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Multimodal Therapies against Pancreatic Ductal Adenocarcinoma: A Review on Synergistic Approaches toward Ultimate Nanomedicine Treatments

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In the last decades, extensive research has been carried out on the understanding and treatment of pancreatic cancer. Despite the significant advances in medicine and nanotechnology, there is an increasing concern about the lack of a standardized therapy with favorable outcomes for patients affected by this malignant disease, whose survival rates are nowadays far alarmingly low. The aim of this review is to offer a comprehensive view on the topic, by drawing upon two strands of research into pancreatic cancer. First, a detailed overview on the tumor genesis, progression, and resulting intricate microenvironment is presented. Thereafter, an extensive insight into the current treatments and their evolution throughout time, with a major focus on nanomedicine approaches, are offered to the reader. With respect to previous studies, a particular emphasis is given here to innovative theranostic approaches. In the light of what is now well established by recent evidence, the focus is given to multimodal treatments involving the combination of different therapies and, in particular, of nanoparticle-based medicine. The challenging purpose is finally of shedding light on future ultimate treatments out of the currently followed well-worn paths.

1. Introduction

Pancreatic cancer (PC) is nowadays among the leading causes of cancer death in the world,[1] with the prospect of surpassing breast, prostate, and colorectal tumors and thus becoming the second main cause of cancer-related death worldwide by 2030.[2] In particular, pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic cancer and represents about 90% of PC cases.[3] Its tumor microenvironment (TME) shows innate and acquired chemoresistance,[4] due the presence of a dense tumor stroma, called desmoplasia, made up of many different cellular types[5] and minimal blood flow within the vessels that surround the tumor site.[6] This configuration therefore causes hypoxia and reduces the chances for drugs to reach and successfully treat the cancer.[7] As a direct consequence, chemoresistance and multidrug resistance (MDR) are the major obstacles to PDAC treatment[8] and must be carefully addressed while designing new therapeutic approaches.

Due to the lack of visible symptoms at early stages, which lead to a very late recognition of the disease, PDAC is often diagnosed when already spread throughout the body and thus not always removable with surgical resection.[9] Nevertheless, surgery preceded and/or followed by systemic treatments remains the only option than can provide a realistic hope for patients,[10–12] whose 5 year survival rate is reported to be less than 9% in the case of this malignancy.[13] Other traditional treatments include I) chemotherapy, in the form of single-agent or multidrug administrations and delivered alone or in combination with other therapies (in this latter case it is called either adjuvant or neoadjuvant chemotherapy[14–16]), or proposed as second-line therapy and palliative treatment;[17–19] II) radiotherapy and its declinations like chemoradiation;[20–22] III) local ablative therapies (radiofrequency ablation, irreversible electroporation, stereotactic body radiation therapy, and high intensity focused ultrasound among many others[23–25]); IV) immunotherapy and cancer vaccines.[26–28]

All these therapies are usually accompanied by severe adverse effects;[29] at late stages and in the presence of recurrence or metastases, curative treatments are typically replaced by palliative care[30] meant to improve patients' quality of life and relieve the pain.

The application of nanotechnology to the medical field, termed nanomedicine,[31–33] is enabling novel treatments to enter preclinical and clinical trials, with the aim of overcoming the limitations shown by conventional therapies: to mention but a few, light- and ultrasound-triggered minimally invasive techniques such as sonodynamic and photodynamic therapy have recently been applied to PDAC.[34,35] In addition to that, an ever-increasing knowledge of this particularly resistant tumor and its hallmarks

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has progressively led to the adoption of innovative and smart approaches: targeted therapies to avoid multidrug resistance and systemic toxicity,[36,37] stromal therapies aimed at a TME and vessel normalization,[38–41] the use of exosomes as nanocarriers for gene therapy.[42–44] Recent advances in nanotechnology are also allowing researchers to develop new platforms, namely nanoparticles, meant to improve drug delivery in the TME surrounding pancreatic cancer cells, while enhancing drug selectivity, providing bigger therapeutic windows, reducing side effects, and enabling real-time tracking abilities.[45]

