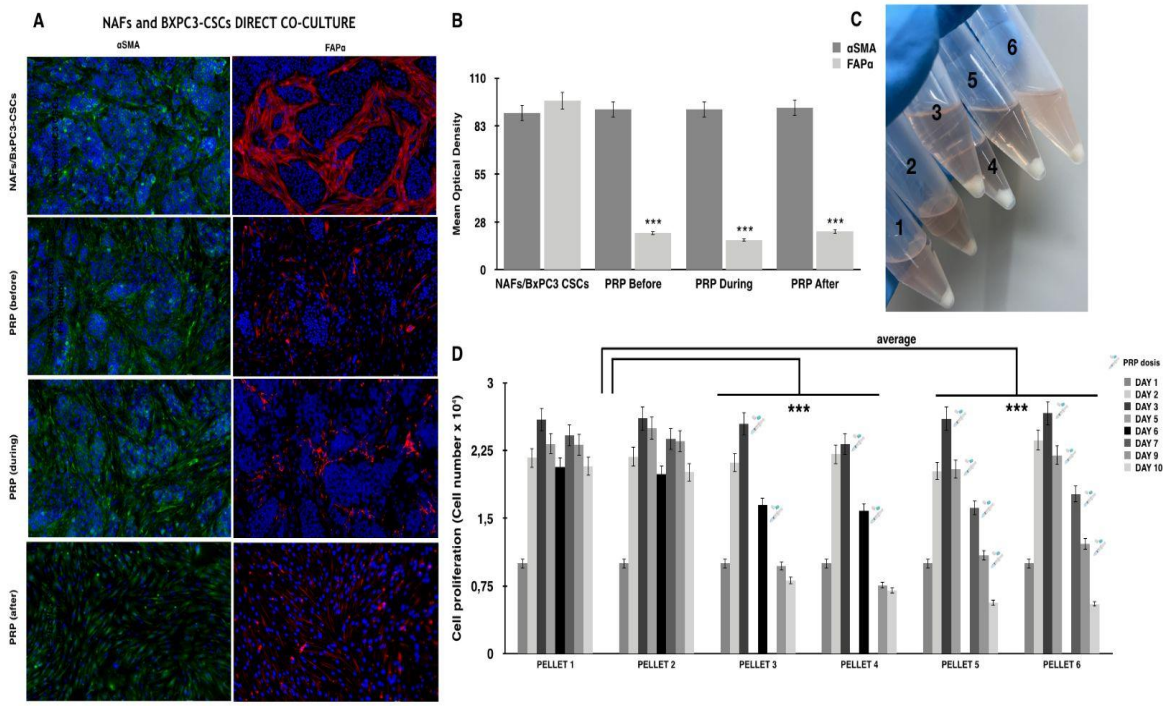


Figure 52. CAFs obtained after direct co-culture undergo a significant impairment in their CAF-like phenotype upon PRP addition. A) Direct co-culture (in a well) of NAFs and BxPC3-CSCs cells. Representative immunofluorescence images of αSMA (green) and FAPα (red) expression in normal and malignant fibroblasts without and with PRP treatment before, during, and after the direct co-culture. Nuclei were counterstained with DAPI (blue). (Original magnification: 10x). B) Quantification of fluorescence intensity normalized by cell nuclei. C) Direct co-culture (pellet formation in a tube): tubes 1-2: controls with no treatment; tubes 3-4: PRP treatment consisting in two doses (T/C 0.07/0.42 mg/mL) every 72-hours; tubes 5-6: PRP treatment consisting in four doses (T/C 0.035/0.21 mg/mL) every 48 hours. D) Graph displaying cell count in the 3D direct co-

culture assays. Proliferation was measured before the addition of new treatment with fresh medium. The experiments were conducted in duplicate. Error bars indicate ± SD *P < 0.05, **P < 0.01, ***P < 0.001.

In the 3D direct co-culture between fibroblasts and BxPC3-CSCs, it was studied cell proliferation after administering two different doses of PRP treatment (Figures 52C-D): i) T/C 0.07/0.42 mg/mL every 72 hours (pellets 3-4) and ii) T/C 0.035/0.21 mg/mL every 48 hours (pellets 5-6). With the highest dose of PRP treatment (two doses separated by 72 hours), a significant inhibition in cell proliferation was observed, resulting in a 62.5% decrease (pellets 3-4) compared to control pellets (1-2). When PRP was administered at lower doses but more frequently (every 48 hours), the proliferation rate decreased by up to 72% (pellets 5-6). These results indicate that PRP decreased the proliferation of two cell populations found within the TME: malignant fibroblasts and CSCs.

1.3 PRP decreased the tumourigenic capacity of CAFs over tumoural cells

Based on the results described above, all subsequent experiments were conducted using NAFs/BxPC3-CSCs indirect co-culture to obtain CAFs.

Given the known interplay between CAFs and tumour cells within the TME, experiments were conducted to investigate whether PRP treatment could influence this intricate interaction (Fig. 53). Initially, disaggregated BxPC3-CSCs were co-cultured with CAFs and treated with PRP. The expression of EMT-related genes in BxPC3-CSCs was assessed after 14 days of co-culture and compared with a control culture of BxPC3-CSCs and a non-treated CAFs-BxPC3-CSCs co-culture.

Figure 53A shows that PRP treatment led to the up-regulation of E-cadherin. Notably, five other genes known to induce EMT were down-regulated following PRP treatment. Specifically, the transcription factor Snail, associated with metastatic cells undergoing EMT, showed a significant down-regulation (8-

fold), as did SLUG expression (7-fold). Furthermore, other EMT markers, including N-cadherin (5-fold), Sox-2 (2-fold), and Vimentin (2-fold), exhibited decreased expression levels after PRP treatment. These findings align with our previous research [813], underscoring PRP's inhibitory effect on EMT in pancreatic tumour cells.

The aforementioned results support the notion that certain factors produced by CAFs can influence neighboring cells within the TME. To substantiate this concept, a series of experiments were conducted using conditioned media obtained as described in the materials and methods section and Figure 47. Specifically, i) RPMI was utilized as the control or normal medium (NM); ii) NAF CM (medium from NAFs culture); iii) NAF+PRP CM (medium from PRP-treated NAFs culture); iv) CAFs CM (medium from CAFs culture); and v) CAFs+PRP CM (medium from PRP-treated CAFs culture).

A wound healing assay was performed on a BxPC3 cell monolayer, grown after dispersion of BxPC3-CSCs, and the different culture mediums were added. As can be observed in Figure 53B-C, cells were able to almost close the gap after 72 hours in culture with NM; the rate and length of cell migration enhanced with NAF CM and it was completed after incubation with CAFs CM, indicating that CAFs cells expel factors that induce the migration of tumour cells. Treatment with PRP dramatically reduces cell migration even in cells that were cultured with CAF CM. Thus, PRP was able to hamper the "instructions" sent by CAFs cells to tumour cells and significantly inhibited cell invasion.

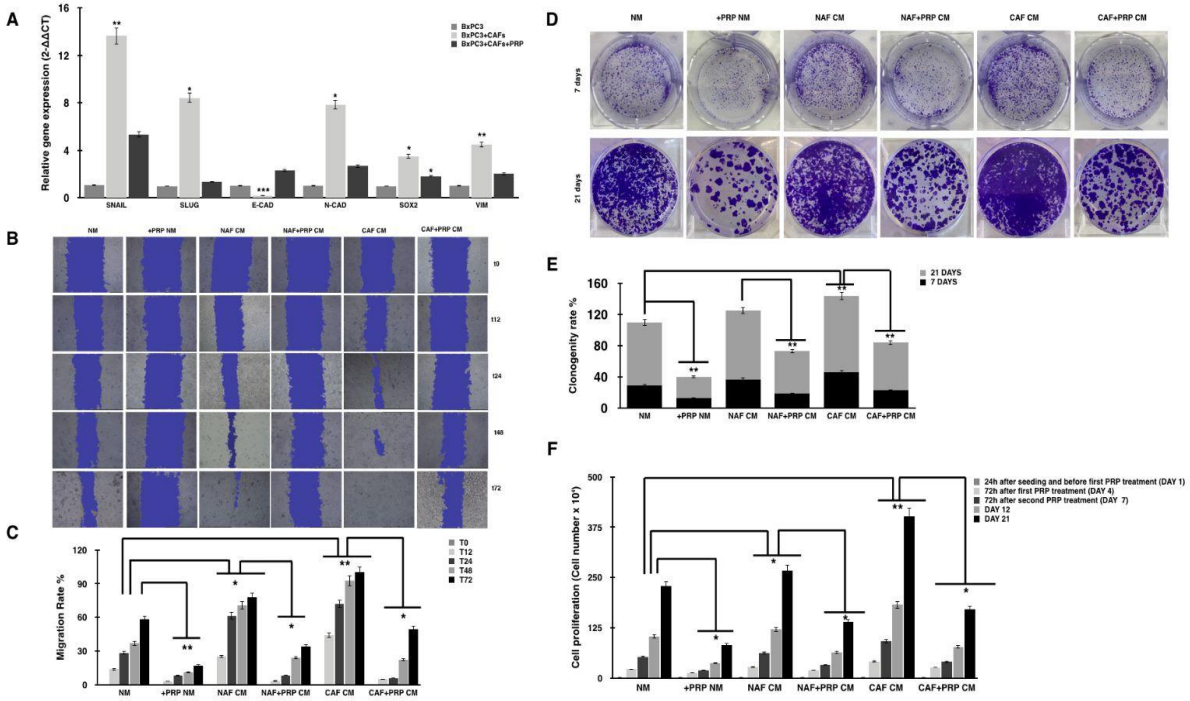


Figure 53. Tumourigenic Capacity of CAFs over tumour Cells. A) Expression of EMT related genes in disaggregated BxPC3-CSCs cells after co-culture with or without CAFs and with or without PRP treatment. B) Quantification of cell migration distances at 0, 12, 24, 48, and 72 hours after scratching by wound healing assay of BxPC3-CSCs cells grown in normal Bx medium (NM), NAFs conditioned medium (NAF CM), and CAFs conditioned medium (CAF CM) without and with IC_{50} PRP treatment. Original magnification 4x. Images analyzed with ImageJTM software. C) Percentage of cell migration determined by the rate of cells moving towards the scratched area over time using ImageJTM software. D) BxPC3-CSCs colony formation assay by crystal violet staining at 7 and 21 days after seeding in NM, NAF CM, and CAF CM without or with PRP treatment. E) Percentage of clonogenicity rate determined by the rate of stained cells at 7 and 21 days using ImageJTM software. F) Alamar blue cell viability and proliferation assay in BxPC3-CSCs under the same conditions as in B) and D). The graph illustrates the fluorescence levels of the redox indicator throughout 21 days after seeding, as a complementary study of D. The experiments were conducted in triplicate. Error bars represent ± SD *P < 0.05, **P < 0.01, ***P < 0.001.

A crystal violet assay to study colony formation rate is shown in Figure 53D-E. Disaggregated BxPC3-CSCs were grown in the different CMs and plate colony formation assay was performed after 7 and 21 days. When compared with NM, the addition of NAF CM increased the number of colonies, an increment that was even higher when cells were cultured with medium from CAFs. In addition, cell proliferation was assessed by Alamar Blue protocol (Fig. 53F); proliferation rate was higher in the tumour cells cultured in CAF CM, in the absence of PRP,

while in the conditions of NAF CM or PRP treatment, the proliferation drastically decreased (50% and 70% respectively). Thus, results indicated that CAFs strongly enhance proliferation of tumour cells and support the inhibitory capacity of PRP.

Taken together, these results indicate that CAFs effectively regulate the EMT process in tumour cells and enhance their invasiveness and proliferation via paracrine signaling that PRP treatment is able to hamper.

1.4 The interaction between CAFs and non-tumoural cells within the TME can be modulated by PRP

The subsequent objective was to assess the impact of CAFs on non-tumour cells within the TME, such as endothelial cells or tissue fibroblasts. To investigate the potential generation of heterogeneous cancer-promoting fibroblasts in the TME through the education of NAFs by CAFs, NAFs were incubated with CAFs CM, with and without PRP treatment. Figure 54A illustrates the results of the RT-qPCR analysis, which evaluated the expression of genes associated with CAFs (FAPα and SDF-1), inflammatory response (IL-1, IL-6, and CXCL8), and immunomodulatory response (EGR2). The gene expression levels in NAFs cultured in CAF CM and NAFs cultured in CAF+PRP CM were compared with those in NAFs and CAFs alone. The results revealed that the expression of all six genes was up-regulated in NAFs cultured with CAFs CM. Specifically, CAF-related genes FAPα and SDF-1 showed a 7-fold and 6-fold up-regulation, respectively, compared to NAFs. Additionally, the expression levels of IL-1, IL-6, CXCL8, and EGR2 were also significantly increased (22-fold, 14-fold, 10-fold, and 5-fold, respectively) compared to NAFs. These findings suggest that factors secreted by CAFs into the medium were sufficient to activate normal fibroblasts, essentially "educating" the NAFs to develop into pro-inflammatory fibroblasts, hereafter referred to as CAFs-educated fibroblasts (CEFs) (Figure 48).

It is noteworthy that when comparing the expression of CAF-specific markers FAPα and SDF1 between CAFs and CEFs, there was lower expression in CEFs, indicating that CEFs exhibit an inflammatory phenotype with some

characteristics of CAFs but do not fully attain a completely pro-tumoural phenotype. Interestingly, when NAFs were cultured in CM from CAFs treated with PRP, gene expression significantly decreased. Specifically, there was a 40% down-regulation of FAP and SDF1, and a 77% decrease in IL-1, 78% in IL-6, 66% in CXCL8, and 65% in EGR2 compared to CEFs. These results suggest that PRP treatment effectively disrupts the paracrine signaling of CAFs aimed at inducing a pro-inflammatory phenotype in surrounding tissue fibroblasts, thereby inhibiting the chain of malignification among fibroblasts within the TME and surrounding tissue that leads to fibrosis. During tumour initiation and progression, the TME requires a robust and expanding network of blood vessels, with endothelial cells playing a crucial role in the process of neo-angiogenesis. In this study, the potential of CAFs was investigated to stimulate the proliferation of human umbilical vein endothelial cells (HUVECs) through paracrine signaling, and to examine whether treatment with pancreatic (pro)enzymes (PRP) can mitigate this effect. Immunofluorescence imaging in Figure 54B-C illustrates HUVECs co-cultured with NAFs and CAFs, stained with specific endothelial markers VE-cadherin and CD31. A notable increase of 14% and 21% in VE-cadherin expression, and 18% and 23% in CD31 expression, is observed in co-cultures with NAFs and CAFs respectively, indicating a pro-angiogenic influence mediated by CAF-derived factors. Interestingly, the addition of the PRP formulation significantly reduces the expression of endothelial markers, with a 57% decrease in VE-cadherin and 64% decrease in CD31 observed in HUVECs co-cultured with NAFs, and a 45% decrease in VE-cadherin and 38% decrease in CD31 observed in HUVECs co-cultured with CAFs.

These findings suggest that both healthy and malignant fibroblasts possess the ability to promote angiogenesis. However, the introduction of PRP into the culture medium effectively suppresses endothelial cell proliferation by 66%, highlighting its potential as a therapeutic intervention to inhibit tumour-associated angiogenesis.

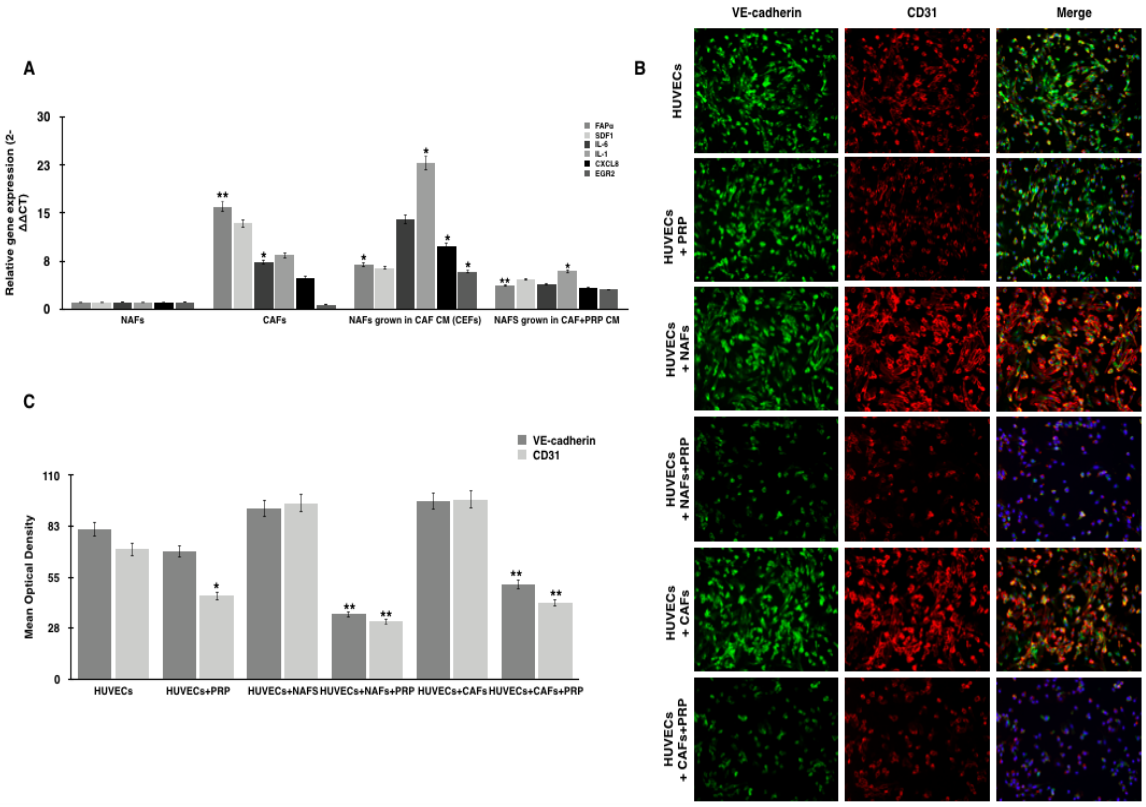


Figure 54. Tumourigenic capacity of CAFs over healthy cells. A) RT-qPCR determination of the expression of the genes: FAPα, SDF-1, IL-1, IL-6, CXCL8 and EGR2 in control NAFs, CAFS, NAFs grown in CAF CM and NAFs grown in CAF+PRP CM. B) Representative images of VE-cadherin and CD31 immunofluorescence labeling in HUVECs after 14 days of different co-culture conditions with or without PRP addition. (Original magnification: 20x). C) Quantification of fluorescence intensity normalized by cell nuclei. The assays were repeated in at least three independent experiments. Error bars indicate ± SD *P < 0.05, **P <0.01, ***P <0.001.

1.5 Pancreatic (pro)enzymes promotes a cancer-restraining CAFs like phenotype

To assess the potential of PRP treatment in "re-educating" malignant fibroblasts, the expression of Meflin was examined, a protein associated with cancer-restraining CAFs that has been shown to enhance the sensitivity of PDAC to chemotherapeutics [826]. In Figure 55A, a substantial expression of Meflin is observed in NAFs, while CAFs exhibit significantly lower expression of this protein, decreasing after quantitative analysis (Fig. 55B) from 97 to 17 MOD.

Interestingly, a significantly higher abundance of Meflin+ CAFs was detected after PRP treatment, with greater Meflin expression in cells that received the treatment during or after the malignification process (about 65 times higher compared to CAFs). Additionally, administering PRP treatment before malignancy was sufficient to achieve a 28-fold increase in Meflin expression. These results suggest that PRP enhances the subpopulation of cancer-restraining CAFs. Furthermore, Meflin gene expression was assessed by RT-qPCR (Fig. 55C). It is evident that PRP treatment significantly enhances the expression of Meflin, supporting the notion of a shift towards a less pro-tumoural phenotype (Meflin+ CAFs) following PRP administration. Specifically, there are notable changes in Meflin gene expression, with increases of 45, 35, and 31-fold compared to non-treated CAFs when the treatment was applied before, during, or after malignification, respectively.

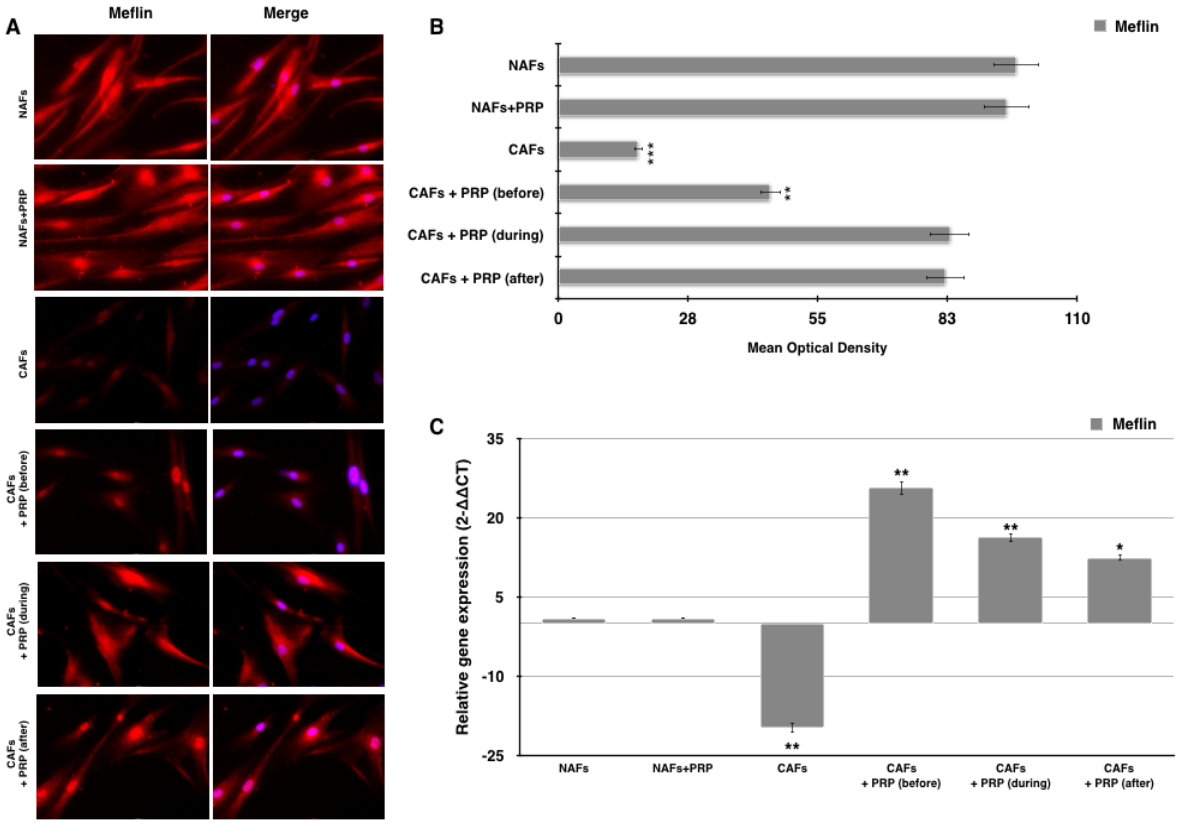


Figure 55. PRP induces over-expression of Meflin. A) Representative immunofluorescence images depicting Meflin expression (in red) corresponding to NAFs and CAFs before, during, and after PRP treatment. Nuclei were counterstained with DAPI (in blue). (Original magnification: 40x). B) Quantification of fluorescence intensity normalized by cell nuclei. C) RT-qPCR analysis of Meflin gene expression in NAFs and CAFs before, during, and after the malignification process with and without PRP treatment. The experiments were conducted in triplicate. *P < 0.05, **P < 0.01, ***P < 0.001.

Of particular interest, the data suggest that PRP not only acts as an inhibitory or preventive treatment against CAF malignification but also serves as a phenotypic reverser of CAFs.

1.6 PRP inhibits the TGF-β pathway in CAFs through the reduction of SMAD2/3 expression and phosphorylation

TGF-β signaling plays a pivotal role in various biological processes that promote tumourigenesis and metastasis, including the malignification of fibroblasts within the TME. The canonical TGF-β pathway involves the expression and subsequent phosphorylation of Smad2/3 proteins [827]. To investigate whether PRP inhibits fibroblast activation and malignification by interacting with the TGF-β pathway, the impact of PRP treatment was assessed on CAFs and compared it with the addition of the TGF-β commercial inhibitor TGF-βRI (LY2157299) (Fig. 56) [828]. First, in Figure 56A the gene expression of Smad2 and Smad3 was analyzed by RT-qPCR in NAFs, CAFs, and PRP or TGF-βRI treated CAFs. Fibroblast malignification led to a significant up-regulation of Smad2 and Smad3 (95-fold and 63-fold respectively) compared to NAFs. PRP treatment significantly reduced the gene expression of both Smad2 and Smad3 by up to 22-fold and 12-fold respectively. Similarly, treatment with TGF-βRI resulted in a reduction of Smad2 and Smad3 gene expression by 59.2-fold and 34.34-fold respectively (Fig. 56F).

Furthermore, immunofluorescent labeling of Smad2/3 proteins demonstrated that PRP treatment tended to decrease the translocation of Smad2/3 into the nucleus. PRP treatment not only prevented Smad2/3 nuclear translocation but also decreased its cytoplasmic expression (Figure 56B-C). TGF-βRI also

reduced the Smad2/3 expression in CAFs and blocked its translocation into the nuclei (Figure 56G-H). Finally, it was analyzed the expression of p-Smad2/3 and total Smad2/3 proteins by Western blotting in PRP and TGF-βRI treated CAFs and compared them with NAFs and non-treated CAFs (Figure 56D-E and 56I-J). The analysis confirmed that PRP repressed the TGF-β pathway by inhibiting Smad2/3 phosphorylation, preventing their translocation into the nucleus, which is necessary for the formation of the functional Smad complex. Treatment with the TGF-β inhibitor also demonstrated a reduction in the expression of Smad2/3 and p-Smad2/3, similar to PRP treatment.