Indeed, a major contribution is now offered by nanoparticle-based theranostics, a promising area of research that focuses on the manipulation and tuning of surface and bulk properties of some materials that can be synthesized at the nanoscale in order to create multimodal platforms able to address, diagnose, and treat tumors.[46–48] The past thirty years have witnessed increasingly rapid advances in the application of nanomedicine and specifically nanoparticles to pancreatic cancer treatment, and a considerable literature has grown up around this topic.[37,49] The aim of this review is to summarize the latest nanomedicine findings concerning PDAC treatment, starting with a thorough preliminary dissertation meant to depict and investigate the plethora of factors influencing tumor pathogenesis and biology, and those contributing to its poor response to current therapies. Afterward, the main alternatives offered by nanomedicine to overcome some of the limitations related to conventional PDAC treatments are presented, with a special focus on novel multimodal approaches and innovative preclinical models benefiting from the latest nanotechnological advances. Finally, potential concrete and evidence-based future outlooks are proposed, in the light of what has been produced so far and of the ever-evolving nature of bionanotechnology.

2. Pancreatic Cancer Diagnosis

PDAC is an exocrine cancer that starts mostly (75%) in the head of the organ, from the epithelium of the pancreatic ducts. It takes more than a decade to metastasize, but early stages are largely asymptomatic and thus difficult to be diagnosed.[50] Moreover, current recommended diagnostics[51] such as computed tomography (CT) and magnetic resonance imaging (MRI), together with other more accurate techniques like abdominal and endoscopic ultrasound are costly and invasive, and thus are not used as screening tests on patients who do not show a genetic predisposition.[52]

The lack of early symptoms is one of the main challenges related to this type of cancer, and researchers are nowadays focusing on the identification of at-risk populations to better target screenings and prevention measures.[30] In addition, selection and recall bias are very common drawbacks of the studies which have led to the identification of some of the well-established risk factors among the selected patients, such as age,[53] sex,[54] diabetes mellitus,[55] smoking,[56] obesity,[57] hereditary and recent pancreatitis,[58] hereditary pancreatic cancer.[59]

3. Molecular Pathology

There is an urgent need to better understand the molecular pathology of pancreatic cancer, and a 2016 comprehensive integrated genomic analysis of 456 bulk tumor tissues of PDACs detected 32 significantly mutated genes, aggregated into 10 molecular mechanisms: KRAS (Kristen RAt Sarcoma virus), TGF-β (Transforming Growth Factor-β), WNT (Wingless/Integrated), Notch pathway, SLIT/ROBO (Slit glycoprotein/Roundabout receptor) signaling, G1/S transition, SWI–SNF (SWitch/Sucrose Non-Fermentable), chromatin modification, DNA repair, and RNA processing.

Expression analyses resolved 4 PC subtypes: squamous, pancreatic progenitor, immunogenic, and aberrantly differentiated endocrine exocrine, each one associated with specific histological characteristics.[60] These mutations are the drivers of the decline from normal mucosa to invasive malignancy, and thus have been thoroughly analyzed to better understand PDAC pathogenesis from pre-existing noninvasive neoplasia.[61] The most frequent genetic abnormalities are mutational activation of the KRAS oncogene, which in turn engages various downstream effectors,[62] inactivation of tumor-suppressor genes including CDKN2A (cyclin-dependent kinase inhibitor 2A),[63] TP53 (tumor protein P53),[64] SMAD4 (small mothers against decapentaplegic homolog 4),[65] and BRCA2 (breast cancer gene 2),[66] telomere shortening.[67] Epigenetic dysregulations such as alterations in DNA methylation and histone modifications, as well as noncoding RNAs were proved to alter gene function in pancreatic cancer,[68] and changes in microRNA expression seem to contribute to cancer development[69] and are hence addressed as diagnostic markers for PC detection.[70]

4. Precursor Lesions

PDAC is thought to develop from precursor lesions, namely pancreatic intraepithelial neoplasia, intraductal papillary mucinous neoplasm, and pancreatic mucinous cystic neoplasm, whose early detection could reduce both the incidence of pancreatic cancer and the mortality rate of patients.[71]

Pancreatic intraepithelial neoplasia (PanIN) is the most common lesion in elderly population, and it represents a promising target for early detection, since it is usually benign and subjected to predictable morphological changes. It is classified into three grades, PanIN-1, PanIN-2, and PanIN-3, and its detected distinctive genetic changes were shown to occur in a certain order during the lesion grade progression from PanIN-1 to PanIN-3. In fact, KRAS2 mutation and telomere shortening seem to appear first, then the inactivation of p16/CDKN2A takes place, and in PanIN-3 lesions, the inactivation of TP53 and MAD4/DPC4 (mothers against decapentaplegic homolog 4/deleted in pancreatic cancer-4) is typically detected. An insight of the order of genetic alterations might be useful in developing early gene-based screening tests, as well as in creating more accurate genetically engineered mouse models.[72]

Intraductal papillary mucinous neoplasm (IPMN) is a visible mucin-producing neoplasm that develops in the main pancreatic duct or branches and can take years to progress into invasive cancer. There are many similarities but also some significant differences in the genetic characteristics of IPMN with respect to PDAC, like a lower prevalence of KRAS2 gene mutations. Most patients are cured with surgical resection, and during the hunt for invasive carcinoma in the course of the histological examination, it is easy to misdiagnose PDAC from IPMN.[73]

Pancreatic mucinous cystic neoplasm (MCN) is a relatively rare lesion, larger in size compared to PanINs, usually slow growing, and noninvasive, with an excellent prognosis and no communication with the main pancreatic ductal system. KRAS mutations are believed to be the main drivers of genetic alterations occurring in low grade MCNs, while alterations in p53 tumor suppressor gene take usually place in invasive MCNs.[74]

Although some of these mechanisms have been well established, the main issue remains the clinical detection of precursor lesions before their degeneration into PDAC, because even modern high-resolution imaging methods lack the resolution and sensitivity required to detect PanINs smaller than 5 mm and to decipher the diagnostic differentiation of lesions such as IPMN and MCN.[75]

5. Tumor Microenvironment and Desmoplasia

The TME is a pivotal regulator of drug resistance, cancer survival, and malignant transformation, and thus it represents a potential target for new therapies against PDAC. In this niche microenvironment, tumor progression is maintained through desmoplasia, namely a fibrous and connective tissue growth in which continuous paracrine signals are exchanged between cells,[76] which was recently found to have both tumor promoting and tumor restraining roles in PDAC.[77]

Distinctive features of the pancreatic TME are I) the presence of a dense stroma, which makes up to 90% of the tumor bulk and is characterized by lack of vascularization, intensive fibrosis, and poor immune infiltration;[78] II) the poor perfused vessels which cause hypoxia and prevent drugs from reaching the tumor site;[79] III) the altered extracellular matrix (ECM),[80] whose excessive deposition provides solid structural foundation and is responsible for the generation of various chemical signals which regulate tumor progression and desmoplasia;[81] and IV) the continuous molecular crosstalk that takes place between pancreatic cancer cells and pancreatic stellate cells.[82]

The altered ECM consists of many noncellular and cellular components. As regards to noncellular ones, hyaluronic acid (HA) is excessively produced and deposited[83] and it negatively impacts the vascular compartment of the tumor, by enhancing the interstitial fluid pressure (IFP) and thus compressing blood vessels and hindering drug delivery in the TME;[84] collagen increases solid stress rather than IFP[85] and especially type V was shown to affect tumor angiogenesis;[86] C-X-C family of chemokines, such as CXCL8 (CXC ligand 8) and CXCL12 (CXC ligand 12) and their corresponding receptors, cooperate to promote angiogenesis and microvessel formation;[87,88] fibronectin is secreted by stellate cells and it has an important role in cell adhesion, migration, and differentiation.[81]

6. Cells in the TME

As regards to cellular constituents, pancreatic stellate cells (PSCs) and cancer-associated fibroblasts (CAFs) are the major components of the TME.

6.1. Pancreatic Stellate Cells

PSCs are activated from their quiescent state by cytokines like interleukins (ILs), tumor necrosis factor-α (TNF-α), and growth factors secreted by damaged and tumor cells as a consequence of inflammation.[89] Their transformation into an activated phenotype is assessed by several parameters, such as the loss of vitamin A, proliferation, cytokine release, and the synthesis of ECM proteins, and their presence in stromal areas has been proved and pointed out as the main source of stromal collagen.[90] PSCs are believed to closely interact with cancer cells as well as with other stromal cell types such as endothelial and immune cells.