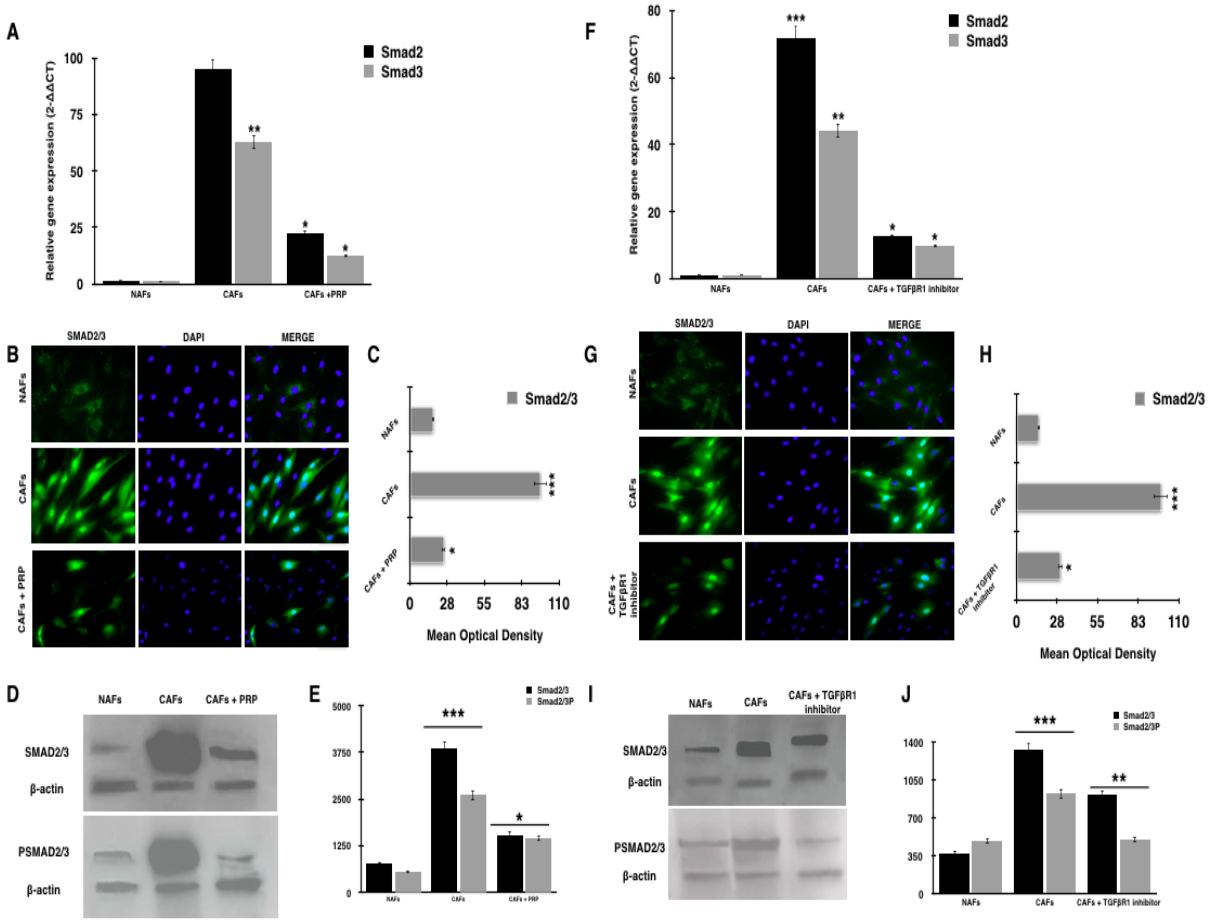


Figure 56. PRP modulates fibroblast malignification by interfering with the TGFβ signaling pathway. A) RT-qPCR analysis of Smad2 and Smad3 gene expression in NAFs, CAFs, and CAFs treated with PRP. Gene expression was normalized to the untreated and non-malignified control. B) Representative immunofluorescence images of Smad2/3 expression (green) in NAFs, CAFs, and CAFs treated with PRP. Nuclei were counterstained with DAPI (blue). (Original magnification: 40x). C) Quantification of fluorescence intensity normalized by cell nuclei. D) Western blot analysis of Smad2/3 and

phosphorylated Smad2/3 (P-Smad2/3) in NAFs, CAFs, and CAFs treated with PRP. β-actin was used as an internal control. E) The relative expression of Smad2/3 and P-Smad2/3 normalized to β-actin was quantified using Image-J software NIH. F-J) Experiments were repeated treating CAFs with the pharmacological inhibitor Galunisertib (TGFβR1 inhibitor). F) RT-qPCR analysis of Smad2 and Smad3 gene expression in NAFs, CAFs, and CAFs treated with TGFβR1 inhibitor. Gene expression was normalized to the untreated and non-malignified control. G) Representative immunofluorescence images of Smad2/3 expression (green) in NAFs, CAFs, and CAFs treated with TGFβR1 inhibitor. Nuclei were counterstained with DAPI (blue). (Original magnification: 40x). H) Quantification of fluorescence intensity normalized by cell nuclei. I) Western blot analysis of Smad2/3 and phosphorylated Smad2/3 (P-Smad2/3) in NAFs, CAFs, and CAFs treated with TGFβR1 inhibitor. β-actin was used as an internal control. J) The relative expression of Smad2/3 and P-Smad2/3 normalized to β-actin was quantified using Image-J software NIH. The assays were repeated in at least three independent batches. Error bars indicate ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

These findings collectively suggest that PRP may impede the involvement of the TGF-β pathway in the transition of NAFs to CAFs by inhibiting Smad2/3 phosphorylation, facilitating Smad2/3 dephosphorylation, or by directly binding and inhibiting the formation of the Smad2/3+4 complex and its translocation into the nuclei (Figure 57).

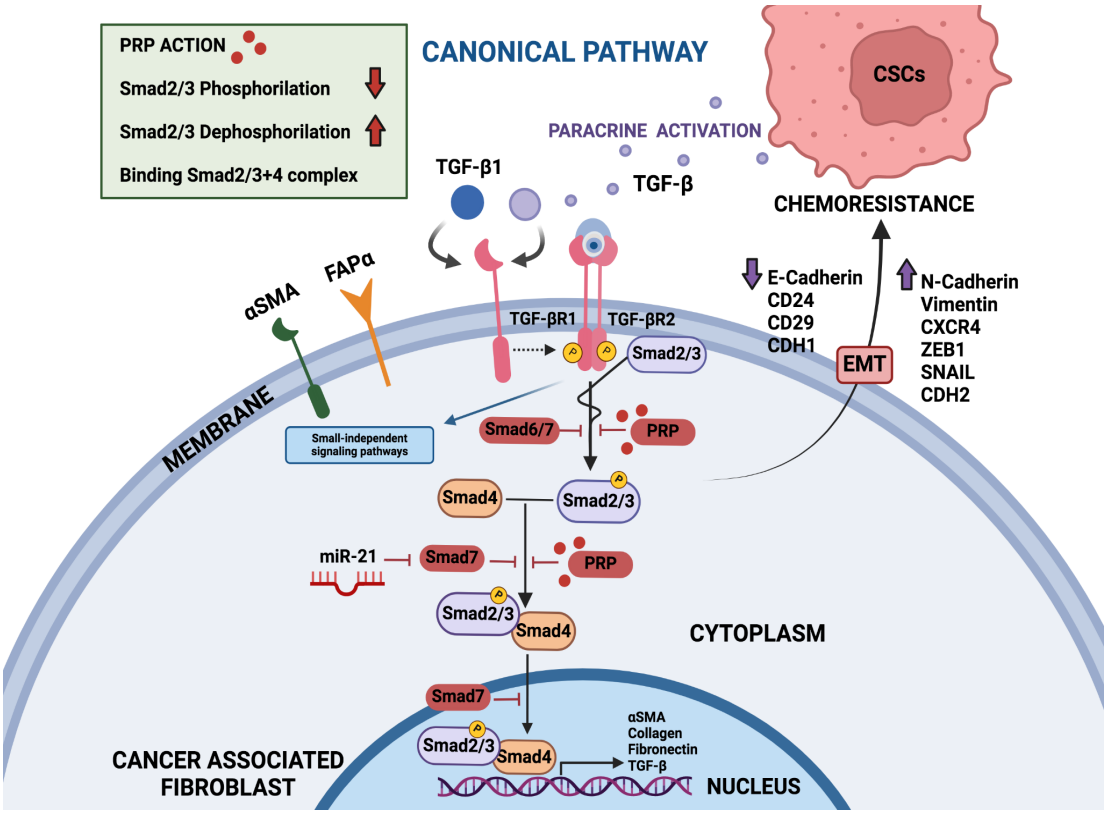
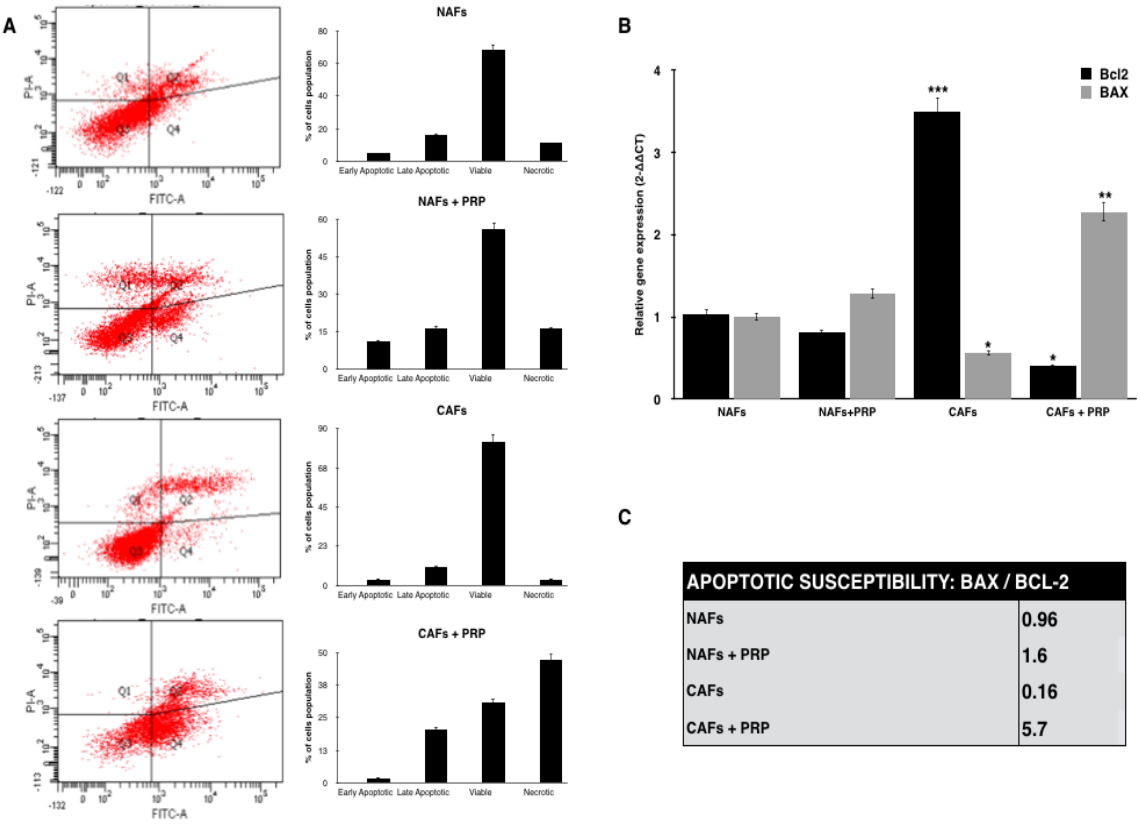


Figure 57. TGFβ signaling pathway in CAFs and potential PRP effect. In the canonical signaling cascade, activated TGF-βR1 leads to Smad2/3 phosphorylation. The p-Smad2/3 complex then binds to Smad4 and translocates into the nucleus, inducing gene transcription. The signaling can be inhibited by a negative feedback loop of Smad7 on TGF-βR1. Potential points of action of PRP include decreasing the phosphorylation of Smad2/3, increasing the dephosphorylation of Smad2/3, or interacting with the Smad2/3-Smad4 complex, affecting its entry into the nucleus. This mechanism also indirectly affects the EMT process in CSCs during crosstalk with the TME.

1.7 PRP does not induce apoptosis in NAFs but increases apoptotic susceptibility in CAFs

Previous findings demonstrated the significant reduction of CAF markers and their tumourigenic functions upon PRP treatment. Here, it is delved deeper into the specific effects of PRP on the CAFs subpopulation. Annexin and PI double staining via cytometry revealed that PRP-treated NAFs did not undergo apoptosis or necrosis in a significant way (Figure 58A). In contrast, PRP induced a significant reduction in the number of CAFs by promoting late apoptosis (21.6 ± 2.3%) and necrosis (44.5 ± 1.07%) (Fig. 58A). Furthermore, it was examined the expression of apoptosis-related genes, specifically Bcl-2 as an anti-apoptotic gene and BAX as a pro-apoptotic gene, through RT-qPCR (Fig. 58B). Consistent with our previous findings, PRP-treated NAFs showed no significant change in the expression of either gene compared to untreated NAFs. However, PRP-treated CAFs exhibited a notable up-regulation of BAX compared to untreated CAFs (2.28-fold increase compared to 0.56-fold). Conversely, the expression of the anti-apoptotic gene Bcl-2 was down-regulated in PRP-treated CAFs, showing a decrease from 3.5 to 0.4 fold compared to untreated CAFs.

Finally, Figure 58C presents a comparative analysis of the "apoptotic susceptibility" value, indicating that CAFs exhibit a more than 35 times increase in this value following PRP treatment. These results underscore the selective apoptotic susceptibility of CAFs to PRP treatment, highlighting its potential to induce programmed cell death specifically in the malignant fibroblast population within the TME.



APOPTOTIC SUSCEPTIBILITY: BAX / BCL-2	
NAFs	0.96
NAFs + PRP	1.6
CAFs	0.16
CAFs + PRP	5.7

Figure 58. PRP increases late apoptosis and necrosis in CAFs. A) Representative images from flow cytometry after annexin V-fluorescein staining of NAFs and CAFs with or without PRP treatment showing the percentage of cells in each state. B) Expression levels of BAX and Bcl-2 genes in NAF and CAF control groups compared to those treated with PRP. C) Table showing the obtained Bax/Bcl-2 protein expression ratio for each experimental condition. The assays were repeated in at least three independent batches. Error bars indicate ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

1.8 *In vivo* study of the effect of PRP on TME

An *in vivo* study was conducted to elucidate the impact of PRP on TME formation. Three groups were established: a control non-treated group (1st group; mice without treatment), a pre-treated group (2nd group; mice pretreatment group), and a pre-treated and treated group (3rd group; mice treated before and after tumour induction). Xenograft tumours were induced by subcutaneous inoculation of BxPC3-CSCs into immunodeficient mice and subjected to the

treatment regimen as described in the materials and methods section and Figure 59A.

After extraction, tumours were sectioned and immunofluorescent analyzed using markers for NAFs (αSMA), fibrosis (FAPα and Smad2/3), and "re-education" (Meflin) (Figures 59B-F). αSMA labeling revealed the typical distribution of fibroblasts surrounding tumour cells, with no significant differences observed between the tested groups (Fig. 59B and Fig. 59F). However, the expression of the CAF marker FAPα was notably reduced in tumours pretreated with PRP (2nd group) compared to untreated tumours (1st group). Interestingly, the presence of CAFs was significantly diminished in tumours receiving double treatment (3rd group) (Fig. 59C and Fig. 59F). The distribution of Smad2/3 within the tumour tissue was also affected by PRP treatment, particularly in tumours from mice treated before and after tumour induction, where a marked decrease in Smad2/3 expression was observed, along with a notable reduction in the fibrotic barrier (Fig. 59D, arrows and Fig. 59F). Consistent with previous findings, the tumour-reversion marker Meflin exhibited high expression in tumour sections from treated mice, with a significant increase observed in the 3rd group compared to the 1st group (28 times increase in 2nd group and 90 times increase in 3rd group) (Fig. 59E and Fig. 59F).

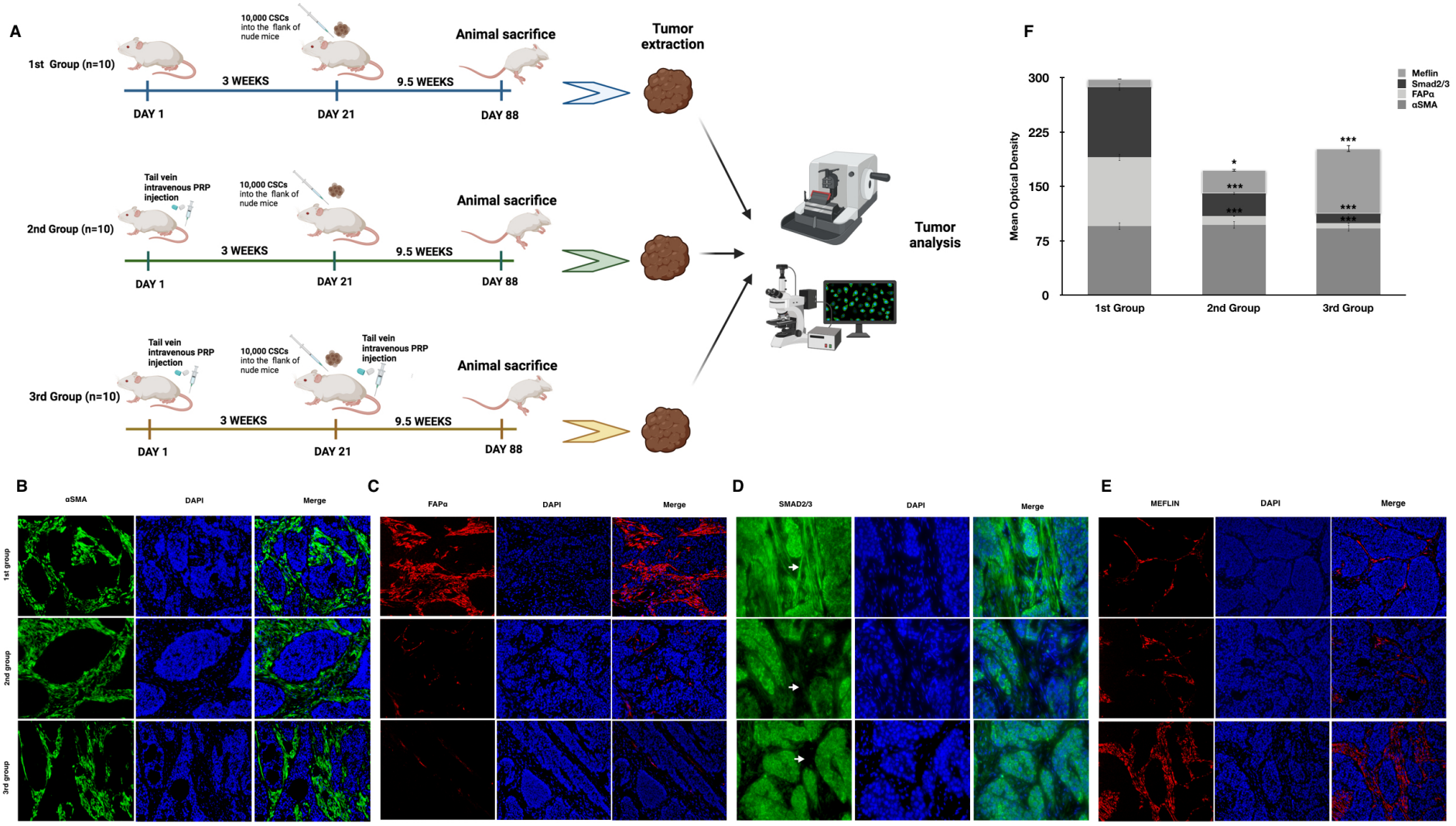

A
1st Group (n=10)
10,000 CSCs into the flank of nude mice
Animal sacrifice
3 WEEKS
9.5 WEEKS
DAY 1
DAY 21
DAY 88
Tumor extraction
2nd Group (n=10)
Tail vein intravenous PRP injection
10,000 CSCs into the flank of nude mice
Animal sacrifice
3 WEEKS
9.5 WEEKS
DAY 1
DAY 21
DAY 88
3rd Group (n=10)
Tail vein intravenous PRP injection
10,000 CSCs into the flank of nude mice
Tail vein intravenous PRP injection
Animal sacrifice
3 WEEKS
9.5 WEEKS
DAY 1
DAY 21
DAY 88
Tumor analysis
F
Mean Optical Density
300
225
150
75
0
Meflin
Smad2/3
FAPα
αSMA
1st Group
2nd Group
3rd Group
B
αSMA
DAPI
Merge
C
FAPα
DAPI
Merge
D
SMAD2/3
DAPI
Merge
E
MEFLIN
DAPI
Merge
1st group
2nd group
3rd group

Figure 59. Xenografts *in vivo* study. The *in vivo* study was designed to determine the antitumour activity of PRP against CAFs within the TME of pancreatic CSCs-induced tumours. BxPC3-CSCs were disaggregated before injection into the flanks of nude mice. The mice were divided into three groups: 1st group: control mice; 2nd group: mice pretreated before tumour induction; and 3rd group: mice treated before and after tumour induction. A) Scheme of the *in vivo* study design to determine anti-tumour activity of PRP. B-E) Representative images of tumour sections staining with the antibodies αSMA (green), FAPα (red), Smad2/3 (green) and Meflin (red) (Original magnification: 20x for αSMA, Meflin, FAPα, and 40x for Smad2/3). F) Quantification of fluorescence intensity normalized by cell nuclei. Error bars indicate ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

In conclusion, PRP demonstrated *in vivo* efficacy in impairing fibrotic tissue formation, reducing the population of CAFs within the TME, resulting in less aggressive and smaller tumours and disrupting the crosstalk between malignant fibroblasts and tumour cells.

CHAPTER 2

EFFICACY OF PRP AGAINST PANCREATIC TUMOUR CHEMORESISTANT CELLS

CHAPTER 2

In this study, it was evaluated the efficacy of the PRP formulation in three pancreatic GEM-chemoresistant (GR) cell lines through different studies (Supplementary 8). An *in vitro* analisys was conducted to evaluate weather the administration of PRP could improve the sensivity of chemoresistant cells to GEM. The results indicated that PRP treatment significantly: (i) reduced GEM IC_{50} values in both wild-type and resintant cells; (ii) decreased proliferation, clonogenicity, migration and invasion of GR tumour cells; (iii) induced apoptosis and necrosis in GR cells; (iv) lowered the expression of metabolic and transporter chemoresistance markers; (v) reduced the expression of EMT markers and increased the expression of epithelial markers in GR cells and (vi) negatively regulated the TGFβ signaling pathway.

2. RESULTS

2.1 Obtaining and Characterization of BxPC3 chemoresistant cell line

In this study the effect of PRP was tested in three pancreatic cancer cell lines resistant to GEM. GEM resistant Suit2.007 and Patu-T (GR-Suit2.007 and GR-Patu-T) were kindly provided by Professor Elisa Giovannetti [829], while chemoresistant BxPC3 (GR-BxPC3) were obtained by exposing the cells to increasing concentrations of GEM (previously explained in material and methods sections and in Figure 28). Resistance index (RI) was evaluated using MTT and SRB assays. GR-BxPC3 showed a statistically significant increase in IC_{50} compared with wild type (WT) BxPC3 with a RI (RI = IC_{50} WT cells/IC_{50}-GR cells) of 0.076 (Table 9 and Figure 60). These results are consistent with the published data for GR-Suit2.007 and GR-Patu-T (0.0023 and 0.00069 respectively) [829]. It can be appreciated that the three chemoresistant cell lines expressed a R^2 over 0.90 and that BxPC3 and Suit2.007 were the most sensitive to GEM, whereas Patu-T displayed the highest level of resistance.

2.2 PRP increases the chemosensitivity of BxPC3, Suit2.007 and Patu-T to GEM

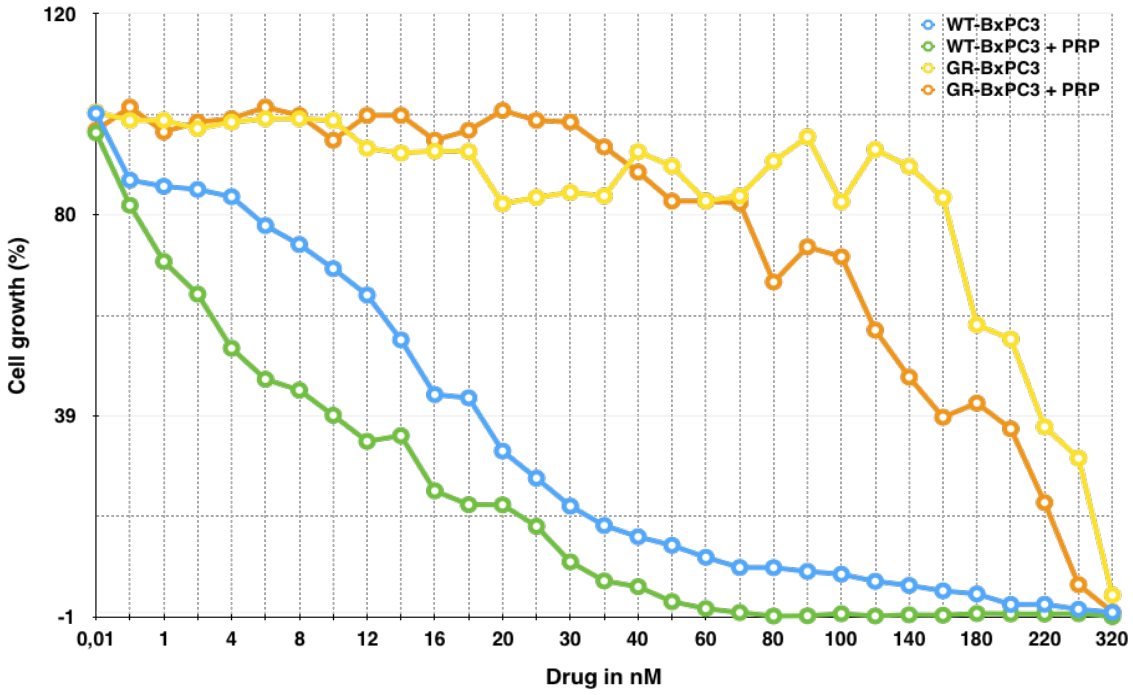
To determine the effect of PRP over WT and GR cells a two-pulse treatment was conducted and changes in the IC_{50} were analyzed. Results are summarized in Table 9 and shown in Figure 60. In WT-BxPC3, the IC_{50} decreased a 56% while the IC_{50} of GR-BxPC3 experienced a decrease of a 33% in its value. In the case of WT-Suit2.007, there was also a decrease of a 49% while the GR-Suit2.007 decreased up to a 34% in its value. Finally, the Patu-T cell line was the most resistant to PRP treatment, with a decrease of 35% in the IC_{50} of the WT cells and a 19% in the GR-Patu-T.