The interaction between PDAC tumor cells and pancreatic stellate cells provokes increased tumor growth and metastases,[91] by creating a supporting niche for the tumor cells and by promoting the endothelial–mesenchymal transition (EMT), a developmental process that leads cells to assume an aggressive mesenchymal phenotype with migratory capacity, invasiveness, elevated resistance to apoptosis, and increased ECM components' production.[92]

The loss of epithelial cell markers such as E-cadherin and the increased expression of mesenchymal markers such as vimentin are some of the hallmarks of EMT,[93] and this process was proved to contribute to chemoresistance in pancreatic cancer[4] and to be linked with the activation of the Notch signaling pathway.[94]

PSCs have also been proved to interact with endothelial cells, inducing proliferation and tube formation of human microvascular endothelial cells, possibly via vascular endothelial growth factor (VEGF) mediation.[95] The process of immune evasion by cancer cells in PDAC is believed to be supported by PSCs, which act as $CD8^+$ (Cluster of Differentiation 8) T-cell sequestration in stromal areas, thanks to the PSC-derived chemokine CXCL12, thus reducing their antitumor effects.[96] Moreover, various other interplays occur between PSCs and mast cells, whose effect is an increase in cell proliferation,[97] and between PSCs and myeloid-derived suppressor cells (MDSCs), whose migration into the tumor suppress immune cell function.[98] Finally, as previously pointed out, PSCs play a fundamental role in hindering drug delivery in the tumor area by producing the thick stroma surrounding cancer cells.

6.2. Cancer-Associated Fibroblasts

CAFs are a heterogeneous cellular population that can originate from resident fibroblasts, bone-marrow-derived cells, and stellate cells.[99] They lack specific cell surface markers and are therefore mostly identified by their elongated morphology or tissue position,[100] they can assume both a tumor supportive or suppressive role, according to the stage of tumorigenesis and many other context-dependent factors,[101] and they are responsible for an active remodeling of the desmoplastic stroma through various paracrine mechanisms, while being highly chemotherapy resistant[102] and showing a unique heterogeneity of subpopulations in PDAC. In fact, recent studies identified at first two subtypes of CAFs. One type, called myCAF and located primarily adjacent to cancer cells, expresses high levels of α-smooth muscle actin and possesses a tumor-suppressing role; the second type,

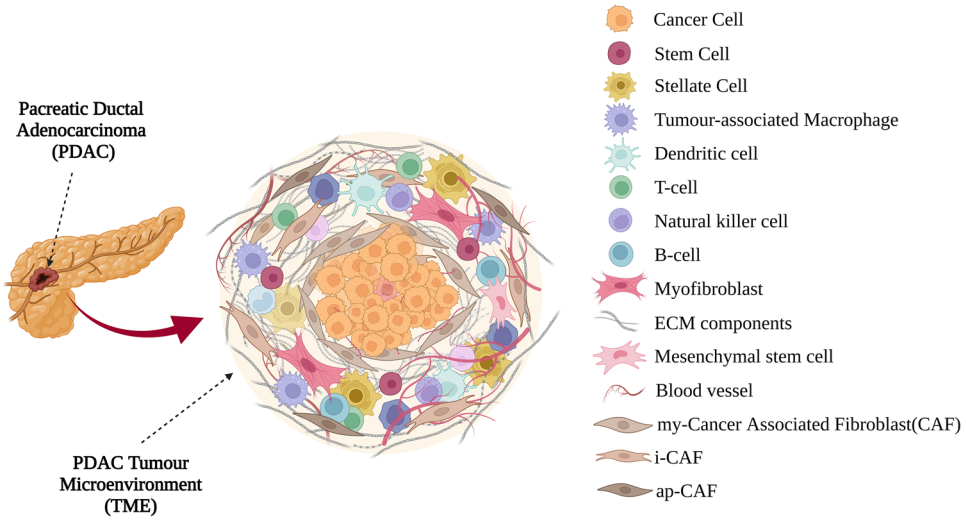

Figure 1. Scheme of the plethora of cells taking part to PDAC TME. Created with Biorender.com.

named iCAFs, is located farther from the cancer cells and is believed to exhibit a tumor-promoting behavior.[103]

A later in vivo study identified a third subtype called antigen-presenting CAFs), with immune-modulatory capacity that might contribute to the inhibition of optimal T-cell response.[104,105]

6.3. Pancreatic Stem Cells

Pancreatic stem cells are self-renewing, immortal cells derived from the bone marrow which inhabit a hypoxic niche composed of different cells such as endothelial cells, immune cells, and cytokines.[71,106] Their proliferation is increased by the hypoxic environment of the tumor site[107] and influenced by inflammatory cytokines such as interleukins. They show intrinsic chemoresistance, since they are highly resistant to a typically used first-line treatment drug, namely gemcitabine,[108] and have been proved to increase their proliferation in the presence of this chemotherapeutic agent.[109] For this reason, many studies have focused on addressing them by other means, like targeting either some signaling pathways such as the hedgehog (Hh) pathway[110,111] or surface markers which are typically exposed by these cells[112] through treatments with kinase inhibitors, antibodies,[113] antibiotics, and immunotherapy.[7,114]

6.4. Other Cell Types

Other important cell types of the pancreatic cancer TME are I) T-lymphocytes, which inhabit the site of invasion and the lymphatic system of the tumor and synthesize cytokine interleukins[115] (whose effect is in turn the activation of inflammatory response, proliferation, and metastasis); II) B-lymphocytes, associated with an invasive front of tumors and expressed in lymphoid structures near the TME;[116] III) tumor-associated macrophages (TAMs), that secrete chemokines and cytokines triggering immune cell recruitment, whose communication with the TME favors the progression of PC;[117] IV) suppressor cells of myeloid lineage,[118] that downregulate cytotoxic T-lymphocyte activation; V) dendritic cells (DCs), which lose their capacity of processing and presenting antigens in PC;[114] VI) tumor-associated neutrophils, involved in premetastatic stages pf PC;[119] VII) vascular endothelial cells and pericytes, activated by growth factors of TME;[120,121] VIII) natural killer (NK) cells, which are however largely excluded from the tumor tissue and hence mostly present in peripheral blood, but nevertheless support immune evasion mechanisms and contribute to the production of immunoregulatory IL-10 cytokines.[122] **Figure 1** reports a schematization of PDAC TME, which includes all the previously mentioned cell types contributing to its desmoplasia.

7. Molecular Markers

Some potential molecular markers have been identified to diagnose and treat PC, and they can be classified into three main classes, based on their biological source: serum markers, tumor markers, and cancer stem cell markers. Serum markers like cancer antigen 19.9,[123] IL-6, IL-8, and IL-10,[124] survivin,[125] mesothelin,[126] and carcinoembryonic antigen (CEA) are expressed on the surface of PC tumor cells and therefore can be useful for early detection and specific targeting, with the employment of human monoclonal antibodies (mAbs),[127] peptide vaccines,[128] or antibody–drug conjugates.[129]

Tumor markers include, among the others: secreted protein acidic and rich in cysteine (SPARC),[130] which is used to target drugs, thanks to its high affinity to albumin; hyaluronic acid,[131] abundant in the TME and associated with PDAC pathogenesis, blood vessel collapse, and EMT,[132] whose depletion proved to improve the tumor condition;[133] mucins, which have a central role in immunosuppression and metastasis,[134] can be used to distinguish between PDAC and its precursor lesions[135] and can be targeted by MUC (mucin) tumor-specific antibodies.[136]

Cancer stem cells usually expose several surface markers, which have been proved to be related to stemness and gemcitabine resistance in PC stem cells;[137] for example, CD44 expression is associated with poor prognosis,[138] high grade of

cancer, radiation therapy resistance, and metastasis,[139] and it is often exploited for targeted drug delivery as well as for tumor visualization.[140]