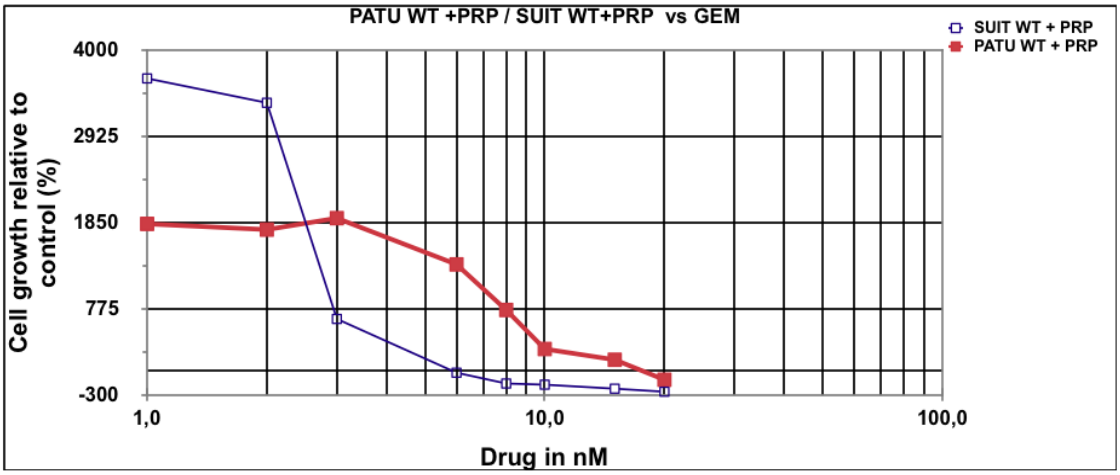
Table 9. Sensitivity of pancreatic cancer cells (WT and GR) to GEM (IC_{50} Values).

Cell type	IC_{50} (nM)	Resistance factor
WT-BxPC3	16	1
WT-BxPC3 + PRP	7	0,44
GR-BxPC3	210	13
GR-BxPC3 + PRP	140	8
WT-SUIT-2 *	7.09	1
WT-SUIT-2 + PRP	3.62	0.51
GR-SUIT-2 *	> 3000	> 423.1
GR-SUIT-2 + PRP	1974	278.42
WT-PATU-T *	8.33	1
WT-PATU-T + PRP	5.4	0.6
GR-PATU-T *	> 12.000	> 1440

GR-PATU-T + PRP	**9736**	**1168.78**

* The results for the Suit and Patu-T cell lines, both WT and GR, after treatment with PRP were compared to the data from untreated cells, which were previously obtained and published in [829].





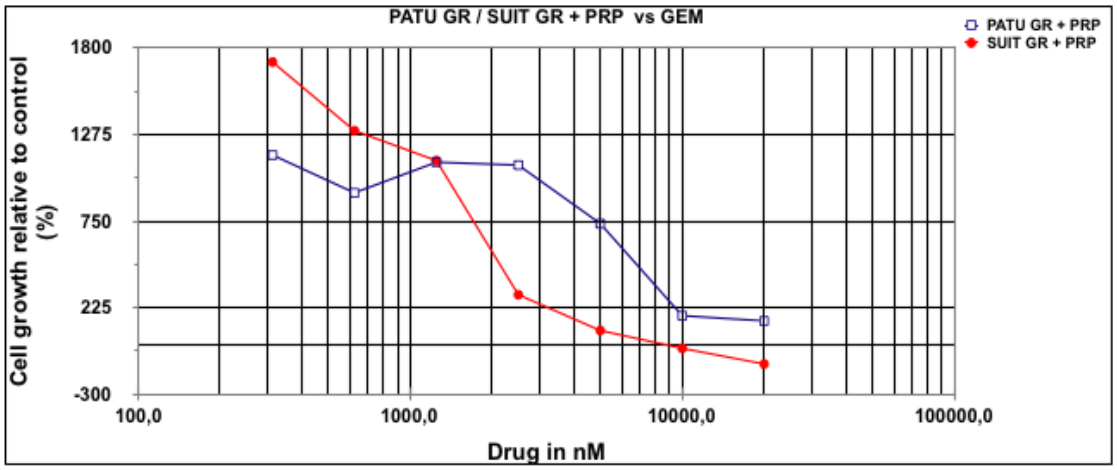


Figure 60. IC_{50} values of pancreatic cell lines BxPC3 (WT and GR), Suit2.007 (WT+PRP and GR+PRP) and Patu-T (WT+PRP and GR+PRP).

2.3 PRP decreases the proliferation, clonogenicity, migration and invasion rate of chemoresistant cells

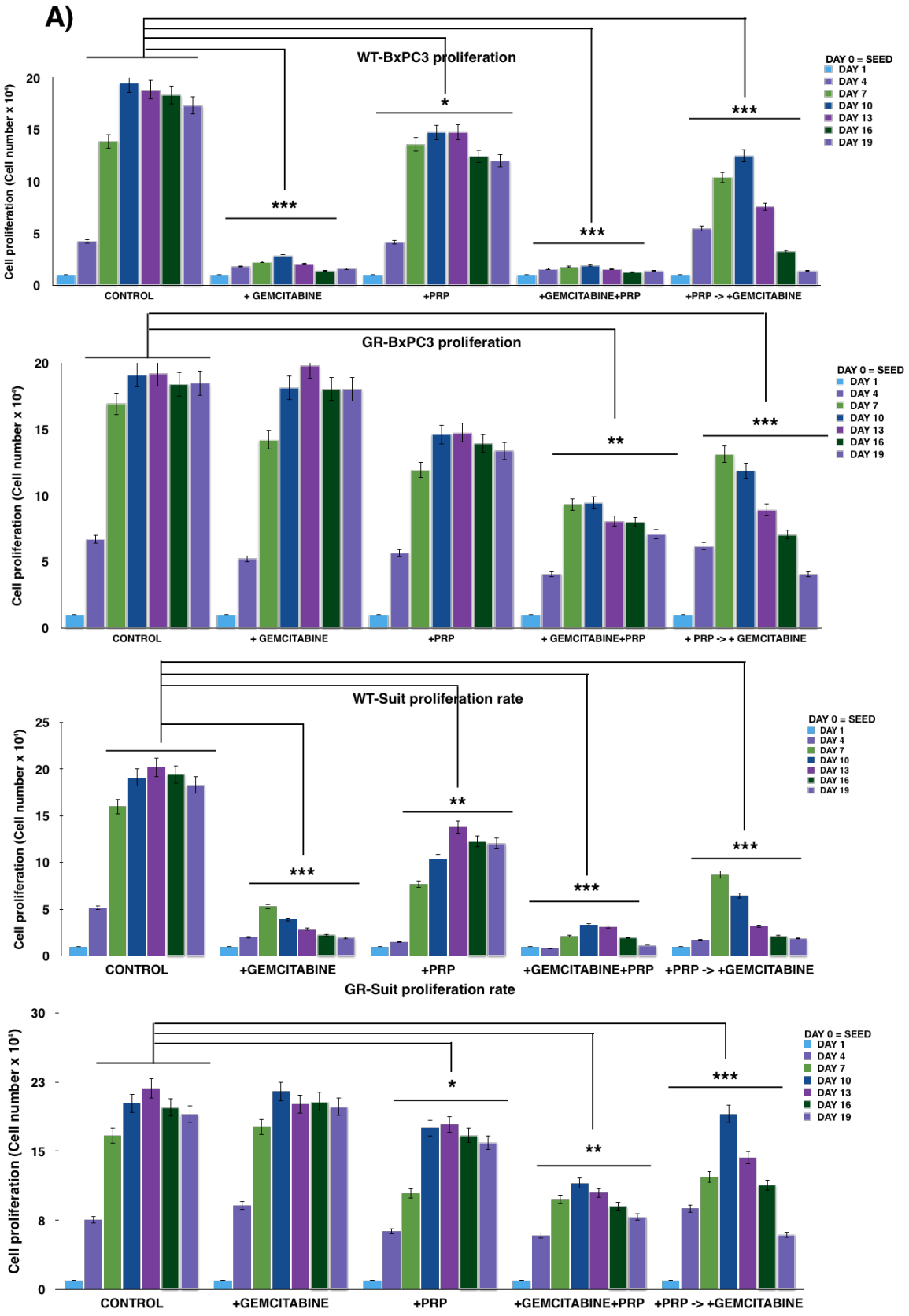
The proliferation rate of the three pancreatic cell lines, both WT and GR, was analyzed over 19 days. Treatment with GEM alone induced limited proliferation in WT cells but had no significant effect on GR cells. In contrast, an adjuvant treatment regimen involving the administration of PRP followed by GEM (PRP→GEM) resulted in reduced cell growth for both WT and GR cells. Although PRP alone did not significantly affect cell proliferation, it enhanced sensitivity to GEM. Notably, the PRP→GEM regimen showed greater inhibition of cell growth compared to the simultaneous administration of both compounds (PRP+GEM) (Figure 61A). Specifically, at day 19, PRP→GEM treatment resulted in a 92% reduction in proliferation for WT-BxPC3 and a 73% reduction for GR-BxPC3. For WT-Suit2.007, proliferation decreased by 87% with PRP→GEM treatment, while GR-Suit2.007 showed a 67% decrease. Lastly, WT-Patu-T exhibited a 71% reduction in proliferation following PRP→GEM treatment, and GR-Patu-T showed a 59% reduction, confirming that GR-Patu-T was the most resistant of the three lines (Figure 61A).

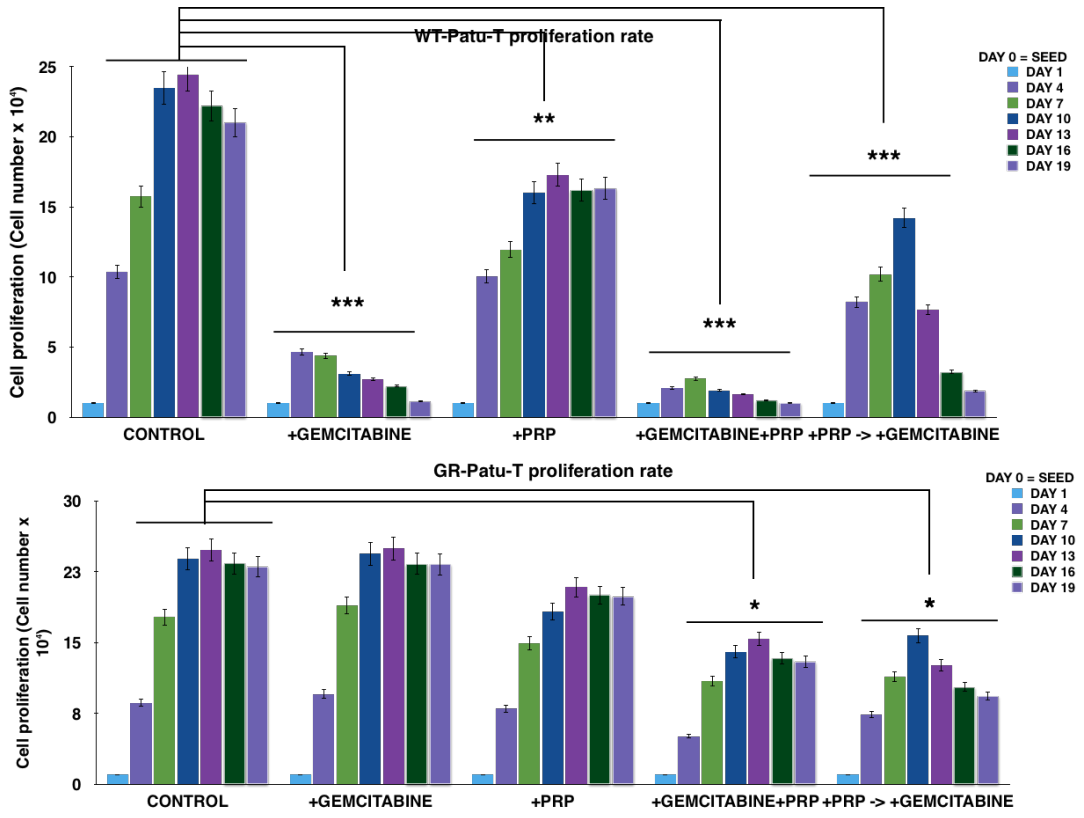
The crystal violet assay was employed to assess the cytotoxicity of each treatment condition and to evaluate cell viability by counting colony formations after 19

days (Figure 61B). The growth-inhibitory effect of the PRP+GEM combination was 91% in WT-BxPC3 and 78% in GR-BxPC3, while GEM or PRP alone had no significant effect on GR-BxPC3. The most pronounced effects were observed with the PRP→GEM treatment, which reduced viability by 95% in WT-BxPC3 and 83% in GR-BxPC3. Similar results were observed in Suit2.007 cells, where GEM or PRP alone had little impact on GR-Suit2.007 cells, but PRP+GEM treatment led to a 92% reduction in viability for WT-Suit2.007 and an 80% reduction for GR-Suit2.007. The PRP→GEM treatment further decreased viability by 96% in WT-Suit2.007 and 85% in GR-Suit2.007. Although the Patu-T line was the most chemoresistant of the three, it also responded to the treatments. Individual treatments with GEM or PRP had minimal effects, but the PRP+GEM combination decreased viability by 68% in WT-Patu-T and 61% in GR-Patu-T. Sequential PRP→GEM treatment led to a 90% decrease in viability for WT-Patu-T and a 60% decrease for GR-Patu-T (Figure 61B).

A wound healing assay was conducted on the three cell lines under the same conditions to evaluate their migration capacity. As shown in Figure 61C, after 48 hours, the three chemoresistant lines had covered 100% of the wound area, with GR-Patu-T closing the wound the fastest, followed by GR-Suit2.007 and then GR-BxPC3. However, when treated with PRP→GEM, the closure was reduced: GR-Patu-T covered only 65% of the wound, GR-Suit2.007 covered 61%, and GR-BxPC3 covered 31% after 72 hours. These results illustrate the inhibitory effect of PRP on the migration of chemoresistant cells.

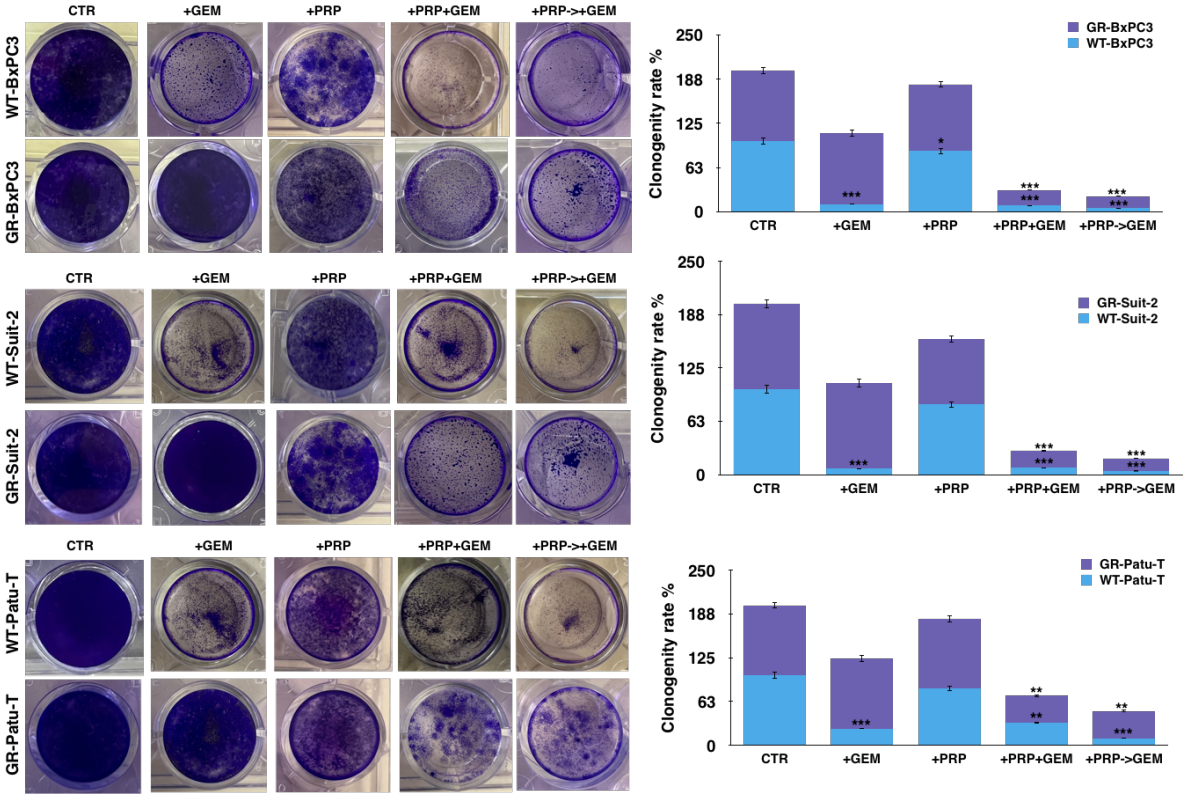
Finally, to evaluate the effect of PRP on the invasion capacity of the pancreatic cell lines, an assay using Matrigel to mimic the ECM was conducted. As shown in Figure 61D, after 5 days, all three chemoresistant cell lines exhibited 100% invasion. However, following PRP→GEM treatment, the invasion rate was significantly reduced, with a rate of only 8% of GR-BxPC3 cells, 11% of GR-Suit2.007 cells, and 12% of GR-Patu-T cells moving through the matrigel layer over time after 5 days. These results demostrate the inhibitory effect of PRP on tumour invasion in chemoresistant pancreatic cells.


A)
WT-BxPC3 proliferation
GR-BxPC3 proliferation
WT-Suit proliferation rate
GR-Suit proliferation rate
Cell proliferation (Cell number x 10⁴)
DAY 0 = SEED
DAY 1
DAY 4
DAY 7
DAY 10
DAY 13
DAY 16
DAY 19
CONTROL
+ GEMCITABINE
+GEMCITABINE
+PRP
+GEMCITABINE+PRP
+PRP -> +GEMCITABINE
+ PRP -> + GEMCITABINE
*
**
***

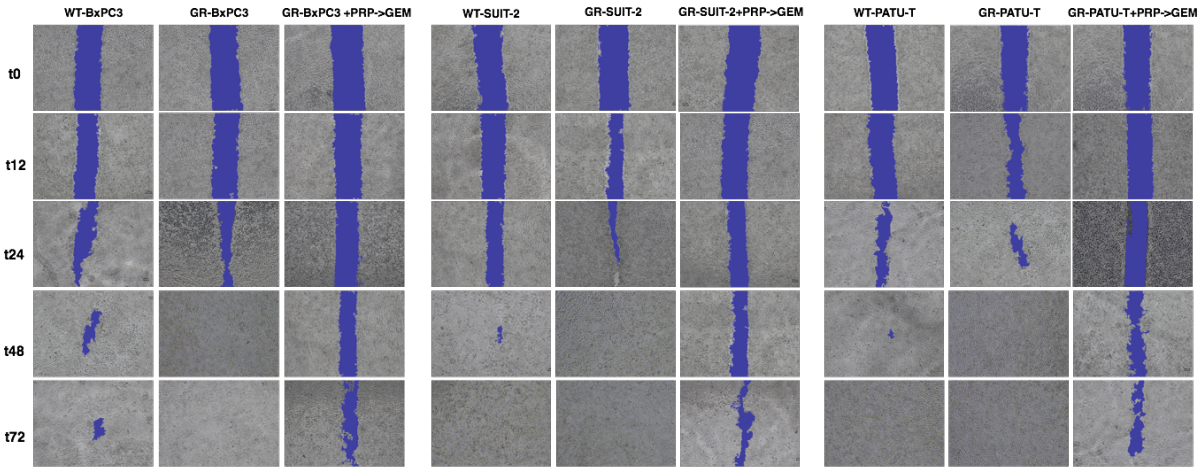

WT-Patu-T proliferation rate
Cell proliferation (Cell number x 10⁴)
DAY 0 = SEED
DAY 1
DAY 4
DAY 7
DAY 10
DAY 13
DAY 16
DAY 19
CONTROL
+GEMCITABINE
+PRP
+GEMCITABINE+PRP
+PRP -> +GEMCITABINE
GR-Patu-T proliferation rate

B.1)

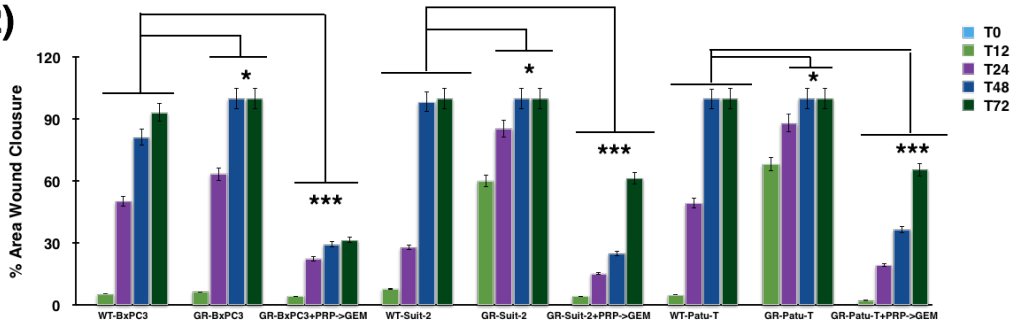
B.2)


CTR
+GEM
+PRP
+PRP+GEM
+PRP->+GEM
WT-BxPC3
GR-BxPC3
WT-Suit-2
GR-Suit-2
WT-Patu-T
GR-Patu-T
Clonogenity rate %
GR-BxPC3
WT-BxPC3
GR-Suit-2
WT-Suit-2
GR-Patu-T
WT-Patu-T

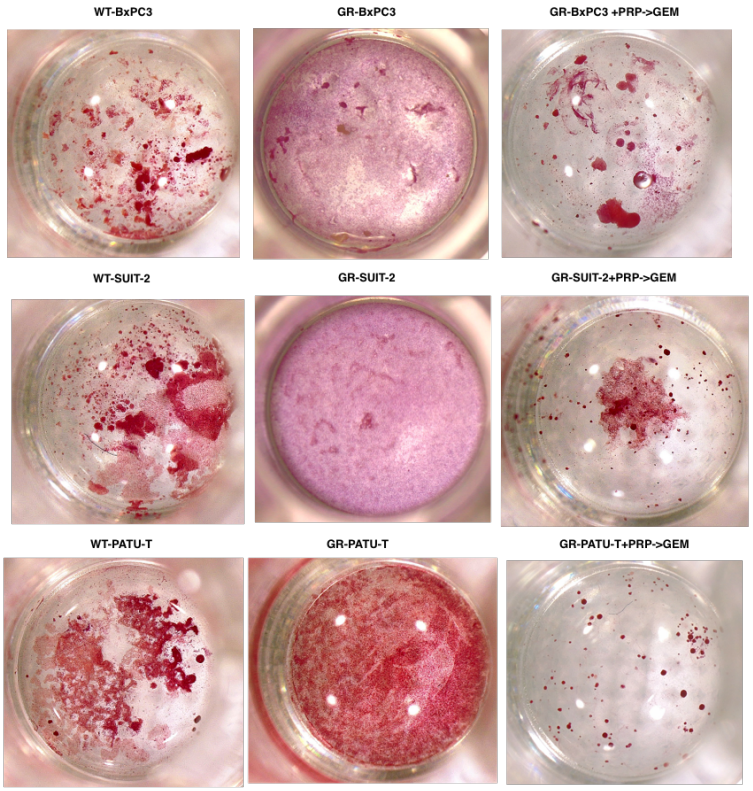
C.1)


WT-BxPC3
GR-BxPC3
GR-BxPC3 +PRP->GEM
WT-SUIT-2
GR-SUIT-2
GR-SUIT-2+PRP->GEM
WT-PATU-T
GR-PATU-T
GR-PATU-T+PRP->GEM
t0
t12
t24
t48
t72

C.2)


% Area Wound Clousure
120
90
60
30
0
*
***
T0
T12
T24
T48
T72
WT-BxPC3
GR-BxPC3
GR-BxPC3+PRP->GEM
WT-Suit-2
GR-Suit-2
GR-Suit-2+PRP->GEM
WT-Patu-T
GR-Patu-T
GR-Patu-T+PRP->GEM

D.1)


WT-BxPC3
GR-BxPC3
GR-BxPC3 +PRP->GEM
WT-SUIT-2
GR-SUIT-2
GR-SUIT-2+PRP->GEM
WT-PATU-T
GR-PATU-T
GR-PATU-T+PRP->GEM

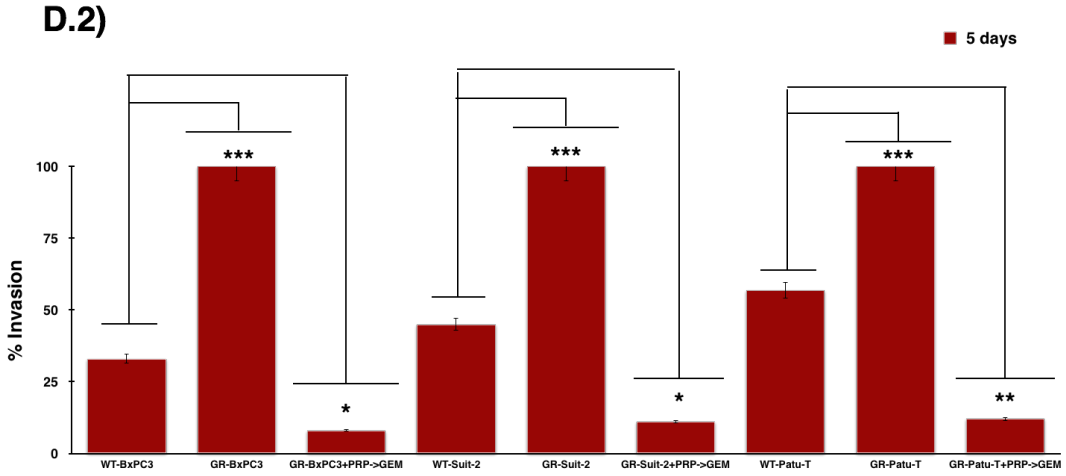


Figure 61. Tumourigenic capacity of pancreatic cell lines. A) Proliferation rate in BxPC3, Suit2.007 and Patu-T cell lines (WT and GR) during 19 days after seeding cells without or with PRP, GEM or coadjuvant treatments PRP+GEM and PRP→GEM. B.1) Colony formation assay by crystal violet staining at day 19 after seeding cells in the same conditions as A). B.2) Percentage of clonogenicity rate determined by the rate of stained cells at 19 days using ImageJ^{TM} software. C.1) Cell migration distances at 0, 12, 24, 48 and 72 hours after scratching by wound healing assay of BxPC3, Suit2.007 and Patu-T cells grown in different conditions without and with treatment. Original magnification 4x. Images analyzed with ImageJTM software. C.2) Percentage of cell migration determined by the rate of cells moving towards the scratched area over time using ImageJ^{TM} software. D.1) Cell invasion property at 5 days after seeding BxPC3, Sui2.007 and Patu-T cells in different conditions without and with treatment. Original magnification 1x. Images analyzed with ImageJ^{TM} software. D.2) Percentage of cell invasion determined by the rate of cells moving through the matrigel layer over time using ImageJ^{TM} software. The experiments were conducted in triplicate. Error bars indicate ± SD *P < 0.05, **P < 0.01, ***P < 0.001.

2.4 PRP and GEM treatment induces apoptosis in resistant pancreatic cancer cells

Here, it was delved into the apoptotic effects of PRP alone or combined with GEM on the chemoresistant cell lines. Annexin and PI double staining *via* cytometry revealed that coadjuvant treatment (2 dosis of PRP followed by 2 dosis of GEM: PRP→GEM) induced cells to undergo apoptosis or necrosis after, promoting a significant reduction in the number of alive chemoresistant cells (Figure 62). In the case of the BxPC3

line, PRP induced a significant 20% reduction in the number of alive WT cells by promoting early apoptosis by 5.1%, late apoptosis by 4.7%, and necrosis by 10.2%. For chemoresistant cells, when they were treated only with PRP it managed to increase early apoptosis by 3.3%, late apoptosis by 3% and necrosis by 5.4%. When the adjuvant treatment PRP→GEM was applied a significant decrease in cell viability (43%) was observed, with a 2.4% increase in early apoptosis, 16.4% in late apoptosis and 24.2% in necrosis (Figures 62A-B).

Furthermore, it was examined the expression of apoptosis-related genes, specifically Bcl-2, anti-apoptotic gene, and BAX, a pro-apoptotic gene (Figure 62C). Consistent with our previous findings, PRP-treated WT-BxPC3 showed significant up-regulation of BAX compared to untreated WT-BxPC3 (2.25-fold). Moreover, the expression of the anti-apoptotic gene Bcl-2 was down-regulated in PRP-treated WT-BxPC3 (0.7-fold). In the case of GR-BxPC3, the post addition of GEM after PRP treatment induced a significant change in which BAX expression increased 10-fold and Bcl-2 decreased 4-fold when compared to GR-BxPC3 untreated cells. This study was completed with the determination of the apoptotic susceptibility: BAX/Bcl-2 (Figure 62D) [832]. It was demonstrated that WT-BxPC3 cells developed a greater susceptibility (3.95 times) to the apoptotic process after being treated with PRP (comparing to untreated WT-BxPC3), the same occurred with GR-BxPC3 whose susceptibility increased 1.47 times after receiving the treatment with PRP and 3.5 times after the PRP→GEM treatment (comparing to untreated GR-BxPC3).

Additionally, the expression of Bcl-2 and BAX in Suit2.007 and Patu-T cell lines was examined (Figures 62E-F). In WT-Suit2.007 cells, PRP treatment led to a significant downregulation of Bcl-2 by 1-fold and a notable increase in BAX expression by 1.5-fold. In GR-Suit2.007 cells, PRP treatment alone or in combination with GEM (PRP→GEM) resulted in a significant decrease in Bcl-2 expression, by 4-fold and 3-fold,

respectively, and an increase in BAX expression by 5-fold with PRP and 6-fold with PRP→GEM (Figure 62E). In Patu-T cells, there were no significant changes in the WT cells. However, in chemoresistant Patu-T cells, Bcl-2 expression was significantly downregulated by 7-fold and 5-fold with PRP and PRP→GEM treatments, respectively, while BAX expression was upregulated by 5-fold with PRP and 2-fold with PRP→GEM (Figure 62F). This study was also completed with the determination of the apoptotic susceptibility: BAX/Bcl-2 (Figure 62D) [832]. WT-Suit-2 cells developed a greater susceptibility (4.16 times) to the apoptotic process after being treated with PRP (comparing to untreated WT-Suit-2), the same occurred with GR-Suit-2 whose susceptibility increased 1.19 times and 1.6 times after receiving the treatment with PRP and the PRP→GEM treatment respectively (comparing to untreated GR-Suit-2). In the case of the Patu-T cell line, it maintained a similar susceptibility between the WT and the WT treated with PRP. In the case of GR, its susceptibility increased slightly from 0.04 to 0.42 when treated with PRP and up to 1.1 when treated with PRP→GEM.

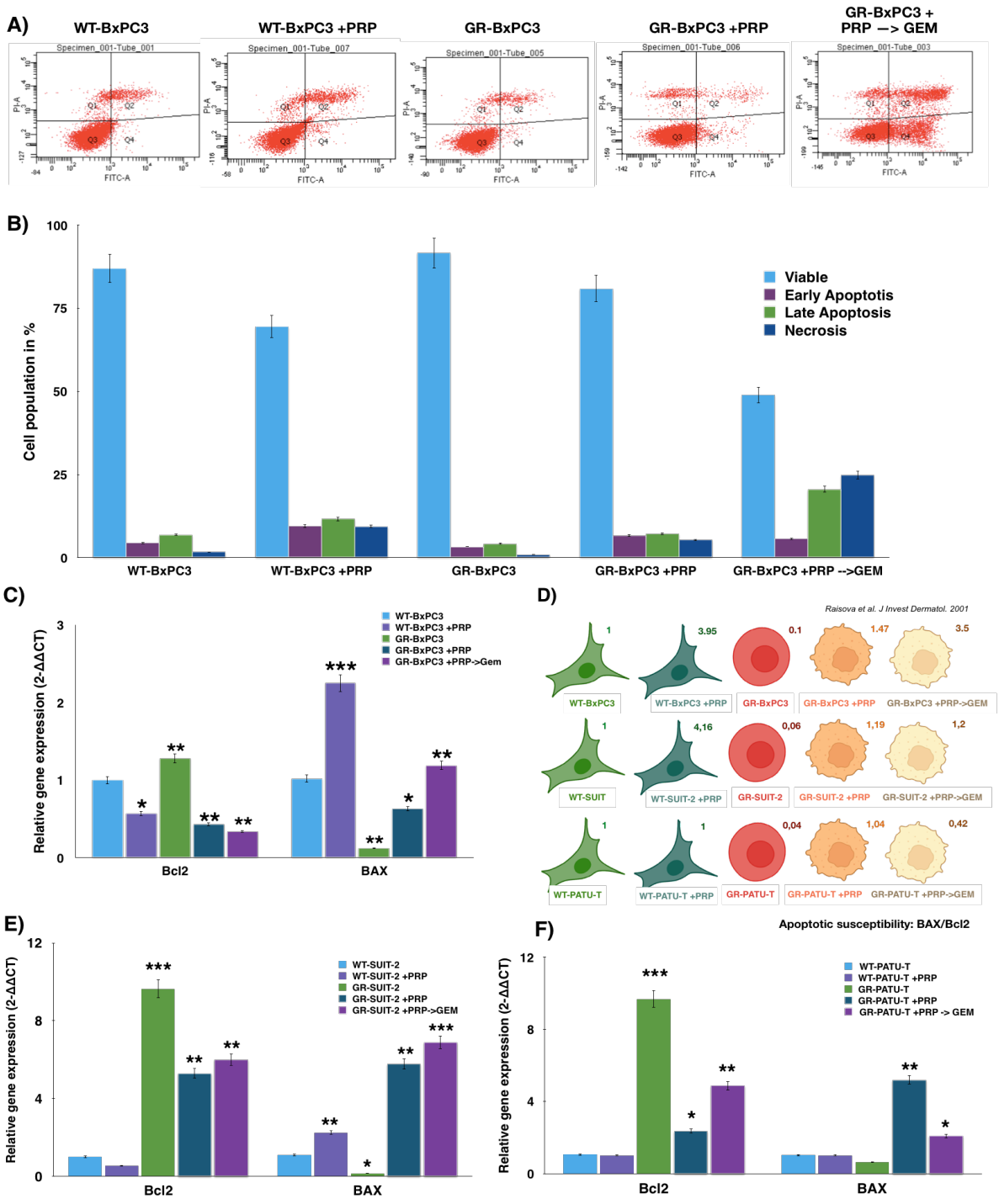


Figure 62. PRP increases apoptosis and necrosis in pancreatic cancer cells. A) and B) Representative images from flow cytometry after annexin V-fluorescein staining of WT and GR-BxPC3 with or without PRP and coadjuvant treatments (PRP+GEM and PRP→GEM) showing the percentage of cells in each stage. C) Expression levels of BAX and Bcl-2 genes in WT and GR-BxPC3 compared to those treated with PRP and coadjuvant treatment (PRP→GEM). D) BAX/Bcl-2 protein expression ratio for each experimental condition in BxPC3 cell line. E) and F) Expression levels of BAX and Bcl-2 genes in WT and GR-Suit2.007 and Patu-T cells compared to those treated with PRP and

coadjuvant treatment. The assays were repeated in at least three independent batches. Error bars indicate ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

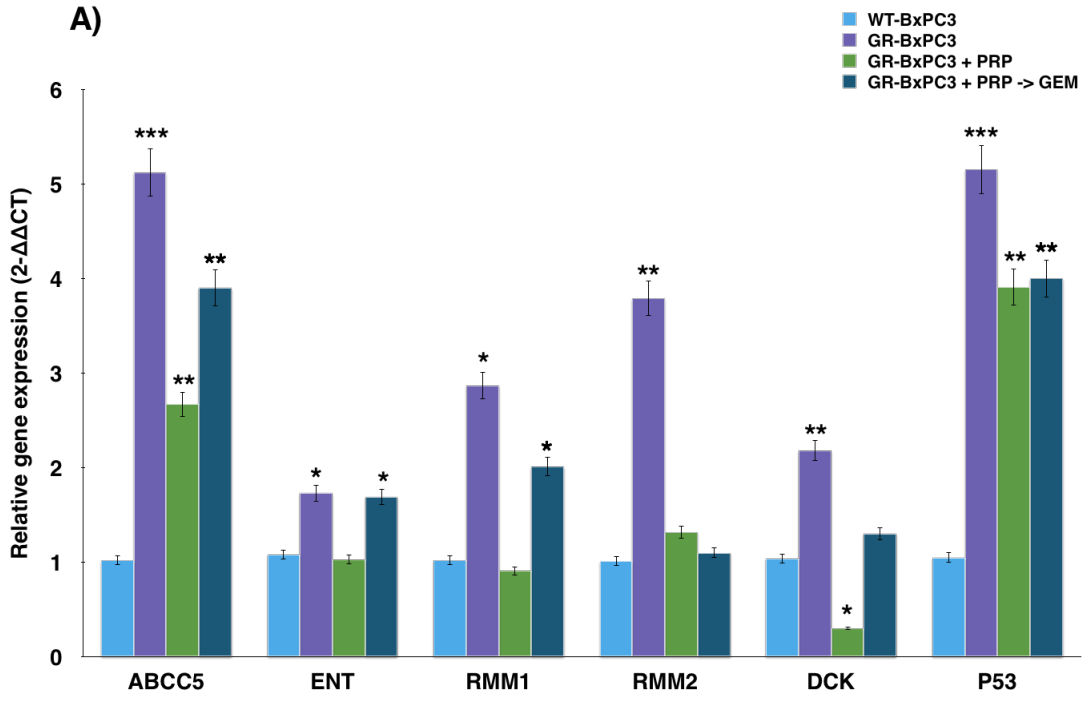
2.5 Overexpression of chemoresistance biomarkers is modulated by PRP

It was next evaluated if PRP could have any effect on the expression of genes related to GEM chemoresistance (Figure 63). By RT-qPCR it was determined gene expression of the exporter ABCC5, the metabolics RMM1, RMM2 and DCK, and the relevant uptake transporter ENT-1 in the pancreatic cell lines studied after the different treatment conditions (CTR, PRP or PRP→GEM) (Figure 63A). ABCC5 showed a 5-fold up-regulation in GR-BxPC3 when compared to WT-BxPC3. Additionally, the expression levels of RMM1, RMM2, DCK and ENT-1 in GR-BxPC3 were also significantly increased (2.9-fold, 3.8-fold, 2.2-fold and 1.7-fold, respectively) when compared to control cells. These findings confirm the acquisition of a chemoresistant phenotype against GEM after the resistant-GEM induction protocol applied (Figure 60A). Interestingly, when GR-BxPC3 were treated with PRP→GEM, chemoresistant related genes expression significantly decreased. Specifically, there was a 2-fold down-regulation of ABCC5, 1-fold down-regulation in RMM1, 2.7-fold down-regulation in RMM2, 1.1-fold in DCK and 0.25-fold in ENT-1, compared to non-treated GR cells. Results suggest that PRP treatment induces chemoresistant cells to be more sensitive to GEM, thereby inhibiting the chemoresistance acquisition phenotype process.

Figure 63B shows GEM sensitive ratio (GSR), which is based on the idea that the resistance/sensitivity to a drug does not depend on the isolated expression of different biomarkers but rather on the relationship between them [824]. For this calculation, the gene expression of the metabolic markers DCK, RMM1, and RMM2, as well as the transport marker ENT-1 were used following the formula: GSR=[ENT1][DCK] / [RMM1][RMM2]. It was observed that GR-BxPC3 cells showed nearly three times less sensitivity to GEM than WT cells. After receiving

PRP treatment, the sensitivity of the GR cells increased by twice, and approximately the same result was shown with the adjuvant treatment PRP→GEM. These values are clearly significant when comparing the resistance between WT and GR cells (treated and untreated).

Same genes were also evaluated in Suit2.007 (Figure 63C) and Patu-T (Figure 63D). The expression levels of ABCC5, RMM1, RMM2, DCK in GR-Suit2.007 were also significantly increased (5-fold, 3.2-fold, 3.8-fold and 2.9-fold, respectively) compared to WT. In contrast, ENT-1 suffered a downregulation in the chemoresistant cells as expected and described in most of the bibliography (1-fold downregulated) [830]. These expression levels were reverted when PRP→GEM treatment was administered since ABCC5, RMM1, RMM2 and DCK decreased 2.5-fold, 1.3-fold, 2.5-fold and 1.7-fold respectively. In contrast, ENT-1 increased 1.3-fold in GR-Suit2.007 after this treatment. On the other hand, GR-Patu-T cells suffered a higher expression in their markers, 7.6-fold in ABCC5, 8.3-fold in RMM1, 6.6-fold in RMM2 and 4.2-fold in DCK. ENT-1 was almost 100% inactivated in these cells. When PRP→GEM treatment was administered, Patu-T seemed to have a higher resistance than Suit2.007 as was expected, ABCC5 only decreased in 1.6-fold, RMM1 in 2.4-fold, RMM2 in 2.5-fold and in 2-fold in DCK, ENT-1 increased 0.7-fold.



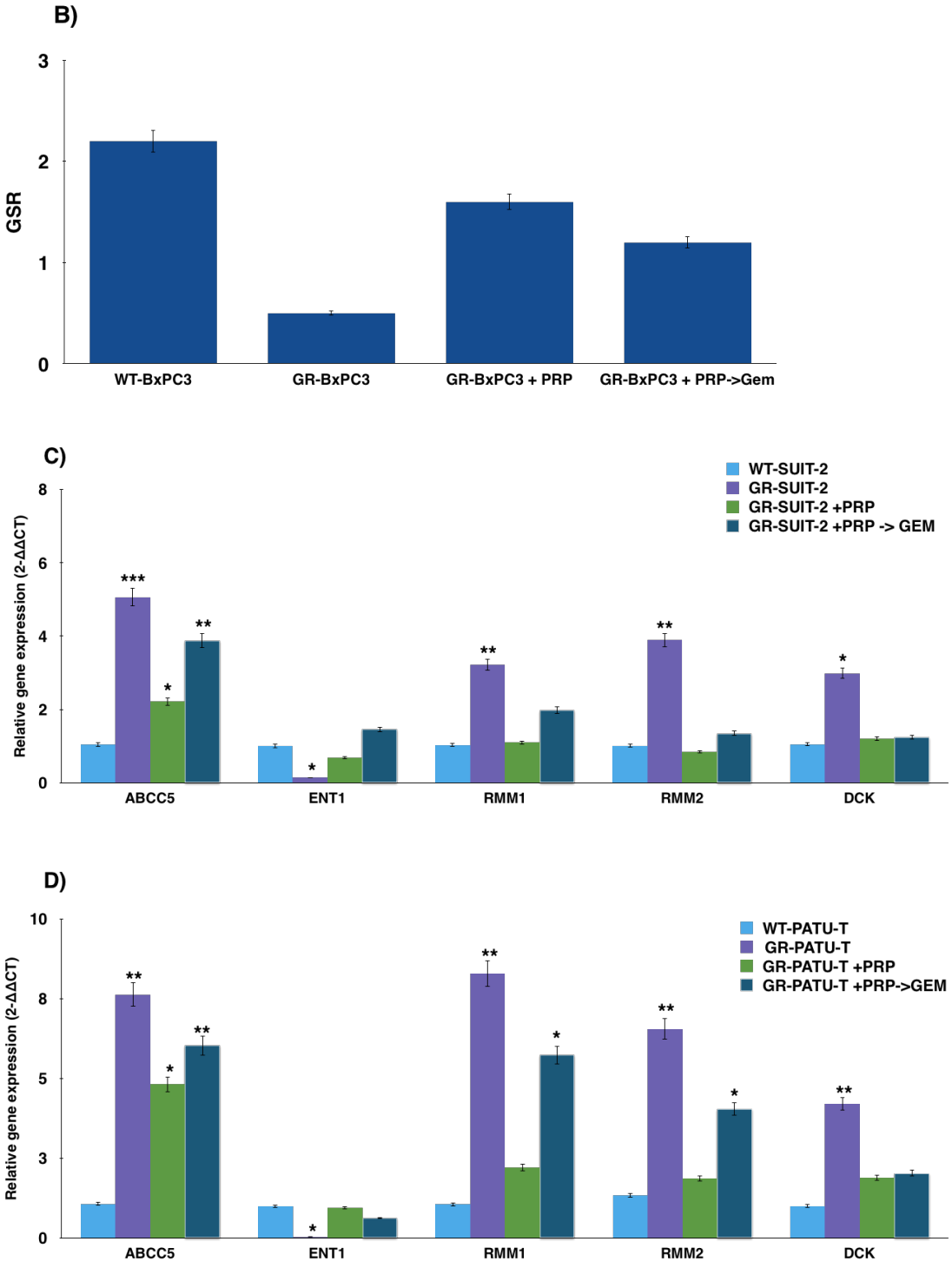
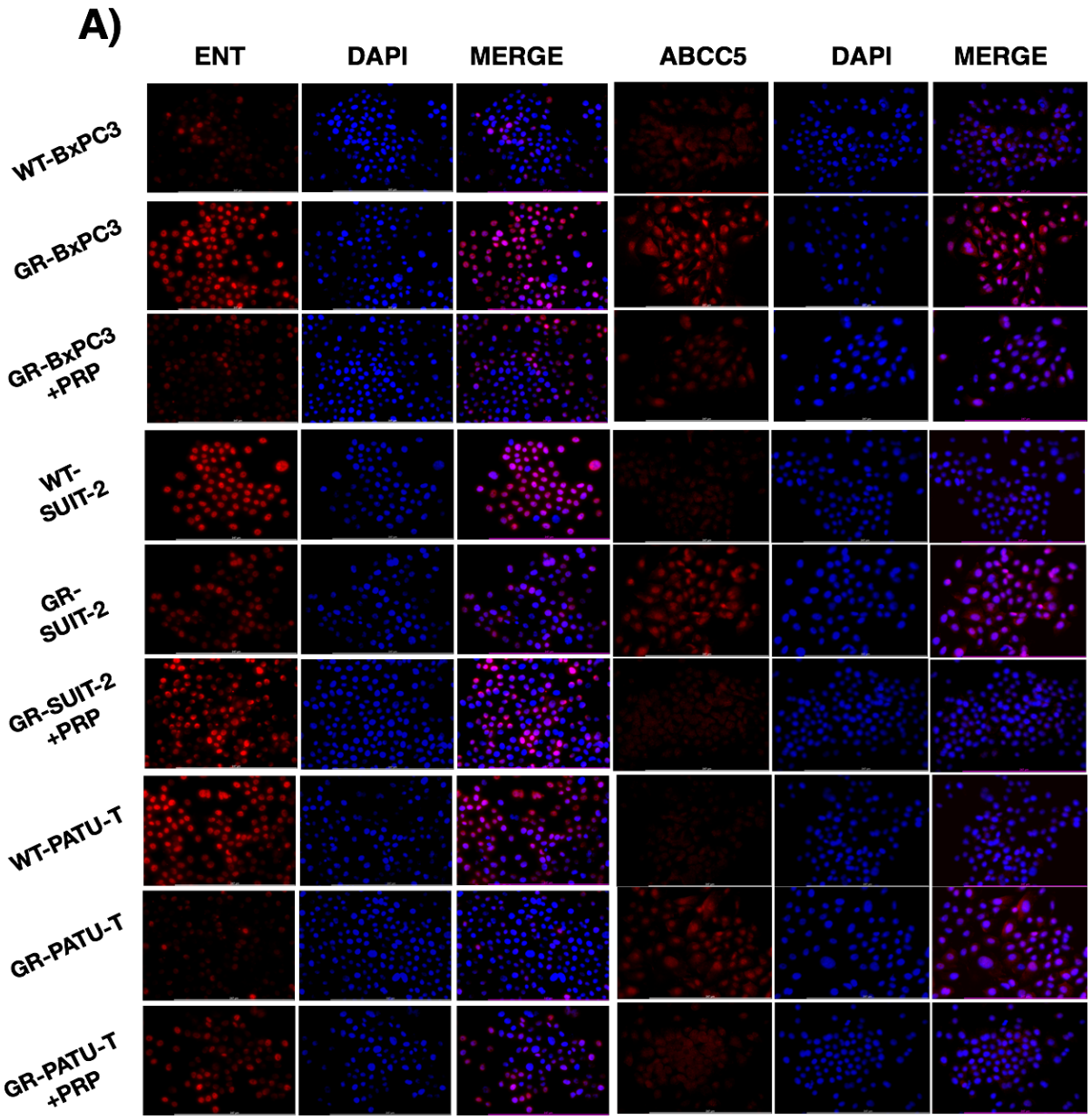
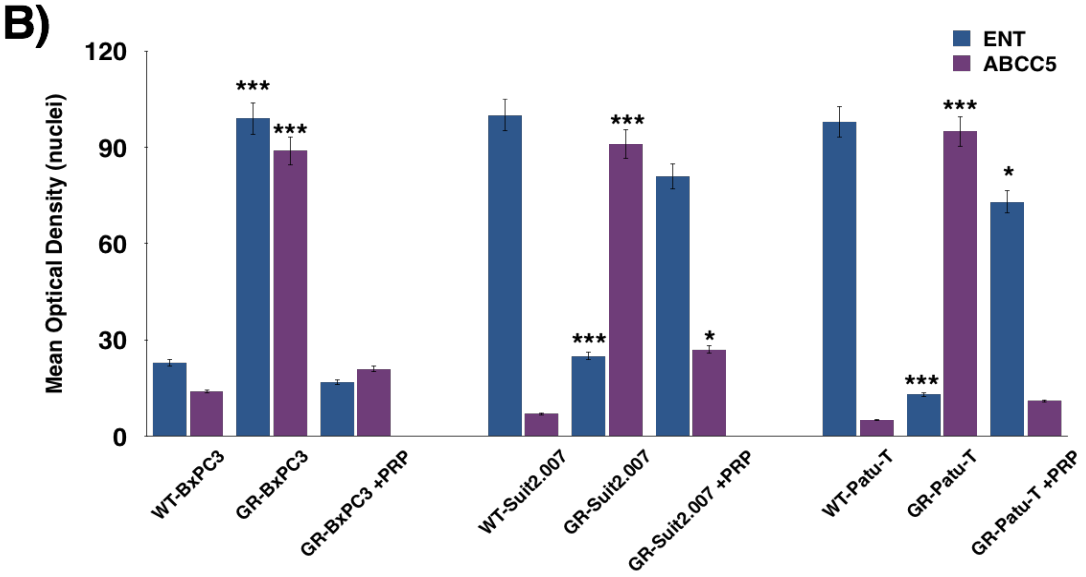


Figure 63. RT-qPCR analysis of markers related to chemoresistant phenotype in GR-BxPC3 (A); GR-Suit2.007 cells (C) and GR-Patu-T cells (D) after PRP or PRP→GEM addition, normalized by expression in WT cells. B) GSR formula application for BxPC3. Error bars indicate ± SD *P < 0.05, **P < 0.01, ***P < 0.001.

2.6 Chemoresistance phenotype of treated cells is affected by PRP treatment

Immunofluorescence and western-blotting analysis were performed in order to confirm previous gene expression results. Figures 64A and 64B show that ABCC5 protein expression (marked in red on the right) was significantly increased in GR-BxPC3, GR-Suit2.007 and GR-Patu-T cells by 75%, 84% and 90% respectively compared to WT-cells. Interestingly, a significant decrease in ABCC5 expression was observed after PRP treatment, with a 60-65% reduction in fluorescence intensity in all three GR-cell types analyzed. ENT-1 expression (marked in red on the left) increased by 76% in GR-BxPC3 while decreased by 75% and 80% in GR-Suit2.007 and GR-Patu-T, all compared to WT. This expression decreased by 82% in GR-BxPC3 when they received PRP or increased by 56% and 60% after they received their PRP treatment in GR-Suit2.007 and GR-Patu-T, respectively. Further, Figures 64C and 64D, show the result of the western blotting analysis performed on the BxPC3 cell line. It could be appreciated that the expression of the metabolic markers DCK and RMM2 significantly increased by almost 50% in GR-BxPC3 compared to WT-BxPC3 whereas it decreased after PRP treatment by 53% DCK and 42% RMM2 when compared with untreated GR-BxPC3. These results confirmed previous data obtained by the RT-qPCR assay.