Other typical markers expressed by PC stem cells are CD133, whose expression is associated with metastasis[141] and whose activity results in a triggering of downstream regulatory signals for stemness properties and EMT;[142] CD24, overexpressed in high-grade PC tumors[143] and advanced PC stages;[144] CXCR4 (C-X-C chemokine receptor type 4), which promotes cancer development, invasion, and metastasis and might be indirectly activated by the hypoxic tumor microenvironment of PC through the expression of CXCL12 by fibroblasts;[145,146] ESA (epithelial surface antigen), related to shorter survival of patients with advanced pancreatic cancer and overexpressed in PC;[147] Oct4, whose knockdown in a 2013 study by Lu et al. resulted in reduced proliferation, chemoresistance, and tumorigenesis in vitro and in vivo.[148] The coexpression of many of these markers has been thoroughly studied over the years, highlighting the considerable complexity of the general framework of pancreatic cancer stem cell markers and of the involved altered molecular pathways.[149]

8. Signaling Pathways

Mutations in the survival pathways involved in PDAC promote tumor progression and resistance against chemotherapeutic drugs. The continuous crosstalk among these pathways leads to the formation of interlinked signaling networks,[8] whose effect is the increase of tumor aggressiveness.

The Notch signaling pathway is only one of the many implicated in the progression of PDAC and in its distinctive chemoresistance.[150] It has been recently found to contribute to EMT[151] since its activation in endothelial cells results in their mesenchymal transformation: in fact, Notch controls the expression of Snail homologs, implicated in the EMT acquisition,[152] and a knockdown of Notch-2, one of its four receptors, in gemcitabine-resistant PC cells resulted in EMT inhibition.[153]

KRAS mutation induces the over secretion of transforming growth factor-β and IL-10 and stimulates various downstream cascades;[154] overexpression of epidermal growth factor receptor (EGFR) and its downstream pathways can result in drug resistance; Hh pathway modulates the stromal environment and is essential for the development of the ECM and the vasculature,[155] and its inhibition was proved to have a proangiogenic role and thus a positive effect on the delivery of drugs to the tumor site.[156]

Signal transducer and activator of transcription 3 (STAT3), a cytoplasmatic transcription factor, is involved in many crucial pathways for tumorigenesis and can be activated by many oncogenes and protooncogenes; it is regulated by cytokines, epidermal and platelet-derived growth factors and contributes to chemoresistance.[157] Phospho-STAT3, a risk factor for pancreatic adenocarcinoma prognosis, is abnormally expressed in this type of cancer and related to tumor size;[158] moreover, it may promote tumor angiogenesis via upregulating the VEGF.[159]

Ephrin receptors form the largest known subfamily of receptor tyrosine kinases, and together with their ligands, they compose an extensive communication system which is involved in many cellular processes along with cancer development and progression. Erythropoietin-producing human hepatocellular (Eph) signaling is bidirectional, since ligands are attached to a membrane and can provoke a forward or a reverse response when they bind to receptors, and it is responsible for tumor promotion and suppression according to mechanisms which have not been clarified yet.[160] There are two subfamilies of Eph receptors, EphA and EphB, and in the last decades, many studies have correlated their expression in cancers with increased/malignancy and poor clinical prognosis; moreover, they appear to be involved in crosstalks with other signaling networks (Akt, MEK/ERK/RSK, namely mitogen-activated protein kinases/extracellular signal-regulated kinases/ribosomal s6 kinase, STAT3) and in feedback loops which contribute to the entangled signaling networks featuring in the tumor microenvironment.[161] With regard to pancreatic cancer, EphA2 overexpression has been correlated with increased invasiveness and metastatic abilities for a long time now,[162] and highlighted as a possible target for therapies.[163] More recently, EphA2 fragments detected in plasma have been proposed as a possible novel PC biomarker,[164] and gemcitabine conjugation with an EphA2 targeting agent has provided encouraging evidence of improved clinical outcomes in xenograft models of pancreatic cancer,[165] reinforcing the idea of using Eph receptors as therapeutic targets in PC, lately pursued in a study by Salem et al.[166]

9. Tumor Vasculature and Hypoxia

Angiogenesis plays an important role in tumor development and progression, and in metastasis spreading as well.[167] PDAC vasculature is characterized by high microvascular density and very poorly perfused vessels, which do not allow drugs to reach and treat the tumor site. Moreover, they help tumor growth through mechanisms such as vessel co-option,[168] vasculogenic mimicry,[169] and vasculogenesis.[170] Pancreatic cancer angiogenesis is activated by genetic and epigenetic alterations, and by the cells and the stromal components of the TME. Moreover, PDAC desmoplasia leads to high IFP, which in turn causes the vasculature collapse and therefore a low drug penetration and uptake,[171] that contributes to cancer resistance to targeting therapies.