A)
ENT
DAPI
MERGE
ABCC5
DAPI
MERGE
WT-BxPC3
GR-BxPC3
GR-BxPC3 +PRP
WT-SUIT-2
GR-SUIT-2
GR-SUIT-2 +PRP
WT-PATU-T
GR-PATU-T
GR-PATU-T +PRP


B)
ENT
ABCC5
Mean Optical Density (nuclei)
120
90
60
30
0
***
***
***
***
*
***
***
*
WT-BxPC3
GR-BxPC3
GR-BxPC3 +PRP
WT-Suit2.007
GR-Suit2.007
GR-Suit2.007 +PRP
WT-Patu-T
GR-Patu-T
GR-Patu-T +PRP

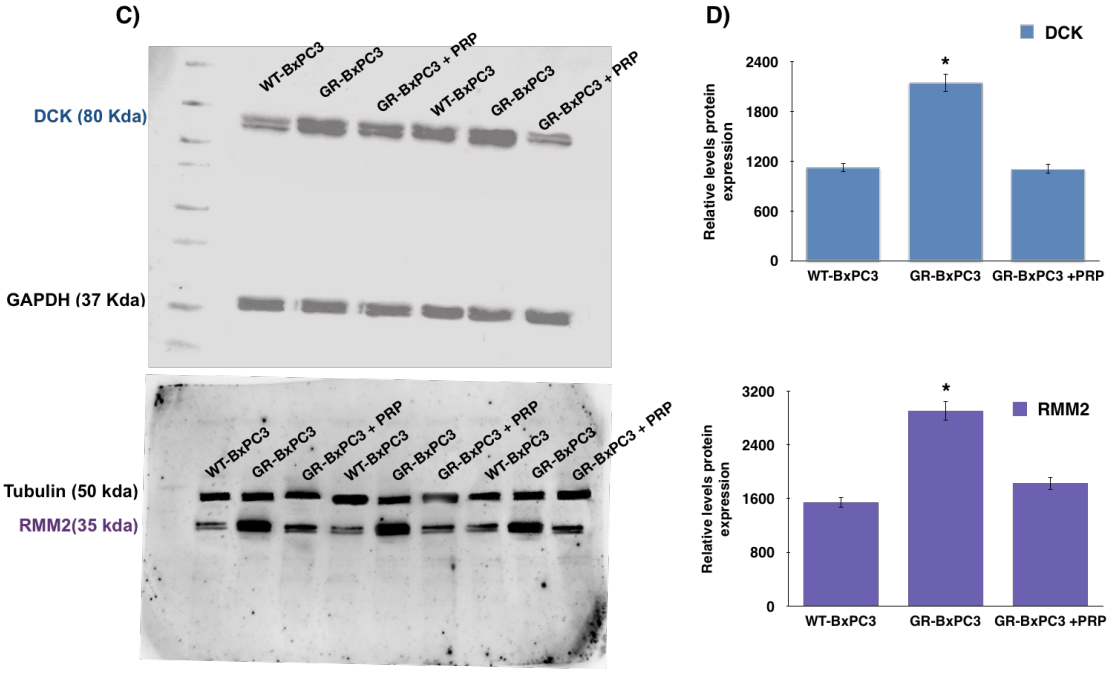
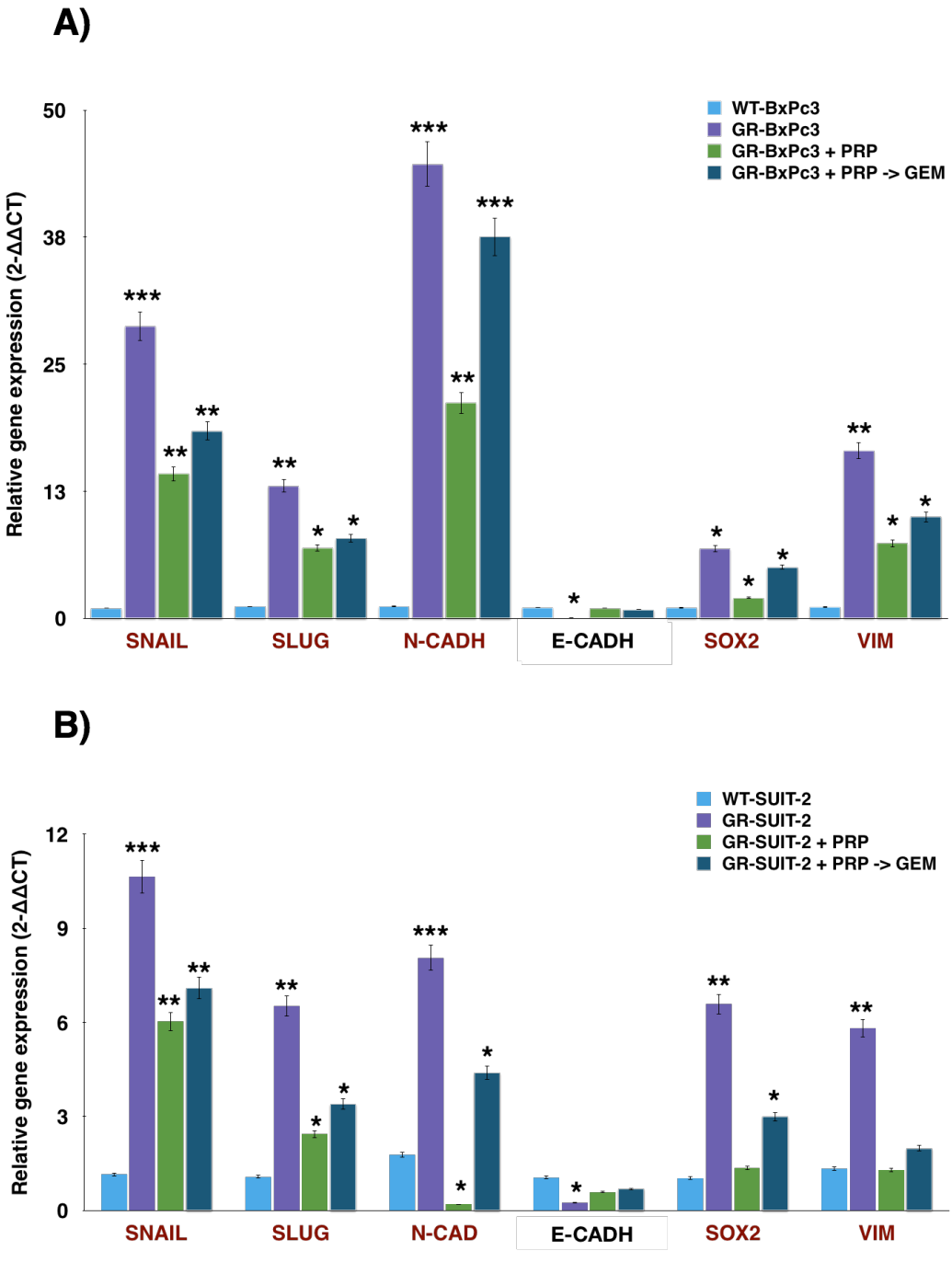


Figure 64. PRP modulates down-expression of biomarkers related to Gem outtake and metabolim. A) Representative immunofluorescence images of ABCC5 and ENT-1 expression (in red) in BxPC3, Suit2.007 and Patu-T cells (WT and GR) (with or without PRP treatment). Nuclei were counterstained with DAPI (in blue). Original magnification: 40x. B) Quantification of fluorescence intensity normalized by cell nuclei. C) Western blotting analysis of RMM2 and DCK in WT- and GR-BxPC3 (with and without PRP treatment). GAPDH and Tubulin were used as internal controls. D) The relative expression of RMM2 and DCK normalized to GAPDH and Tubulin were quantified using Image-J software NIH. The assays were repeated in at least three independent batches. Error bars indicate ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

2.7 EMT pathway is modulated by PRP in chemoresistant cells

EMT plays a pivotal role in the acquisition of a chemoresistant phenotype (Figure 25). Figure 65 showed a slight down-regulation of E-cadherin in GR-BxPC3, GR-Suit2.007 and GR-Patu-T of 1-fold, 1.3-fold and 1.7-fold respectively compared to WT-cells, and PRP→GEM treated cells suffered an up-regulation of 1.8-fold, 1.5-fold and 1.2-fold compared to untreated GR-cells (Figures 65A, 65B and 65C respectively). Notably, five other genes known to induce EMT were down-regulated following PRP→GEM treatment in GR cells. Specifically, the transcription factor Snail, associated with metastatic cell undergoing EMT,

showed a significant downregulation in GR-BxPC3, GR-Suit2.007 and GR-Patu-T when coadjuvant treatment PRP→GEM was administered (10-fold, 3-fold and 7-fold, respectively) comparing to the GR cells without treatment. Furthermore, other EMT markers including Slug (6-fold, 3-fold and 1-fold), N-cadherin (7-fold, 4-fold and 2-fold), Sox-2 (2-fold, 4-fold and 2-fold) and Vimentin (7-fold, 4-fold, 2-fold), exhibited decreased expression levels after PRP→GEM treatment in GR-cells. These findings align with our previous research [567,813], underscoring PRP's inhibitory effect on EMT in pancreatic tumour cells.



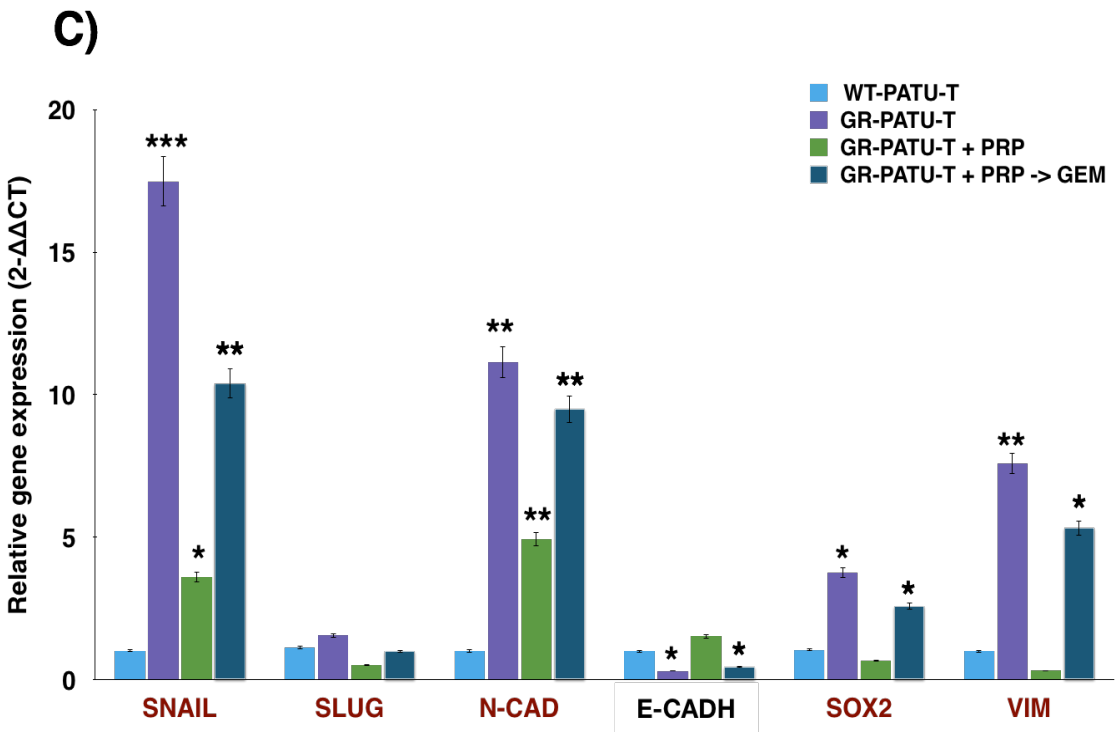


Figure 65. RT-qPCR analysis of markers related to EMT phenotype in GR-BxPC3 (A); GR-Suit2.007 cells (B) and GR-Patu-T cells (C), normalized by expression in WT cells, after PRP or PRP→GEM addition. The experiments were conducted in triplicate. Error bars indicate ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

2.8 PRP inhibits SMAD2/3 expression and phosphorylation in chemoresistant pancreatic cancer cells

TGF-β signaling plays a pivotal role in various biological processes that promote tumourigenesis and metastasis, including the acquisition of a chemoresistance phenotype in tumour cells. The canonical TGF-β pathway involves the expression and subsequent phosphorylation of Smad2/3 proteins [831]. To investigate whether PRP inhibits this process by interacting with this pathway (and how it could interact), the impact of PRP treatment on BxPC3, Suit2.007 and Patu-T (WT and GR) cell lines was assessed and compared with the addition of the TGF-β commercial inhibitor TGF-βR1 (LY2157299 or Galunisertib) (Figures 66 and 67). Figure 66A shows Smad2 and Smad3 gene expression results in WT-BxPC3 and GR-BxPC3 cells with or without PRP treatment.

Chemoresistance acquisition led to a significant up-regulation of Smad2 and Smad3 (95-fold and 49-fold respectively) compared to the WT-cell. Of interest, PRP treatment significantly reduced the gene expression of both Smad2 and Smad3 77-folds and 45-folds respectively, when compared to chemoresistant untreated cells. Similarly, treatment with TGF-βR1 resulted in a reduction of Smad2 and Smad3 gene expression of 187-folds and 169-folds respectively (Figure 66F). Furthermore, immunofluorescent labeling of Smad2/3 proteins demonstrated that PRP treatment tended to decrease the translocation of Smad2/3 into the nucleus (Smad2/3 was labeled in green and nucleus in blue). PRP treatment not only prevented Smad2/3 nuclear translocation but also decreased its cytoplasmic expression (Figure 66B-C). In parallel, TGF-βRI also reduced the Smad2/3 expression in treated GR-BxPC3 and blocked its translocation into the nuclei (Figure 66G-H). Finally, it was analyzed the expression of p-Smad2/3 and total Smad2/3 proteins by Western blotting in PRP and TGF-βRI treated GR-BxPC3 and compared them with WT- and GR-cells (Figure 66D-E and 66I-J). In the case of Smad2/3 and p-Smad2/3, there is a clear overexpression in GR-BxPC3 cells of more than 40% and 60% respectively, while the application of PRP represented a reduction of 82% and 90% (Figures 66D and E). In comparison, the commercial inhibitor Galunisertib was able to inhibit the expression of Smad2/3 and p-Smad2/3 by 90% and 92% respectively (Figures 66I and J).

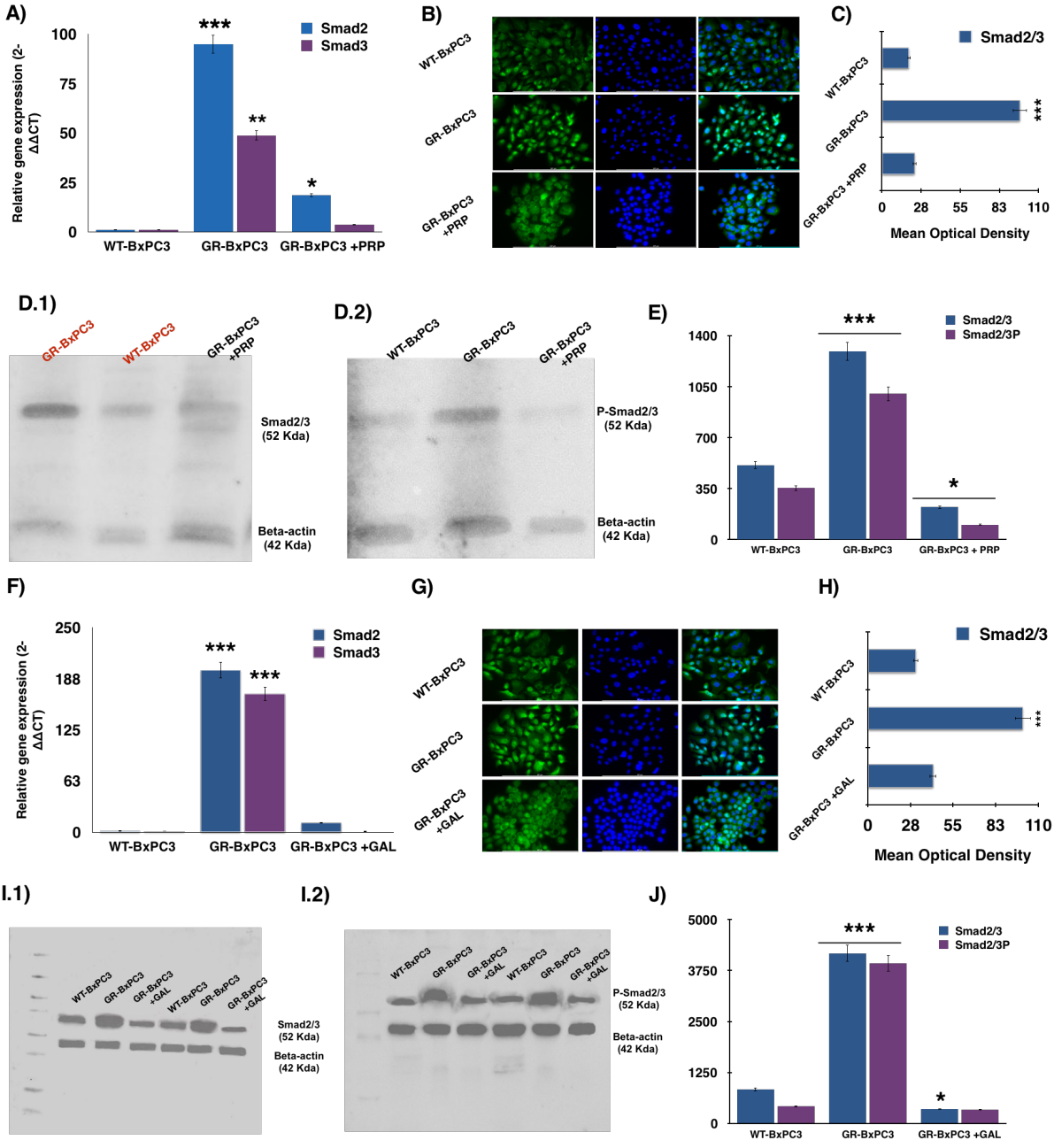


Figure 66. PRP modulates chemoresistance acquisition by interfering with the TGF-β pathway in BxPC3 pancreatic cell line. A) RT-qPCR analysis of Smad2 and Smad3 gene expression in WT-BxPC3, GR-BxPC3 and GR-BxPC3+PRP. Gene expression was normalized to the untreated cells control. B) Representative immunofluorescence images of Smad2/3 expression (green) in WT-BxPC3, GR-BxPC3 and GR-BxPC3+PRP. Nuclei were counterstained with DAPI (blue). Original magnification: 40x. C) Quantification of fluorescence intensity normalized by cell nuclei. D.1) Western blot analysis of Smad2/3 and D.2) Phosphorylated Smad2/3 (p-Smad2/3) in WT-BxPC3, GR-BxPC3 and GR-BxPC3+PRP. β-actin was used as an internal control. E) The relative expression of Smad2/3 and p-Smad2/3 normalized to β-actin was quantified using Image-J software NIH. F-J) Experiments were repeated treating GR-BxPC3 with the pharmacological

inhibitor Galunisertib (TGFβR1 inhibitor) instead of PRP. The assays were repeated in at least three independent batches. Error bars indicate ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Furthermore, the expression of Smad2 and Smad3 in Suit2.007 and Patu-T cell lines was also analyzed via RT-qPCR and immunofluorescence (Figure 67). Chemoresistance acquisition led to a significant up-regulation of Smad2 and Smad3 (21-fold and 31-fold respectively) in GR-Suit2.007 compared to the WT-cell. PRP treatment significantly reduced the gene expression of both Smad2 and Smad3 by up to 17-fold and 29-fold respectively compared to chemoresistant untreated (Figure 67A). In the case of GR-Patu-T, the upregulation was by 38-fold and 27-fold when the chemoresistance was acquired and the reduction was by 22-fold and 21-fold after the treatment in the GR-cell for Smad2 and Smad3 respectively (Figure 67C). The immunofluorescence analysis also corroborated the translocation and phosphorylation of Smad2/3 complex into the nuclei in the chemoresistant cells, on the other hand, Smad2/3 expression and translocation decreased when PRP was administered in both GR-cell lines (Figures 67B and 67D).

These findings collectively suggest that PRP may impede the involvement of the TGF-β pathway in the chemoresistant phenotype acquisition process by inhibiting Smad2/3 phosphorylation, facilitating Smad2/3 dephosphorylation, or by directly binding and inhibiting the formation of the Smad2/3+4 complex and its translocation into the nuclei (showed in Figure 57). These results were corroborated by the fact that TGF-β inhibitor treatment also induced a reduction in the expression of Smad2/3 and p-Smad2/3 similar to PRP treatment.

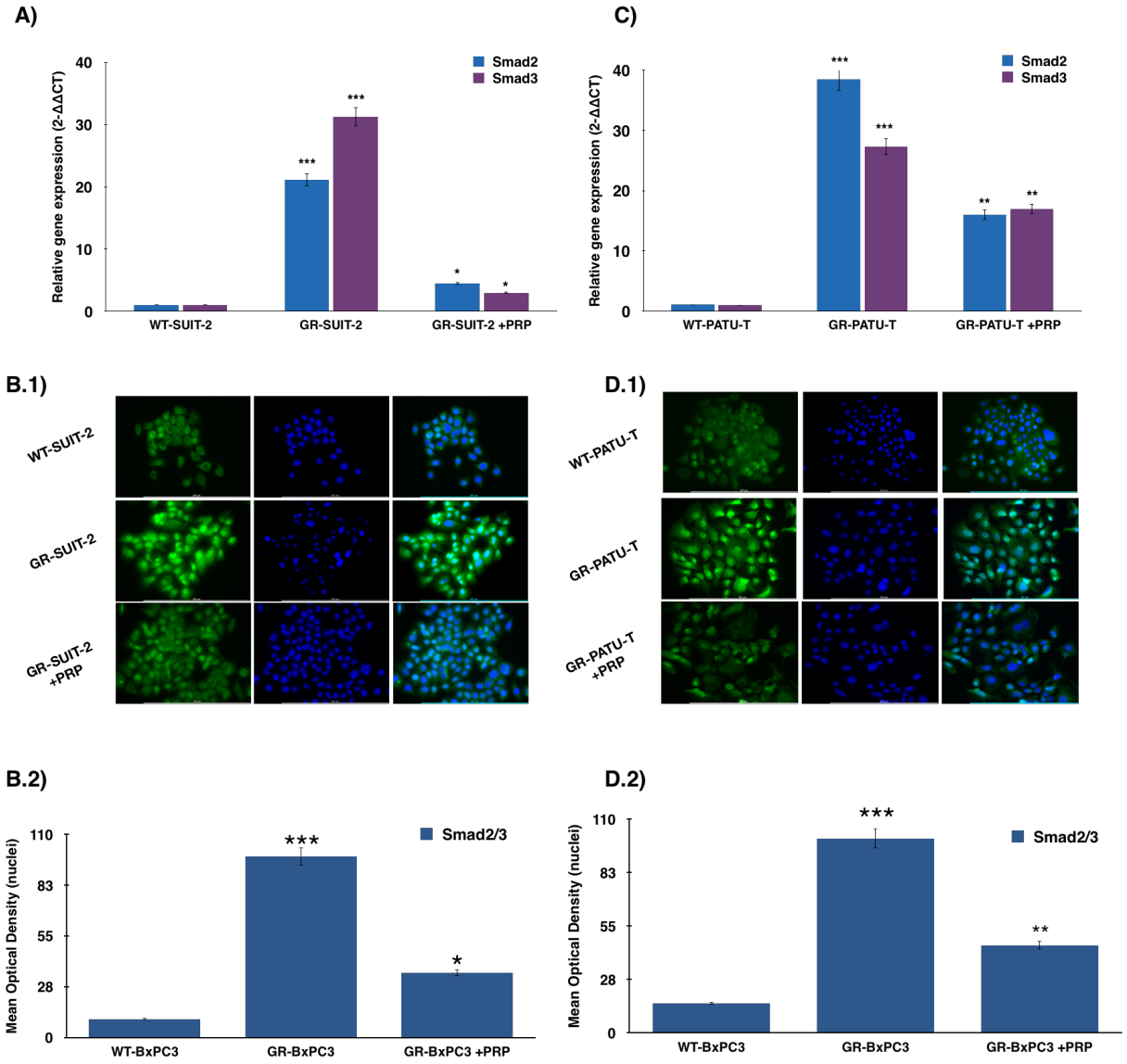


Figure 67. PRP modulates chemoresistance acquisition by interfering with the TGF-β pathway in Suit2.007 and Patu-T pancreatic cell lines. A) RT-qPCR analysis of Smad2 and Smad3 gene expression in WT-Suit, GR-Suit and GR-Suit +PRP. Gene expression was normalized to the untreated cells control. B.1) Representative immunofluorescence images of Smad2/3 expression (green) in WT-Suit, GR-Suit and GR-Suit+PRP. Nuclei were counterstained with DAPI (blue). Original magnification: 40x. B.2) Quantification of fluorescence intensity normalized by cell nuclei. C) RT-qPCR analysis of Smad2 and Smad3 gene expression in WT-Patu-T, GR-Patu-T and GR-Patu-T +PRP. Gene expression was normalized to the untreated cells control. D.1) Representative immunofluorescence images of Smad2/3 expression (green) in WT-Patu-T, GR-Patu-T and GR-Patu-T +PRP. Nuclei were counterstained with DAPI (blue). Original magnification: 40x. D.2) Quantification of fluorescence intensity normalized by cell nuclei. The assays were repeated in at least three independent batches. Error bars indicate ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

DISCUSSION

DISCUSSION

Despite recent advances in the last years, the incidence of cancer is continuously increasing [1-5]. Currently, the basis of cancer therapy relies on radiotherapy and chemotherapy notwithstanding their undesirable side effects. For this reason, new less invasive treatments are being investigated with the aim of improving the anti-tumour efficiency while reducing the side effects of current treatments [64,65]. PDAC is one of the most aggressive and lethal forms of cancer, accounting for approximately 90% of all pancreatic malignancies. Characterized by its late diagnosis and rapid progression, PDAC often presents at an advanced stage, severely limiting treatment options and resulting in a very poor prognosis. The five-year survival rate for PDAC patients remains dismally low, often less than 12% [62,63].

The TME plays a crucial role in the development, progression, metastasis, and recurrence of PDAC. This consists of various cellular components, including CAFs, immune cells, MSCs, and ECM proteins, which create a supportive niche that promotes tumour growth and survival. These features make TME crucial in cancer appearance, progression, recurrence and metastasis [127]. Given its multifaceted role in supporting tumour dynamics, targeting the TME has become a critical focus in developing more effective and durable therapeutic strategies for PDAC.

On the other hand, chemoresistant cells represent an important subset within tumours with a crucial role in cancer recurrence and metastasis [146,502]. In cases where most of the disease is eradicated and chemotherapy is administered, followed by relapse, the tumour cells may not have been completely eradicated and develop chemoresistance [502,529]. This new cell population is responsible of treatment failures, which ultimately lead to tumour growth. Furthermore, chemoresistant cells have demonstrated increased resistance to apoptosis [510]. The drug resistance and tumour recurrence exhibited by chemoresistant individuals can be attributed mainly to the overexpression of ABC efflux membrane proteins, to the low-expression of entry proteins such as ENT, to

the development of a drug metabolism, more active, as well as its ability to remain in a rest state [829,830].

The anticancer effect of Trypsinogen and Chymotrypsinogen has been proven, showing an impact on tumour cell proliferation, cell migration, cell invasion, angiogenesis and tumour progression. Further, the pro-enzyme formulation has also shown to impact on the subpopulation of CSCs derived from a human pancreatic cell line. In fact, treatment of the pancreatic-derived CSCs resulted in inhibition of the EMT process; suppression of CSCs related genes and *in vivo* impaired tumour initiation [567,813]. Other studies *in vitro, in vivo* and even with patients have also concluded that pro-enzymes treatment could be a valuable strategy to suppress tumour progression and even stop metastasis [567]. This represents an important starting point to evaluate its effect on the TME as well as chemoresistant cells.

First of all, this Thesis aimed to examine the impact of the PRP formulation on the intricate molecular interplay between pancreatic cancer cells and stromal cells. Using the pancreatic cell line BxPC3 it was observed that PRP treatment attenuates the capacity of both non-stem cells and CSCs and inhibits the conversion of NAFs into CAFs. This attenuation is evidenced by the diminished expression of CAF-associated cytokines such as SDF1 [833], along with CAF-specific markers like FSP1 [834] and FAPα [835]. Notably, while αSMA has been traditionally identified as a CAF-specific marker, in previous studies [833,836], our findings demonstrate that NAFs also expressed αSMA. This divergence in CAFs recognized markers could be attributed to the unique expression profiles of TME-related cells, such as CAFs, which are influenced by their precursor cell type and the molecular factors/pathways governing their "malignant education" [837]. Fibroblast activation is intricately linked to the intrinsic molecular dynamics of each cancer subtype. Indeed, various distinct CAF subpopulations with specific expression profiles and functional behaviors have been delineated within the same type of cancer, including PDAC [838], lung adenocarcinoma [839], and hepatocellular carcinoma [840]. Moreover, the relative abundance of each CAF subpopulation may fluctuate during different stages of tumour

progression [841].

Furthermore, the direct treatment of CAFs with PRP prompted the lowering of CAFs-related markers (SDF1, FSP1, and FAPα), whereas NAFs-associated markers like αSMA expression were not significantly altered compared with untreated controls. This is a relevant fact since it has been recently documented important functional disparities between FAPα- and αSMA-expressing CAFs in PDAC [841]. Interestingly, a study showed that there are two non-overlapping CAF subpopulations, with FAPα+ CAFs exhibiting pro-tumourigenic properties, whereas αSMA+ CAFs present an anti-tumour behavior [841]. Additionally, the authors used the high ratio αSMA/FAPα-expressing CAFs as a good prognosis factor related to increased overall survival in PDAC patients [841]. Similarly, the abundance of αSMA+ CAFs has been directly correlated with non-recurrent PDAC, while the increased presence of FAPα+ CAFs has been related to peritoneal metastases [842]. In the same line, Chiavarina and colleagues established different CAF subgroups in hepatocellular carcinoma based on specific expression profiles and their particular parental cell type, including hepatic stellate cells, vascular smooth muscle cells, and hepatic portal fibroblasts [840]. This work remarked that hepatic portal fibroblast-derived CAFs exhibited an anti-tumour activity through the secretion of prolargin [840]. Thus, these studies suggest the relevance of CAF phenotypic diversity/plasticity in the context of cancer.

Here, we show that PRP treatment triggered the upregulation of Meflin in treated CAFs, which may be considered as a relevant event since it has been previously noted that this factor could be categorized as a tumour-restraining CAFs biomarker and that the abundance of Meflin-expressing CAFs was positively correlated to tumours with well-differentiated histology, favorable clinical outcomes, and earlier stages of disease progression in PDAC patients [843]. In this context, pharmacologically enhancing Meflin expression in CAFs has been proposed as a strategy to convert them into tumour-suppressive cells, aiming to sensitize PDAC to chemotherapy [844]. Additionally, it was observed that NAFs exhibited an elongated, spindle-like morphology, contrasting with the

star-like shape of CAFs obtained after co-culture with monolayer-BxPC3 and CSCs-BxPC3 cells. This finding is consistent with previous studies reporting similar morphological changes during CAF transformation, as observed in NAFs co-cultured with gastric cancer cells [836]. Notably, treatment with the PRP formulation also induced slight morphological changes in CAFs towards a more elongated shape, although not identical to the morphology associated with NAFs (yet observable in most treated CAFs).

Furthermore, a distinct response to PRP treatment between CAFs generated by BxPC3 non-stem cells and those generated by BxPC3 CSCs (more resistant) was observed. This difference could be attributed, at least in part, to the close relationship between CSCs and a specific CAF subpopulation, termed myofibroblast-like CAFs, as suggested in previous studies [837], which might exhibit differential reactions to the PRP mixture. Nevertheless, although these CSC-induced CAFs appeared slightly more resistant to PRP-reprogramming effects, the results were still significant, indicating promising therapeutic potential for the pancreatic pro-enzymes formulation.

Therefore, these findings support the idea that the PRP formulation may prompt the phenotypic re-education of treated CAFs, transitioning from a tumour-supportive phenotype to a more tumour-restrictive behavior. Indeed, the phenotypic plasticity of CAFs in response to certain external cues has been well-documented. For example, the transition from a proinflammatory-like phenotype towards a myofibroblast-like phenotype induced by TGF-β has been demonstrated in PDAC-related CAFs [845]. Importantly, our results demonstrate that the potential to induce the CAFs phenotypic switch can also be extrapolated to the *in vivo* level, as shown by the decreased FAPα and Smad2/3 expression and increased Meflin staining in the mouse xenograft model. Here, it was observed a reduction in the amount of fibrotic tissue surrounding the tumours in mice that were treated with PRP. This can be attributed to the significant decrease in TGFβ-1, which is closely linked to the transformation of healthy fibroblasts into CAFs [846]. The TME can also lead to the development of new CSCs from non-stem cancerous cells by releasing factors such as IL6, HGF, or TGFβ-1 [127].

Treatment with PRP before, during and after the malignification process may decrease TGFβ-1 levels, which could impede the engraftment of CSCs, formation of their niche, and even subpopulation activation.

Interestingly, our results also indicate that the PRP compound selectively induces CAF apoptosis without significantly affecting NAF viability. This finding may be associated with alterations in the BAX/Bcl-2 expression ratio in non-treated and treated CAFs compared to that exhibited by NAFs. In this context, other studies on colorectal cancer have demonstrated the alteration of the BAX/Bcl-2 expression ratio in stromal cells within the TME, leading to aberrant growth rates [847]. Such expression changes in pancreatic CAFs, particularly the overexpression of the anti-apoptotic factor Bcl-2, may contribute to the generation of extensive fibrosis associated with pancreatic cancer by preventing CAF apoptosis and growth control. Importantly, PRP may counteract this circumstance through the enhancement of pro-apoptotic BAX along with the inhibition of Bcl-2 expression in treated CAFs, while no significant changes were observed in NAFs exposed to the PRP mixture.

Reinforcing the therapeutic potential of PRP, the results also demonstrate a decreased capacity of CAFs pre-treated with PRP to promote cancerous cell proliferation, migration, the EMT process and even to induce the pro-tumourigenic "education" of other NAFs. In this regard, a study on gastric cancer revealed that NAFs educated by CEFs presented an alternative protumoural phenotype characterized by the absence of αSMA and a high expression of pro-inflammatory cytokines, including IL-1b, IL-6, IL-33, LIF, or CRLF1, along with an upregulation of CXCL8 [848]. Specifically, our results indicate that treatment of CAFs with PRP drastically reduced their ability to potentiate the expression of CXCL8 together with pro-inflammatory cytokines like IL1 or IL6 in NAFs [848], suggesting a reduced capacity of treated CAFs to induce the generation of CEFs. Indeed, these findings are consistent with previous investigations describing the anti-inflammatory role of pancreatic (pro)enzymes [785]. In a similar context, it is noteworthy to indicate that treated CAFs have a significantly lower ability to induce the proliferation of endothelial cells like HUVECs [848], which may be

especially relevant given the well-established protumoural role of angiogenesis in tumour progression. Moreover, additional functional properties of CAFs have been indicated by other researchers, highlighting their interaction with other TME-derived cells, including TAMs. For instance, the reprogramming of blood monocytes towards an immune-suppressive and protumourigenic phenotype by triple-negative breast cancer-derived FAPα-expressing CAFs has recently been identified [477,849]. Similarly, the polarization of M0 macrophages into tumour-promoting M2 TAMs by CAF-derived CM has been demonstrated in studies of hepatocellular and cervical cancer [850,851]. Importantly, the authors also showed that M2 TAMs generated by CAFs exhibited a particular secretome different from that of M2 TAMs directly induced by cancerous cells, thus reflecting a similar broad heterogeneity compared to that of their own CAFs [850]. In fact, it has been revealed that TAMs polarization can be specifically influenced by a particular CAF phenotype or subgroup [850].

Several therapeutic approaches have been described to mitigate tumour-associated fibrosis, as it may exert tumour-supportive roles beyond pro-tumourigenic chemical signaling. A dense fibrous ECM, directly correlated with the abundance of CAFs, can decrease intra-tumour blood perfusion, thereby hindering the delivery of anti-cancer drugs [851]. Additionally, CAFs themselves may promote a chemoresistant phenotype in PDAC cells through various mechanisms, including the secretion of exosomes containing specific miRNAs [852]. Therapeutic strategies targeting TME fibrosis can be classified into three main groups: (i) targeting ECM-related molecules; (ii) directly targeting CAFs; and (iii) targeting upstream pro-fibrotic signals [127,853]. According to this classification, PRP treatment fit into the three groups, as it interferes in the TGF-β pathway, induces the apoptosis/reprogramming of CAFs and reduces the capacity of cancerous cells to generate CAFs, likely by altering the pro-fibrotic signals derived from pre-treated tumour cells. Indeed, this event holds special relevance, as other studies have suggested that solely targeting the TME of PDAC may not be sufficient to effectively combat this disorder [854,855].

It is important to highlight the reprogramming, but not drastically cytotoxic effects of PRP on the general fibroblast population. For instance, total

blockade of Sonic Hedgehog signaling in a mouse model of PDAC led to the general depletion of all CAF subpopulations along with stromal fibrosis, resulting in more aggressive pancreatic tumours with heightened proliferation [856]. Similarly, research has demonstrated adverse outcomes, such as accelerated tumour progression, increased cancer aggressiveness, and reduced overall survival in mouse models of pancreatic cancer following the complete eradication of all CAF subpopulations [857]. Özdemir and colleagues proposed the drawbacks of eliminating all CAF subpopulations, including tumour-suppressive CAFs, which could elucidate the conflicting outcomes observed with anti-fibrotic cancer therapies [857]. Supporting this notion, other studies have argued that reprogramming or normalizing the TME, rather than eliminating it entirely, might be more beneficial, as observed in pancreatic tumours [858]. A recent study has emphasized the reprogramming of CAFs, tumour-infiltrating immune cells, the ECM, and the normalization of tumour-associated vasculature as a promising therapeutic strategy for long-term cancer management [859,860].

We aimed to understand how PRP treatment induces the reprogramming of CAFs, focusing on inhibiting the TGF-β pathway. This approach aligns with studies by Lan et al., showing promising outcomes of anti-TGF-β drugs as therapy for cancer-associated fibrosis [861]. Our findings are consistent with previous research from our laboratory, where PRP treatment altered the TGF-β pathway in BxPC-3 CSCs, reducing their aggressiveness [567]. Specifically, it was observed significant reductions in fibrotic desmoplasia associated with pancreatic tumours in nude mice following PRP treatment. Furthermore, our results indicate that PRP treatment partially inhibits the canonical TGF-β/Smad axis in CAFs. These findings suggest that PRP may impede the involvement of the TGF-β pathway in the transition of NAFs to CAFs by inhibiting Smad2/3 phosphorylation, facilitating Smad2/3 dephosphorylation, or by directly binding and inhibiting the formation of the Smad2/3+4 complex and its translocation into the nuclei. This partial inhibition is significant considering the functional switch observed in this pathway during tumour progression, transitioning from a tumour-suppressive to a tumour-supportive role [862,863]. Thus, modulating rather than completely

inhibiting this pathway may be crucial, similar to preserving specific CAF subpopulations within the TME [127]. This partial inhibition may explain the reprogramming of CAFs towards a less pro-tumoural phenotype.

Treated CAFs showed reduced abilities to induce tumour cell proliferation, migration, and the EMT processes closely linked to the TGF-β pathway. Our data also suggest a molecular link between cancer cells and CAFs *via* the TGF-β pathway, indicating a potential therapeutic target for both cell types with a single formulation. Moreover, other pro-fibrotic pathways involved in CAF transformation/activation, such as platelet-derived growth factor (PDGF), hedgehog (HH), or connective tissue growth factor (CTGF), may partially contribute to the observed reprogramming effects of PRP treatment [864-866]. To conclude, quiescent fibroblasts expand upon activation in response to stimuli released by damaged organs and emerging inflammation, generating growth factors and ECMs to autoregulate their expansion as well as to regulate inflammation and immunity. For decades, activated fibroblasts were thought to be cancer cells' accomplices in promoting tumour growth. However, fibroblasts appear to act as involuntary regulators of tumour growth, either positively or negatively, depending on the origin of the fibroblast on one hand, and on the other hand, the context in which it is found, such as the stage of tumour progression. To make things worse, CAFs exhibit remarkable heterogeneity and plasticity, allowing CAFs from the same tumour to arise through different pathways, have different phenotypes, and have functionally different effects on tumour cells and the microenvironment. Nonetheless, it is clear that controlling, reducing, or re-educating the CAFs subpopulation within the tumour is critical to improving current treatments. CAFs are appealing drug targets for a variety of reasons, including: (i) they are genetically more stable than neoplastic cells, making them less prone to developing resistant phenotypes due to high mutation rates and clonal selection; (ii) they have epigenetic differences from normal stromal cells; (iii) they are responsible for the structure of the tumour ECM; and, finally, (iv) CAFs support neoplastic cells throughout the disease spectrum, from pre-invasive lesions to the metastatic focus. Thus, by targeting CAFs, different stages of the disease, as well as numerous pathophysiological processes such as angiogenesis,

EMT, and immune evasion, could be influenced. All in all, the true role of tumour stroma in the invasion and metastasis of cancerous lesions remains unclear. In fact, experimental findings have supported both a protective and a permissive role against tumour development depending on the stage of tumour progression. Therefore, it is more than possible that fibroblasts are both "heroes" and "villains", and it is necessary to investigate, in depth, the different factors involved in these interactions during the different tumour stages and to determine what causes fibroblasts to switch from one team to another and at what time. This opens up avenues for future studies related to PDAC-TME.

Treatment resistance in pancreatic cancer is a significant challenge in the field of oncology, as it concerns all available therapies, from chemotherapy to target therapy and immunotherapy. As these cancers have limited treatment options, shedding light on these hidden mechanisms of treatment resistance can pave the way for groundbreaking advancements in the fight against cancer. The molecular mechanisms of resistance are numerous, complex, and often intertwined, as they include processes that are inherently connected: genetic alterations, altered drug metabolism and efflux, changes in the tumour microenvironment, and phenotypic transformations [460-480]. Unraveling the complexities of these mechanisms is essential to develop strategies to overcome or prevent resistance, ushering in a new era of personalized medicine, where tailored treatments can effectively combat chemoresistant cancers.

Despite the existence of multiple current therapies that are being employed to combat different levels of chemoresistance (Tables 1–4), it is imperative to emphasize the role of collaborative interdisciplinary research in the pursuit of overcoming treatment resistance in PC. Integrating insights from diverse scientific disciplines, including genomics, pharmacology, immunology, and computational biology, can provide a more comprehensive understanding of the intricate network of factors contributing to resistance [121,122]. Furthermore, advancements in diagnostic tools and technologies, such as liquid biopsies and high-throughput sequencing, hold promise in identifying early markers of treatment resistance. These tools not only enhance our ability to monitor the

dynamic changes in the molecular landscape of tumours but also enable timely adjustments to therapeutic interventions. Combination therapies, modulation of the TME, new drugs and personalized medicine approaches are being explored as potential solutions [120-122].

Here we have assessed the effect of the pancreatic pro-enzymes treatment on PC chemoresistant cells and the effectiveness of a treatment based on the combination of PRP and GEM (PRP→GEM). The development and characterization of chemoresistant cell lines represent a critical step in understanding and combating chemotherapy resistance in PC. In this study, it was successfully developed GR cell lines from the BxPC3 pancreatic cancer model and evaluated their resistance mechanisms using various biological assays. By exposing BxPC3 cells to progressively increasing concentrations of GEM, it was observed a significant increase in the IC_{50}, with a RI of 0.076, consistent with existing literature on the establishment of chemoresistant models [130,678]. The GR cell lines Suit2.007 and Patu-T were kindly donated by Prof. Elisa Giovanetti, demonstrating notable RIs, reinforcing the robustness of our model for further experimentation. The observed decrease in IC_{50} values for both WT and GR cells after PRP treatment underlines the potential of PRP as an adjuvant therapy. In fact, PRP significantly increased their sensitivity to the chemotherapeutic treatment. These results complement our original hypothesis that PRP may affect the sensitivity of chemoresistant PC cells.

Furthermore, it was discovered that treatment with PRP triggered a decrease in the rates of proliferation, clonogenicity, invasion and migration in chemoresistant cells once they are treated with PRP followed by treatment with GEM, which can be considered a relevant event since it has been observed previously that these characteristics are typical of tumour cells and are accentuated in chemoresistant patients, significantly affecting their outcomes [867]. Furthermore, it was observed that these characteristics were more increased in the Patu-T cell line, which made it the most chemoresistant of the three lines, and, then, this is indicative that certain there are tumour cells with several degrees of drug resistance associated to different aggressiveness than others. This finding

is consistent with previous studies, reflecting the complexity and variability of cancer biology [678].

Another difference found between chemoresistant lines is the resistance mechanism they develop against the drug in question [130]. The modulation of genes related to chemoresistance by PRP provides mechanistic insights into its effects. Interestingly, ENT-1 is a transporter that mediates the uptake of GEM metabolites into pancreatic carcinoma cells. This means that, contrary to what might be expected, an increase in ENT-1 would allow for enhanced uptake of nucleosides into these cells. This phenomenon can be explained by the fact that BxPC3 cells seem to develop resistance to this drug not through the transport mechanism but rather through the metabolic pathway since the metabolic markers also experienced an increase in expression, thereby enhancing the metabolism of GEM within the cell and its elimination [868,869]. This mechanism is further strengthened by the increase in ABCC5, which, being an efflux transporter, also aids in the expulsion of this metabolite, among other drugs [870-873]. These results suggested that GR-BxPC3 cells are able to develop chemoresistance in a "metabolic way", as later was corroborated with the application of the formula for estimating the "Gemcitabine Sensitive Ratio" [824]. On the other hand, Suit2.007 and Patu-T cells were able to develop chemoresistance through a "transport way". The significant overexpression of key resistance genes, such as the ABC transporters in GR-Suit and GR-Patu suggests a key alteration in the efflux transporter as a resistance mechanism, which is supported by the closure of the ENT-1 transporter preventing thus the entry of GEM into the cell [874,875]. The application of PRP causes a metabolic block within GR-BxPC3, which represents an increase of this active metabolite, while in GR-Suit2.007 and GR-Patu-T cells it represents a decrease in efflux and an increase in the expression of the influx transporters, which also influences accumulation of active GEM inside the cell.

In addition, PRP was able to reduce the rates of proliferation, clonogenicity, invasion and migration in the GR-cells, which are features characteristics of these cells as demonstrated in ovarian, pancreatic and lung cancer lines [876,877]. The fact that the Patu-T cell line stood out as the most

chemoresistant, presenting the lowest susceptibility to treatment with PRP and being the least affected in its proliferation, clonogenicity, invasion and migration rates, suggests that different cell lines may have adaptations or unique signaling pathways that confer greater or lesser resistance not only to chemotherapy but also to the modulatory effects of PRP, and therefore have to be evaluated comparatively [878].

The induction of apoptosis in chemoresistant and non-chemoresistant tumour cells by PRP, as evidenced by increased apoptotic markers and susceptibility, further supports the potential of PRP as an adjuvant therapy. The significant up-regulation of pro-apoptotic BAX and down-regulation of anti-apoptotic Bcl-2 in PRP-treated cells indicate a shift towards apoptosis and necrosis, effectively reducing the viability of chemoresistant and cancer cell populations [880]. In this context, other studies on myeloid leukemia have demonstrated the alteration of the BAX/Bcl-2 expression ratio in chemoresistant cells, leading to aberrant growth rates [881]. Such expression changes in pancreatic tumour cells, in particular the overexpression of the antiapoptotic factor Bcl-2, may contribute to the generation of extensive chemoresistant cell populations capable of avoiding the apoptotic process and controlling their growth [880,881]. Importantly, PRP may counteract this circumstance through the enhancement of pro-apoptotic BAX along with the inhibition of Bcl-2 expression in treating both tumour cells, chemoresistant and non-chemoresistant.

EMT is a biological process in which epithelial cells lose their cell polarity and adhesion properties, acquiring mesenchymal traits such as increased migration, invasiveness, and resistance to apoptosis. This transition plays a significant role in the progression and metastasis of various cancers, including PDAC [484-489]. EMT is also closely associated with the acquisition of chemoresistance, making it a critical area of study for understanding tumour biology and improving therapeutic strategies [879]. During EMT cancer cells convert into CSCs, which are very resistant to standard therapy with standard chemotherapy. These EMT-induced cells exhibit increased efflux of chemotherapeutic drugs through upregulation of drug transporters, enhanced DNA

repair mechanisms, and activation of antiapoptotic pathways, all of which contribute to their survival and proliferation despite the treatment [130]. The data obtained revealed that PRP treatment resulted in downregulation of key transcription factors and markers that induce EMT. Specifically, the transcription factor Snail, known to drive EMT and promote metastasis, was significantly reduced in all three cell lines after receiving PRP→GEM treatment, as was similarly the case with the markers Slug, N-cadherin, Sox-2 (a marker associated with stemness and CSC characteristics) and Vimentin. In addition, E-cadherin, a gene suppressed by the Notch pathway and an epithelial biomarker, was up-regulated after PRP→GEM treatment. The changes observed on these EMT markers in the GR-cells was consistent with our previous research, which demonstrated PRP's inhibitory effect on EMT in pancreatic tumour cells [813].

Moreover, this study provided compelling evidence that PRP exerts a significant inhibitory effect on the TGF-β pathway, a key regulator in the acquisition of the chemoresistant phenotype in pancreatic cancer cells [481]. The canonical TGF-β pathway involves the phosphorylation and activation of Smad2/3 proteins, which then translocate to the nucleus to regulate the expression of target genes associated with these processes [469]. Importantly, treatment with PRP markedly reduced the expression of Smad2 and Smad3 in all GR-cell lines. The analysis demonstrated that PRP treatment not only decreased the cytoplasmic expression of Smad2/3 but also inhibited their translocation into the nucleus, thereby preventing the formation of the active Smad complex required for the transcriptional regulation of TGF-β target genes. This was validated by Western blotting, which showed that PRP treatment inhibited the phosphorylation of Smad2/3 proteins in GR-cells. The effects of PRP were again comparable to those of the commercial inhibitor (TGF-βR1, LY2157299), confirming that PRP effectively disrupts the TGF-β pathway in a good manner. The inhibition of the TGF-β pathway by PRP was consistent across all cell lines tested. This inhibition is significant considering the functional switch observed in this pathway during chemoresistant phenotype acquisition [192,200]. By targeting and inhibiting the TGF-β pathway, PRP not only sensitizes chemoresistant cells to chemotherapy but also disrupts the signaling pathways

crucial for cancer progression and metastasis.

Reinforcing the therapeutic potential of PRP, this study demonstrates that PRP can sensitize chemoresistant pancreatic cancer cells to GEM, reduce their proliferative and migratory capabilities, and modulate key resistance mechanisms and EMT and TGF-β pathways. These findings suggest that PRP has the potential to be developed as an effective adjuvant therapy, addressing one of the significant challenges in pancreatic cancer treatment: chemotherapy resistance. Future studies should focus on elucidating the precise molecular mechanisms underlying these effects and evaluating the therapeutic potential of PRP *in vivo*. This approach could provide a novel therapeutic strategy for improving treatment outcomes in patients with chemoresistant PC.

Future perspectives

The promising findings in this Thesis regarding the PRP formulation and its effects on the TME and chemoresistant pancreatic cancer cells open up several potential avenues for future research and clinical applications. Here, they are some suggested directions:

1. Mechanistic Studies: Further elucidation of the molecular pathways through which PRP exerts its effects will be critical. This includes in-depth studies on the modulation of the TGF-β pathway, EMT, and apoptotic signaling in various cellular contexts. Advanced techniques such as single-cell RNA sequencing could be used to explore the heterogeneity of cancer and stromal cell responses to PRP.

2. Combination Therapies: Given the success of PRP→GEM in sensitizing chemoresistant cells to GEM, exploring other combinatorial approaches with different chemotherapy agents or targeted therapies could

enhance treatment outcomes. Preclinical studies should investigate synergistic effects and define the most effective combinations to combat resistance.

3. TME Targeting: Since PRP affects both tumour cells and the surrounding stromal cells, future research should also focus on its role in reprogramming the TME. Studies should evaluate how PRP can influence other components of the TME, such as immune cells and endothelial cells, potentially enhancing its anti-tumour efficacy.

4. Biomarker Development: Identifying biomarkers that predict response to PRP treatment will be essential for personalized medicine approaches.

5. Longitudinal Studies on Resistance Evolution: Understanding how cancer cells evolve resistance during sequential PRP and chemotherapy treatments can provide insights into adaptive changes. Longitudinal studies might reveal critical windows for intervention and help develop strategies to prevent or overcome resistance.

6. Interdisciplinary Collaborations: The complexity of treatment resistance and tumour biology underscores the need for interdisciplinary research efforts. Collaborations integrating genomics, pharmacology, engineering, and computational biology could accelerate the development of more effective therapies.

7. *In Vivo* Studies in Orthotopic Patient Derived Xenotransplant (PDX) Models: Conducting *in vivo* studies is crucial for assessing the efficacy and safety of PRP in a more complex, physiological context that closely mimics human cancer. These preclinical studies should focus on examining tumour growth inhibition, metastasis reduction, TME response and survival benefits in animal models of PC based on PDX. Evaluating the effects of PRP in combination with standard chemotherapy regimens in these models will provide valuable insights into its potential as an adjuvant therapy.

8. Pharmacokinetics and Biodistribution: Future *in vivo* studies should also investigate the pharmacokinetics and biodistribution of PRP components to understand their systemic effects and accumulation in target tissues. This information is essential for optimizing treatment protocols, dosing schedules, and delivery methods.

9. Optimization of Delivery Methods: Developing efficient delivery systems for PRP is crucial for maximizing its therapeutic potential. Research should explore various delivery methods, such as direct tumour injection or systemic administration, and consider novel delivery vehicles like nanoparticles or hydrogels to enhance PRP stability, durability and targeting.

10. Combination with Immunotherapy: Considering the impact of PRP on the TME and immune cells, investigating its use in combination with immunotherapies could reveal synergistic effects. Such studies could identify novel strategies to enhance anti-tumour immunity and overcome immunosuppressive barriers.

11. Clinical Trials and Validation: To translate preclinical findings into clinical practice, it is paramount to conduct well-designed clinical trials. These studies should focus on evaluating the safety, optimal dosing, and efficacy of PRP as an adjuvant therapy in patients with pancreatic cancer. Understanding the clinical impact could pave the way for incorporating PRP into existing treatment protocols.

12. Organoid Models for Personalized Treatment: The use of patient-derived organoids can revolutionize our understanding of PC and treatment responses. By creating 3D organoid cultures that closely mimic the TME, researchers can assess the efficacy of PRP and other therapeutic agents in a more personalized manner. These models allow for the exploration of tumour heterogeneity and heterotypic interactions within the TME, providing insights into how different tumour subtypes respond to PRP. Furthermore, organoids can

be utilized to identify biomarkers that predict individual responses to treatment, ultimately guiding personalized therapy strategies and improving clinical outcomes for patients with pancreatic cancer.

By pursuing these future directions, the therapeutic potential of PRP can be thoroughly explored and potentially expanded, offering hope for better management of chemoresistant pancreatic cancer and potentially other aggressive tumours. These prospects signal an exciting new chapter in cancer therapy development, aimed at overcoming some of the most challenging barriers in oncology. These efforts aim to translate laboratory findings into actionable cancer therapies, ultimately improving outcomes for patients with pancreatic cancer and potentially other malignancies.

CONCLUSIONS

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1. The crosstalk between cancer-associated fibroblasts and tumour cells has a significant role in the tumour progression and dissemination. Thus, therapeutic strategies targeting the tumour microenvironment are key to control tumour spread.
2. A treatment based on the pancreatic pro-enzyme formulation, PRP, has a significant impact on tumour microenvironment by promoting the phenotypic-reversion of CAFs into normal fibroblasts.
3. PRP effectively reduces tumourigenicity, endothelial cell proliferation, and cancer cell proliferation and migration, contributing to a less aggressive phenotype in PDAC cells.
4. Apoptosis and necrosis processes are found to be stimulated after treatment with PRP specifically in CAFs.
5. The PRP formulation modulates relevant CAFs-related molecular pathways such as EMT and TGF-β/Smad.
6. PRP formulation *in vivo* affects the tumour microenvironment by reducing its fibrotic nature. This effect could be linked to the ability of PRP treatment to impair the communication between cancerous cells and the surrounding stroma through the alteration of the expression of a wide variety of cytokines.
7. Chemoresistant pancreatic cells were successfully obtained after culturing with increasing concentrations of Gemcitabine over a period of approximately 18 weeks, reaching a resistance level of 160nM.
8. Chemoresistant BxPC3 cells appear to up-modulate the expression of the ENT-1 influx transporter and overexpress metabolism markers, such as RMM1, RMM2 or DCK; while the Suit2.007 and Patu-T cell

lines appear to have a resistance mechanism more focused on preventing the entry of the drug into the cell.

9. PRP formulation is an efficient treatment against the chemoresistant pancreatic cell lines being able to re-sensitize them to Gemcitabine.
10. PRP significantly affects the rate of proliferation, invasion, migration and clonogenicity in tumour and chemoresistant cells when applied adjuvantly to Gemcitabine.
11. Apoptosis and necrosis processes are found to be stimulated after treatment with the combination of PRP and Gemcitabine in tumour cells and chemoresistant cells.
12. The EMT and TGF-β pathways, involved in the acquisition of the chemoresistant phenotype, are potentially affected by PRP.
13. PRP has the potential to be developed as an effective adjuvant therapy addressing chemotherapy resistance, which encourages promoting preclinical and clinical trials in the near future.

CONCLUSIONES

1. La comunicación entre los fibroblastos asociados al cáncer y las células tumorales tiene un papel significativo en la progresión y diseminación del tumor. Por lo tanto, las estrategias terapéuticas dirigidas al microambiente tumoral son clave para controlar la propagación del tumor.
2. Un tratamiento basado en la formulación de proenzimas pancreáticas, PRP, tiene un impacto significativo en el microambiente tumoral al promover la reversión fenotípica de los CAFs a fibroblastos normales.
3. El PRP reduce efectivamente la tumorigénesis, la proliferación de células endoteliales, y la proliferación y migración de células cancerosas, contribuyendo a un fenotipo menos agresivo en las células de PDAC.
4. Se ha encontrado que los procesos de apoptosis y necrosis son estimulados después del tratamiento con PRP específicamente en los CAFs.
5. La formulación de PRP modula vías moleculares relacionadas con CAFs relevantes como EMT y TGF-β/Smad.
6. La formulación de PRP *in vivo* afecta el microambiente tumoral al reducir su naturaleza fibrotica. Este efecto podría estar vinculado a la capacidad del tratamiento con PRP para alterar la comunicación entre las células cancerosas y el estroma circundante a través de la alteración de la expresión de una amplia variedad de citoquinas.
7. Se obtuvieron con éxito células pancreáticas quimiorresistentes después de cultivar con concentraciones crecientes de Gemcitabina durante un período de aproximadamente 18 semanas, alcanzando un

nivel de resistencia de 160nM.

8. Las células quimiorresistentes BxPC3 parecen up-modular la expresión del transportador de entrada ENT-1 y sobreexpresar marcadores de metabolismo, como RMM1, RMM2 o DCK; mientras que las líneas celulares Suit2.007 y Patu-T parecen tener un mecanismo de resistencia más centrado en prevenir la entrada del fármaco en la célula.
9. La formulación de PRP es un tratamiento eficiente contra las líneas celulares pancreáticas quimiorresistentes, siendo capaz de re-sensibilizarlas a la Gemcitabina.
10. El PRP afecta significativamente la tasa de proliferación, invasión, migración y clonogenicidad en células tumorales y quimiorresistentes cuando se aplica como tratamiento adyuvante a la Gemcitabina.
11. Se ha encontrado que los procesos de apoptosis y necrosis son estimulados después del tratamiento con la combinación de PRP y Gemcitabina en células tumorales y quimiorresistentes.
12. Las vías de EMT y TGF-β, involucradas en la adquisición del fenotipo quimiorresistente, se ven potencialmente afectadas por PRP.
13. El PRP tiene el potencial de ser desarrollado como una terapia adyuvante efectiva para abordar la resistencia a la quimioterapia, lo que fomenta la promoción de ensayos preclínicos y clínicos en un futuro cercano.

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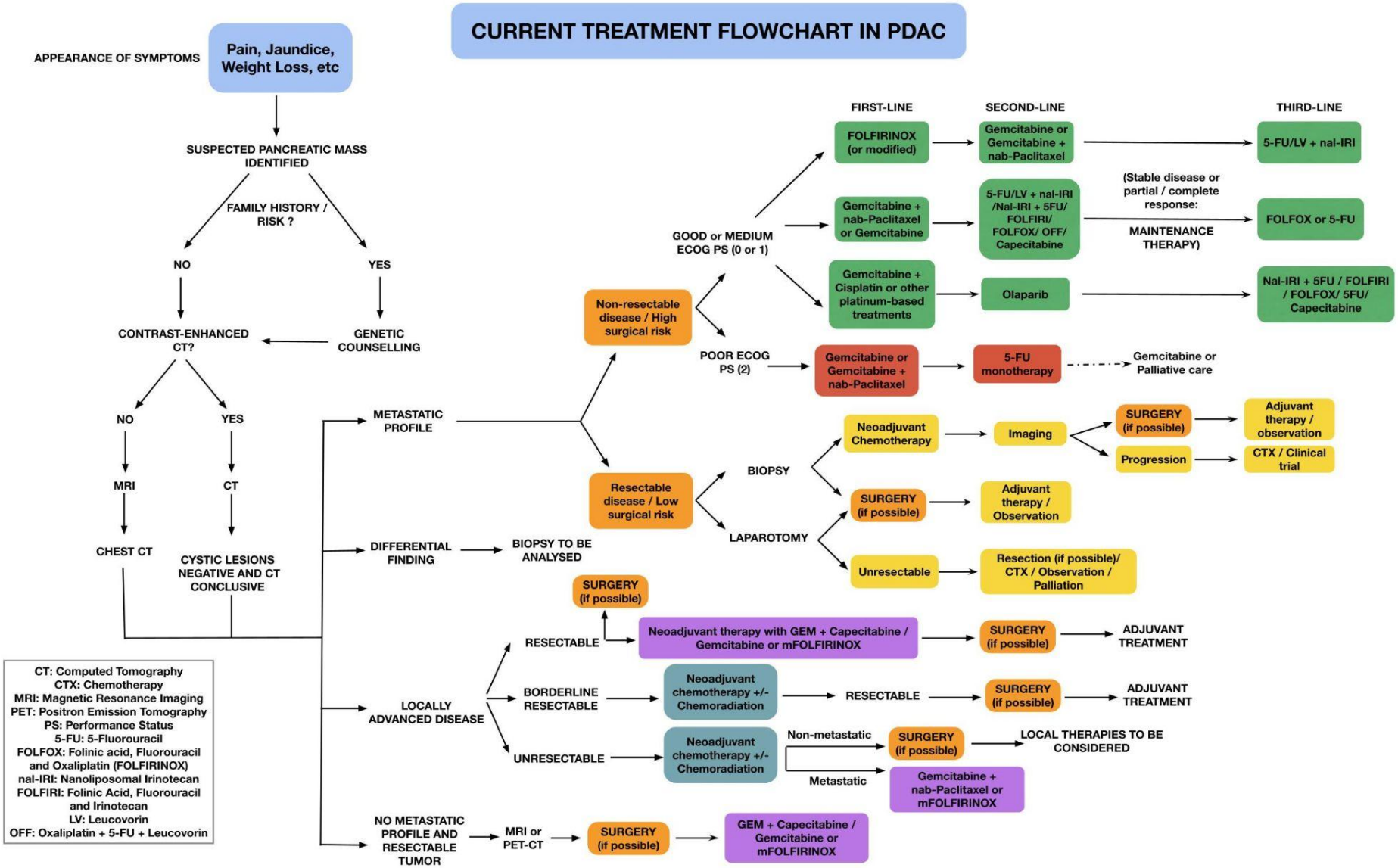
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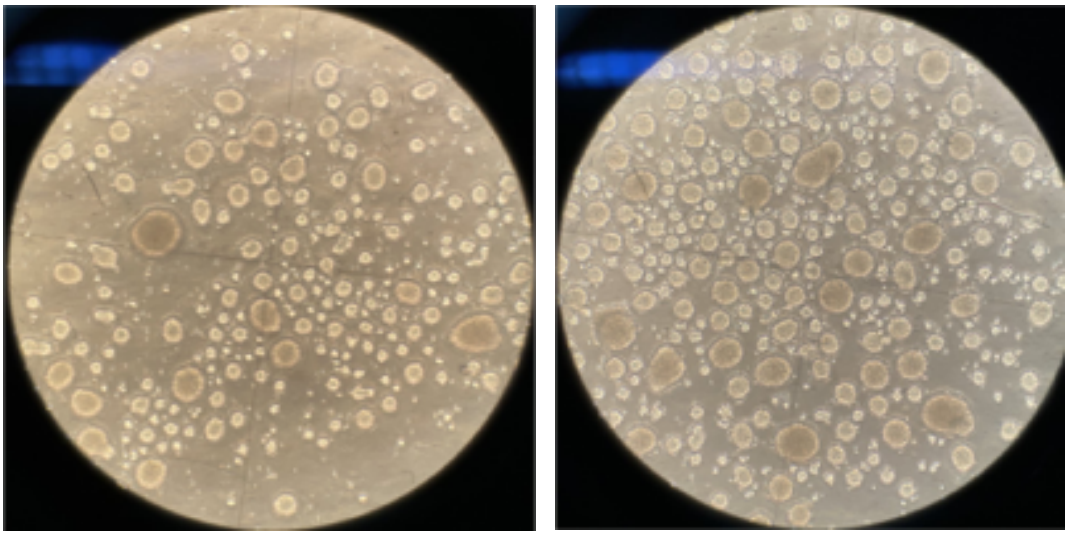
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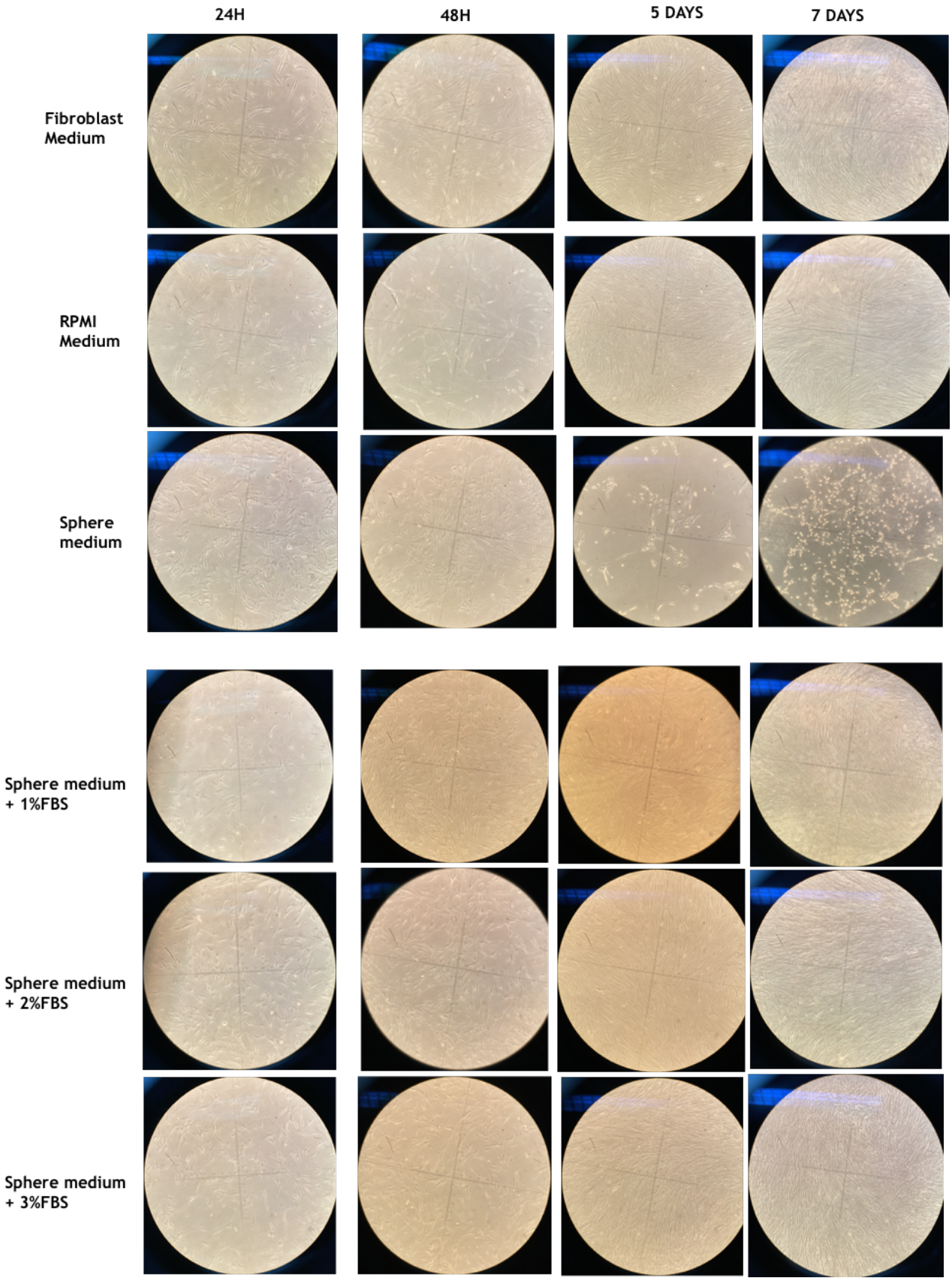


Supplementary 1. Current treatment flowchart in PDAC. Generalized treatment guidelines for patients with PDAC. PDAC usually presents with vague clinical symptoms, including poorly localized pain, jaundice, or unintentional weight loss. When PDAC is suspected, each patient's clinical history and risk probability are evaluated. Patients are usually diagnosed using computed tomography (CT) of the chest, abdomen, and pelvis to evaluate the extent of disease or ultrasound with or without a biopsy. After confirmatory diagnosis, the patient's surgical candidacy is determined based on a combination of imaging studies, symptom burden, surgical risk, and comorbidity profile. After analysis, PDAC can be classified into: metastatic profile, differential finding, locally advanced diseased and non-metastatic profile and resectable tumour. For operable disease, the type of surgery is determined by the anatomic location of the tumour, as well as several additional factors described in this thesis, with most patients receiving a Whipple operation or a distal pancreatectomy. Regardless of whether a patient receives surgical treatment, current guidelines recommend chemotherapy and the precise regimen administered is based primarily on ECOG performance status and comorbidity profile. At the point of advanced stage or recurrent tumour, personalized medicine options could be useful. *Lencioni G., et al. Seminars in Cancer Biology (2024).*

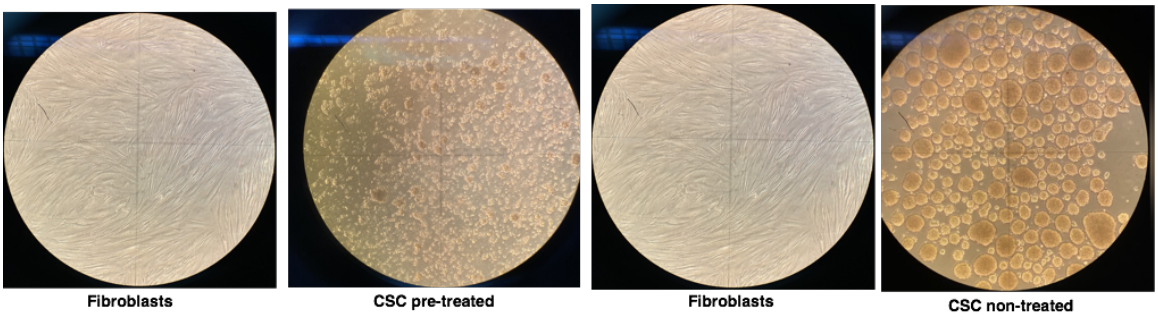
Supplementary 2. Primary (left) and secondary (right) sphere formation. The sphere-forming assay with BxPC3 cell line. Representative bright-field images of primary spheres (left panel) and secondary spheres, after 72 hours, with CSC-like properties (right panel). 10x magnification. Secondary spheres will be ready to be used 72 hours post-formation.

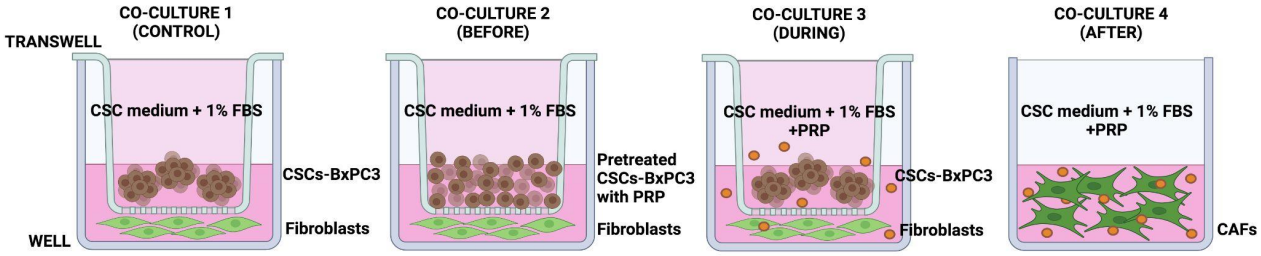


Supplementary 3. Medium test before co-culture system. Given that the fibroblasts and the BxPC3 CSCs were going to share medium during the 14 days of co-culture, some previous tests were carried out to find out in which medium there was more optimal growth of both cell types. It was verified that the fibroblasts were not capable of growing in the medium of the spheres when it did not have FBS, so it was decided to try adding 1%, 2% and 3% of FBS, being lower amounts than those normally used in the culture medium (5%) since the spheres do not require FBS in their medium. Finally, it was determined that 1% FBS was a sufficient amount for the growth of fibroblasts without affecting the population of tumour spheres. Fibroblast medium (DMEM) and BxPC3 medium (RPMI) were used as controls.

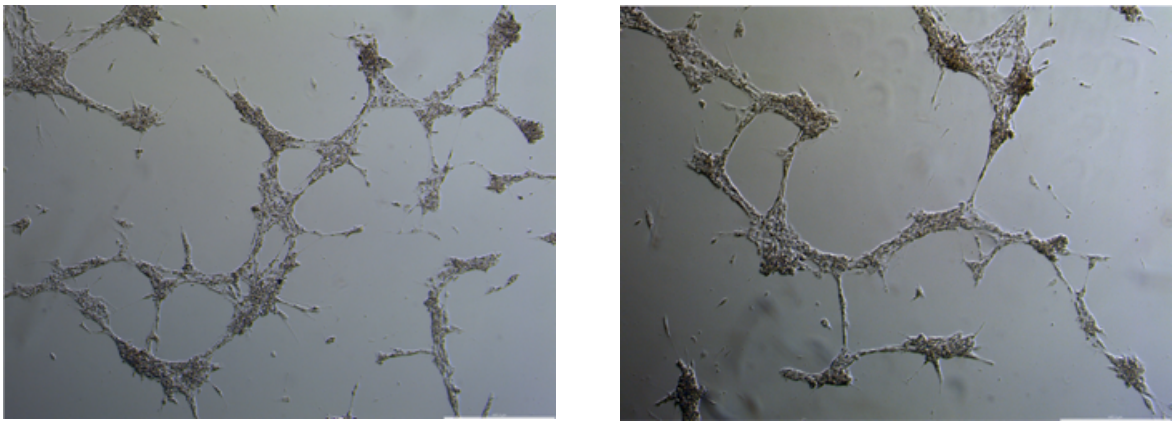

24H
48H
5 DAYS
7 DAYS
Fibroblast Medium
RPMI Medium
Sphere medium
Sphere medium + 1%FBS
Sphere medium + 2%FBS
Sphere medium + 3%FBS

Supplementary 4. Indirect 2D co-culture system and fibroblast malignification. Once the secondary spheres were obtained, they were co-cultured with fibroblasts (above and below the transwell respectively) in three conditions to be studied: spheres pretreated with PRP and then cocultured with fibroblasts (before); treatment administered during coculture (during); and PPR administrated after the coculture over the malignant fibroblasts (CAFs) (after). The used medium was the one selected in Supplementary 3. Non-treated co-culture was used as control. After 14 days of culture, CAFs were obtained with a much more widened and stellate morphology (photos in bright-field 5x and optic microscope 10x).

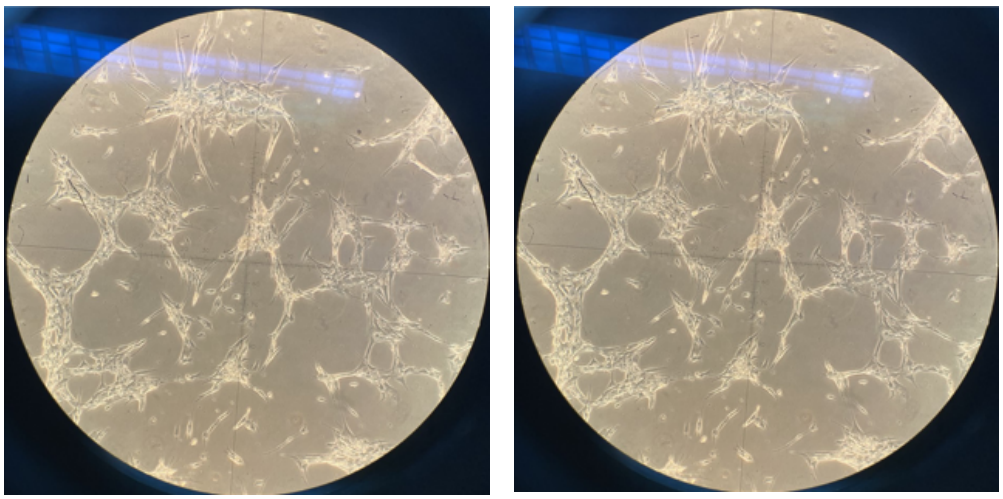




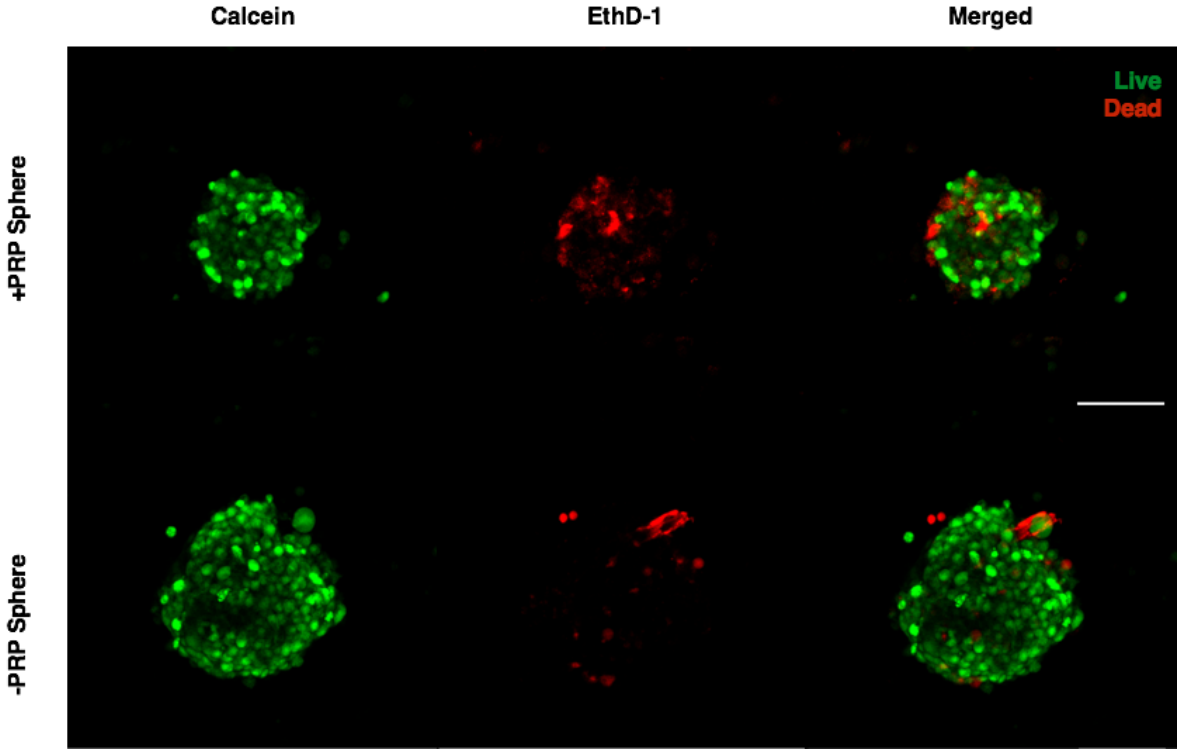
CAFs in bright-field 5x



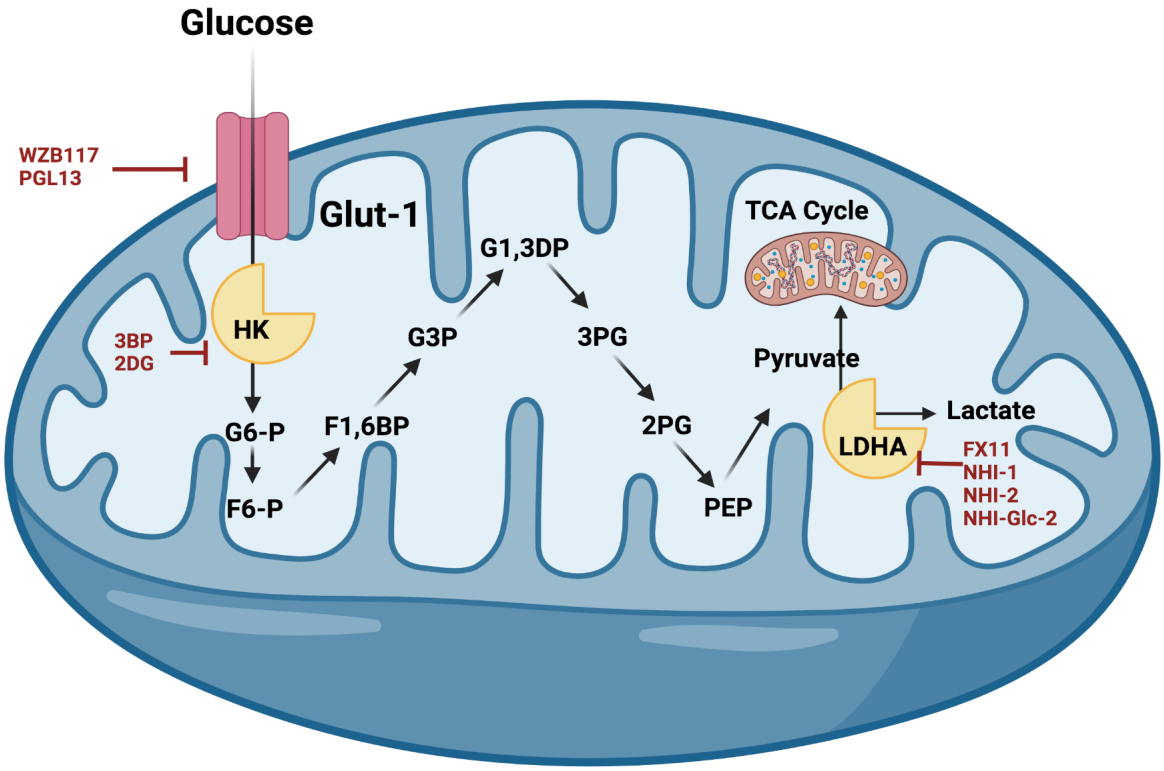
CAFs in Optic microscope 10x



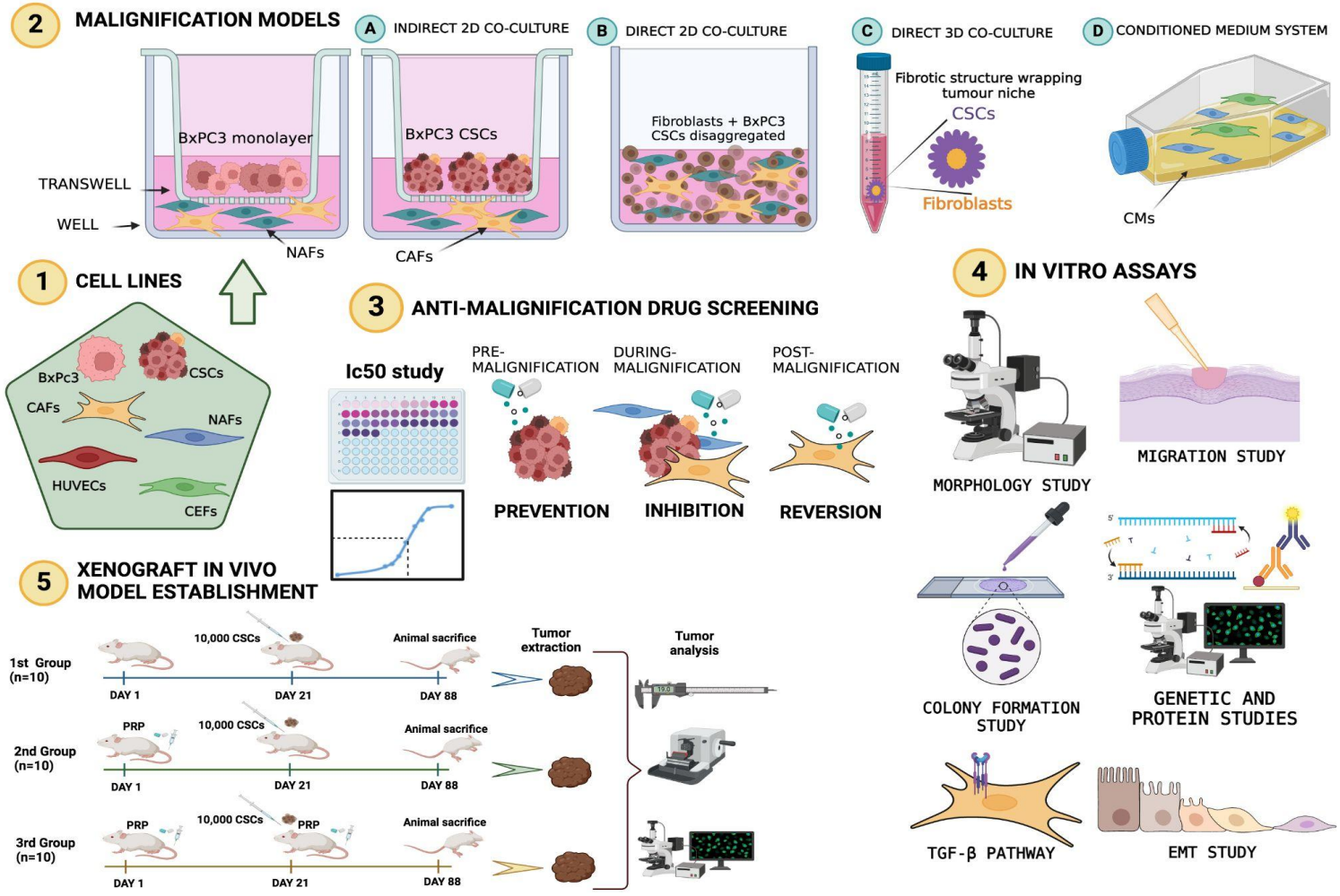
Supplementary 5. LIVE/DEAD staining in BxPC3 secondary spheres. Secondary spheres from BxPC3 were obtained as explained in the Methodology section. Spheres were treated with or without (control) PRP and stained with the Live/Dead Kit: Calcein (Green = LIVE), EthD-1 (Red=DEAD), and their overlay. The samples were examined under the confocal microscope (Nikon Eclipse Ti-E A1, USA) with a 10x magnification. Scale bar: 100 μm.



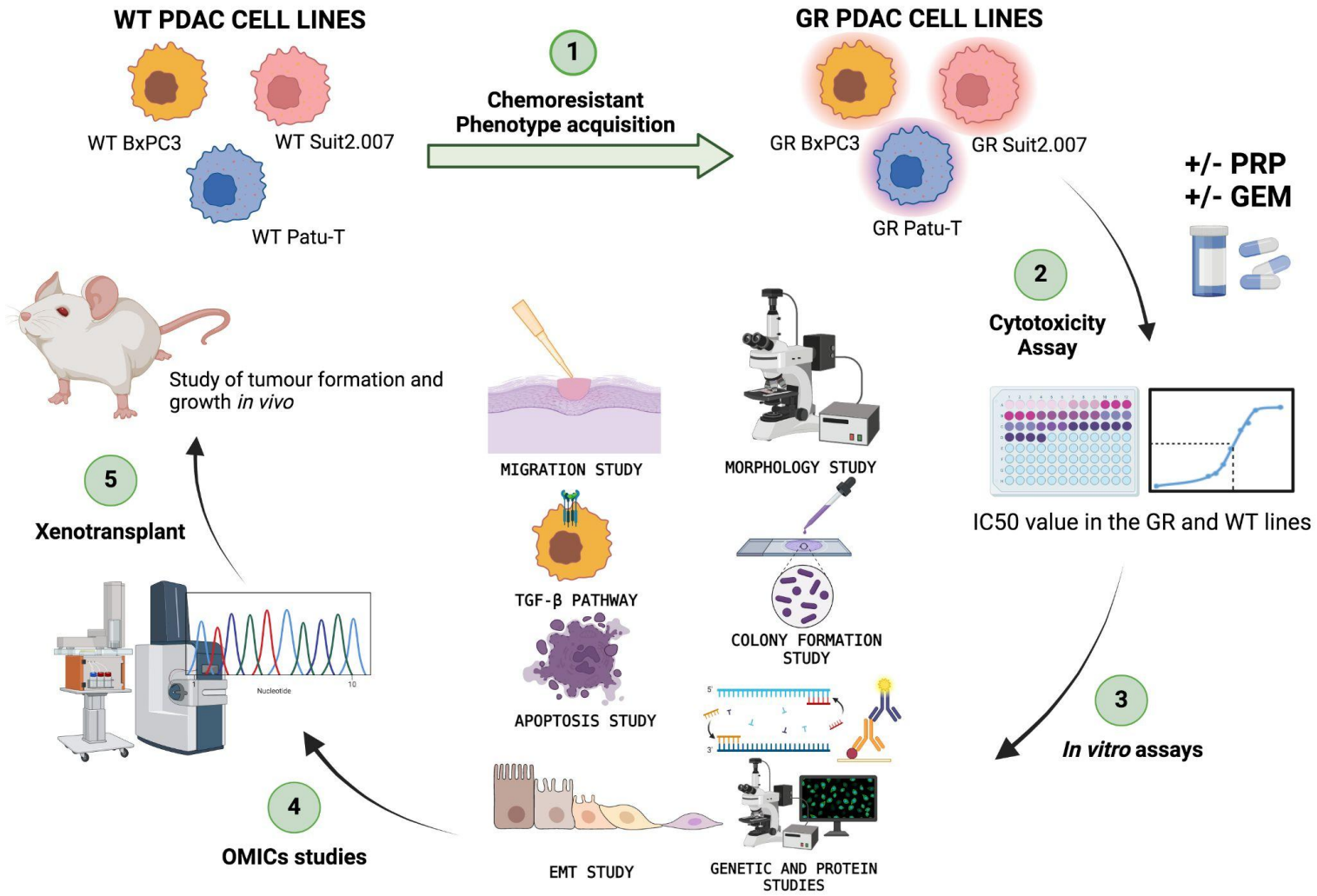
Supplementary 6. Based on Warburg's studies. Diagram of selected components of the glycolysis pathway and inhibitors, specifically small molecules targeting glucose transporter 1 (GLUT-1), hexokinase (HK) and lactate dehydrogenase (LDH-A), that have been studied or are currently under evaluation as potential new anticancer treatments. Image created with Biorender.



Supplementary 7. Graphical Abstract Chapter 1. Image created with Biorender.



Supplementary 8. Graphical Abstract Chapter 2. Image created with Biorender.



ANNEX

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Review

Dual Role of Fibroblasts Educated by Tumour in Cancer Behavior and Therapeutic Perspectives

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Abstract: Tumours are complex systems with dynamic interactions between tumour cells, non-tumour cells, and extracellular components that comprise the tumour microenvironment (TME). The majority of TME's cells are cancer-associated fibroblasts (CAFs), which are crucial in extracellular matrix (ECM) construction, tumour metabolism, immunology, adaptive chemoresistance, and tumour cell motility. CAF subtypes have been identified based on the expression of protein markers. CAFs may act as promoters or suppressors in tumour cells depending on a variety of factors, including cancer stage. Indeed, CAFs have been shown to promote tumour growth, survival and spread, and secretome changes, but they can also slow tumourigenesis at an early stage through mechanisms that are still poorly understood. Stromal–cancer interactions are governed by a variety of soluble factors that determine the outcome of the tumourigenic process. Cancer cells release factors that enhance the ability of fibroblasts to secrete multiple tumour-promoting chemokines, acting on malignant cells to promote proliferation, migration, and invasion. This crosstalk between CAFs and tumour cells has given new prominence to the stromal cells, from being considered as mere physical support to becoming key players in the tumour process. Here, we focus on the concept of cancer as a non-healing wound and the relevance of chronic inflammation to tumour initiation. In addition, we review CAFs heterogeneous origins and markers together with the potential therapeutic implications of CAFs "re-education" and/or targeting tumour progression inhibition.

Keywords: tumour microenvironment; cancer-associated fibroblast; cancer cell; magnification; inflammation; metastasis

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1. Introduction

According to the American Cancer Society, in 2022 there will be an estimated number of 1.9 million new cancer cases diagnosed and 609,360 cancer deaths in the United States. According to these data, cancer is, and will continue to be, one of the worst diseases in the world. Numerous people continue to pass away from cancer every year, despite major recent improvements in our understanding of the molecular and genetic causes of the disease [1]. This failure is primarily due to cancer cells' resistance to current therapies, which causes tumour recurrence and metastasis, which account for more than 90% of cancer-related fatalities [2].

According to the "seed and soil" hypothesis, a suitable host microenvironment (the soil) is necessary for the best growth of tumour cells (the seed) [3]. Many researchers have concentrated primarily on tumour cells over the past 40 years. Emerging data suggest that tumours are made up of tumour parenchyma and stroma, two separate but interdependent

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Review

A Brief Review on Chemoresistance; Targeting Cancer Stem Cells as an Alternative Approach

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† These authors contributed equally to this work.

Abstract: The acquisition of resistance to traditional chemotherapy and the chemoresistant metastatic relapse of minimal residual disease both play a key role in the treatment failure and poor prognosis of cancer. Understanding how cancer cells overcome chemotherapy-induced cell death is critical to improve patient survival rate. Here, we briefly describe the technical approach directed at obtaining chemoresistant cell lines and we will focus on the main defense mechanisms against common chemotherapy triggers by tumor cells. Such as, the alteration of drug influx/efflux, the enhancement of drug metabolic neutralization, the improvement of DNA-repair mechanisms, the inhibition of apoptosis-related cell death, and the role of p53 and reactive oxygen species (ROS) levels in chemoresistance. Furthermore, we will focus on cancer stem cells (CSCs), the cell population that subsists after chemotherapy, increasing drug resistance by different processes such as epithelial-mesenchymal transition (EMT), an enhanced DNA repair machinery, and the capacity to avoid apoptosis mediated by BCL2 family proteins, such as BCL-XL, and the flexibility of their metabolism. Finally, we will review the latest approaches aimed at decreasing CSCs. Nevertheless, the development of long-term therapies to manage and control CSCs populations within the tumors is still necessary.

Keywords: chemoresistance; DNA-damaging drugs; cancer stem cells; drug metabolism; p53; reactive oxygen species; drugs pumps; DNA repair; differentiation therapy

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1. Introduction

Currently, chemotherapy is still considered an irreplaceable front-line therapeutic strategy to combat almost all types of cancers, but multidrug resistance represents a common hurdle that deeply compromises clinical outcomes. Therefore, it is key to identify new resistance biomarkers and to analyze their predictive potential in order to guide treatment regimens [1]. Furthermore, a better understanding of the underlying drug tolerance mechanisms may be critical in achieving alternative therapies to improve oncological patients' prognosis. However, it is challenging since a high heterogeneity of chemoresistance markers expression profiles has been observed between different tumors and even between different cell lines of the same tumor type [2]. In this regard, the development of drug-resistant cell lines is essential to study chemoresistance mechanisms. Figure 1 summarizes the procedure to obtain in vitro chemoresistant cell lines by mimicking the conditions experienced by cancer patients during chemotherapy. The use of increasing concentrations of chemotoxic agents represents a common experimental procedure with the aim of establishing stable, drug-tolerant tumor cell lines in vitro [3]. Although this method is very reliable and reproducible, some potential limitations should be kept in mind: first, since only a small fraction of the bulk population of cells will show chemoresistance, it is recommended to start with a large population of cells (a minimum of 10^6 cells); second, it is very important to use the same freshly prepared drug stock for long-term use; third,

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REVIEW Open Access

Deciphering the performance of macrophages in tumour microenvironment: a call for precision immunotherapy

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Abstract

Macrophages infiltrating tumour tissues or residing in the microenvironment of solid tumours are known as tumour-associated macrophages (TAMs). These specialized immune cells play crucial roles in tumour growth, angiogenesis, immune regulation, metastasis, and chemoresistance. TAMs encompass various subpopulations, primarily classified into M1 and M2 subtypes based on their differentiation and activities. M1 macrophages, characterized by a pro-inflammatory phenotype, exert anti-tumoural effects, while M2 macrophages, with an anti-inflammatory phenotype, function as protumoural regulators. These highly versatile cells respond to stimuli from tumour cells and other constituents within the tumour microenvironment (TME), such as growth factors, cytokines, chemokines, and enzymes. These stimuli induce their polarization towards one phenotype or another, leading to complex interactions with TME components and influencing both pro-tumour and anti-tumour processes.

This review comprehensively and deeply covers the literature on macrophages, their origin and function as well as the intricate interplay between macrophages and the TME, influencing the dual nature of TAMs in promoting both pro- and anti-tumour processes. Moreover, the review delves into the primary pathways implicated in macrophage polarization, examining the diverse stimuli that regulate this process. These stimuli play a crucial role in shaping the phenotype and functions of macrophages. In addition, the advantages and limitations of current macrophage based clinical interventions are reviewed, including enhancing TAM phagocytosis, inducing TAM exhaustion, inhibiting TAM recruitment, and polarizing TAMs towards an M1-like phenotype. In conclusion, while the treatment strategies targeting macrophages in precision medicine show promise, overcoming several obstacles is still necessary to achieve an accessible and efficient immunotherapy.

Keywords Tumour-associated macrophages, Cancer cell, Tumour microenvironment, Polarization, Immunity, Immunotherapy

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REVIEW

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Treatment resistance in pancreatic and biliary tract cancer: molecular and clinical pharmacology perspectives

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ABSTRACT

Introduction: Treatment resistance poses a significant obstacle in oncology, especially in biliary tract cancer (BTC) and pancreatic cancer (PC). Current therapeutic options include chemotherapy, targeted therapy, and immunotherapy. Resistance to these treatments may arise due to diverse molecular mechanisms, such as genetic and epigenetic modifications, altered drug metabolism and efflux, and changes in the tumor microenvironment. Identifying and overcoming these mechanisms is a major focus of research: strategies being explored include combination therapies, modulation of the tumor microenvironment, and personalized approaches.

Areas covered: We provide a current overview and discussion of the most relevant mechanisms of resistance to chemotherapy, target therapy, and immunotherapy in both BTC and PC. Furthermore, we compare the different strategies that are being implemented to overcome these obstacles.

Expert opinion: So far there is no unified theory on drug resistance and progress is limited. To overcome this issue, individualized patient approaches, possibly through liquid biopsies or single-cell transcriptome studies, are suggested, along with the potential use of artificial intelligence, to guide effective treatment strategies. Furthermore, we provide insights into what we consider the most promising areas of research, and we speculate on the future of managing treatment resistance to improve patient outcomes.

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KEYWORDS
Biliary tract cancer; chemoresistance; chemotherapy; immunoresistance; immunotherapy; pancreatic cancer; resistance mechanisms; target therapy

1. Introduction and current therapeutic options

The term, biliary tract cancers (BTCs), refers to malignancies that arise in the bile ducts, including intrahepatic bile ducts, extrahepatic bile ducts, and the gallbladder. Overall, BTCs are relatively rare compared to other cancers, accounting for approximately 3% of all gastrointestinal malignancies, but with various incidence rates around the world [1]. In the United States, the estimated annual incidence of BTC is around 2,500 to 3,000 cases [2]. The risk factors for BTC include chronic inflammation of the bile ducts (such as in primary sclerosing cholangitis), gallstones, liver fluke infections, and genetic alterations [1].

Pancreatic cancer (PC) is a highly aggressive malignancy that arises in the tissues of the pancreas. It is more common than BTC, being the 12th most common cancer worldwide and accounting for approximately 2% of all new cancer cases [3]. In the United States, PC is the 10th most common cancer, and it is the third leading cause of cancer-related deaths, having an estimated annual incidence of around 60,000 cases. The risk factors for PC include smoking, obesity, chronic pancreatitis, diabetes, family history of PC, as well as some genetic alterations [4].

1.1. Treatment algorithm in biliary tract cancer (BTC)

Despite the rarity of this disease, recent years have witnessed a substantial shift in the treatment paradigm of advanced BTC, with the introduction of both immunotherapy and target therapies (Table 1). Following the results of the phase-3 TOPAZ-1 trial, standard first-line treatment has become the combination of immune-chemotherapy with anti-programmed death-ligand 1 (PD-L1) durvalumab plus

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Title: Unravelling the complexities of resistance mechanism in pancreatic cancer: insights from *in vitro* and *ex-vivo* model systems

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Curriculum Vitae

María Belén Toledo Cutillas was born the 30th of May 1995, in Murcia, Spain. She grew up in a town called Cieza (Murcia), spending her spare time mainly with her sister and some cousins, practicing sports and learning languages. During her youth, she developed a love for reading and collecting refrigerator magnets from all her travels. In 2013, she started her bachelor's degree in Biotechnology at the University of Murcia. In the later years of her undergraduate studies, she developed a true interest and fascination with cancer biology, largely thanks to her mentor, Prof. Dr. Álvaro Sánchez Ferrer, from the Department of Biochemistry and Molecular Biology A (University of Murcia). There, she began her research journey as an intern for three years and later completed her Final Degree Project on breast cancer and PARP applications. In 2018, she began her master's degree in Regenerative Biomedicine at University of Granada, where she started living. During her master's program, she had the opportunity to work with Prof. Dr. Sara Rodríguez Heras and her group at the Pfizer–University of Granada–Junta de Andalucía de Genómica e Investigación Oncológica (GENYO), who instilled in her their passion for studying Retroelement Biology, particularly LINE-1 in lung cancer. In 2019, as the global pandemic of the COVID-19 virus began, she completed a master's degree in Immuno-Oncology remotely at the Center for Biosanitary Studies (CEB) in Madrid to expand her knowledge of cancer, while also taking the opportunity to learn German through an online course (and practicing boxing at home). In 2020, she moved back to Granada to begin her Ph.D. in pancreatic cancer (PC) at the Centro de Investigación Biomédica (CIBM), under the mentorship of Prof. Dr. Macarena Perán (University of Jaén), Prof. Dr. Juan Antonio Marchal (University of Jaén), and Prof. Dr. Elisa Giovannetti (Vrije Universiteit Amsterdam). Her project focused on the analysis of the tumor microenvironment, chemoresistance in PC, and the effects of treatment based on pancreatic proteinases (PRP) on it. In 2023, she had the opportunity to

carry out a long stay in the laboratory of Prof. Dr. Giovannetti at the CCA center in Amsterdam, where her stay lasted one year. During this time, she worked with various PC chemoresistant lines and delved into the world of proteomics analysis. The results of her Ph.D. project are described in this thesis. In the future, she plans to continue adding magnets from different destinations she visits to her refrigerator (along with a good book to accompany her).