Microvessel density measurements in pancreatic cancers with respect to normal tissues suggested the existence of an active angiogenic process in the central tumor,[172] while other evidences[6] highlighted the presence of hypovascular tumor stroma and hypervascular normal pancreas tissue, suggesting the existence of a vast heterogeneity of vascular distribution within PDAC. According to Saiyin et al. the presence of basal microvilli, hairy-like microvasculature detected in aggressive and metastatic PDAC, could be exploited as a promising therapeutic target for treatment since they are correlated with high glucose uptake in pathological conditions.[173]

The role of pericytes was thoroughly studied and reviewed due to their effect in maintaining impaired microvessel integrity,[174] and their poor presence in tumor vessels was highlighted in different studies.[175] For example, Gilles et al. proved that an increase of pericyte coverage resulted in enhanced tumor perfusion and reduced hypoxic area in PDAC.[176]

Overall, these studies agreed on the importance of a normalization of the tumor vasculature as a potentially effective therapeutic approach, to both relieve hypoxia and enhance drug delivery.[177]

10. Mouse Models

Over the course of the last decades, several experimental mouse models have been generated in order to faithfully reproduce and thus better understand PDAC tumor. The currently available mouse models of pancreatic cancer include cancer-cell-line-based heterotopic and orthotopic xenograft in immunocompromised mice, patient-derived xenografts, transgenic mice (genetically engineered mouse models, GEMMs), and organoid models. They are generally classified according to the manner or tumor induction, the site of tumor implantation, and the histopathological characteristics.[178]

The first experiments with spontaneous tumor animal models involved the use of a chemical viral induction, or the application of experimental genetic techniques in rats[179] and hamsters.[180] More recent attention has focused on the introduction of oncogenes (especially mutant KRAS genes) into mouse embryonic or somatic cells using transgenic, gene knock-in, and gene knock-out techniques to transfer specific genes into mice via retrovirus. Exploiting this technique, Hingorani et al. created the first prenatal GEMM (called KC) that developed the full spectrum of PanIN lesions, which progressed toward PDAC with age,[181] and observed that some of these mice developed metastatic tumors after a long latency. Since this model developed PanINs shortly after birth and therefore showed a different PDAC etiology from human patients, a second generation of GEMMs was established by Guerra et al.[182] These postnatal models allowed temporal control of KRAS activation in the pancreas, but they proved that mature acinar cells were resistant to transformation by oncogenic KRAS. Nevertheless, in the presence of pancreatitis-induced inflammation, the progression in PanINs and PDACs with high penetrance was observed. Later on, tissue damage and proliferation of acinar cells were proved to be an effect of inflammation[183,184] and the mechanism of inhibition of oncogene-mediated senescence[185] in high-grade PanIN-2/3s and in low-grade ones in the presence of acute or chronic pancreatitis was suggested among other inflammatory pathway activations (such as STAT3/Socs3) as a possible cause for PDAC development.[186] Based on these results, many other mouse models combining KRAS mutations with other common genetic alterations such as CDKN2A (INK4A/ARF, inhibitors of cyclin-dependent kinase 4/alternative reading frame), TP53, and SMAD4, were implemented to induce PDAC.[187]

A notable example derived from the KC strain is the KPC mouse model, developed by Hingorani et al., which expresses a mutated form of TP53 and better mimicked the PDAC TME from a pathological and immunological point of view.[188] In fact, this mouse model I) retraced the progression, metastasis, and stromal complexities of the tumors; II) offered a very fast PDAC progression (20–24 weeks); III) exhibited high penetrance and gemcitabine resistance;[156,189] IV) developed a dense desmoplasia and poor vasculature, closely mimicking the dynamics of TME; V) provided samples of tissues, serum, and tumor cell lines exploitable in further researches; VI) was used to develop gene specific knockout models, suitable to study the effect of certain genes on the pathogenesis; VII) produced an intact immune system, hence allowing the study of immune response in PDAC; VIII) offered autochthonous tumors. Therefore, the application of KPC mouse models considerably boosted the understanding of biomarker development,[188,190–192] the role of tumor stroma,[132,189,193,194] and signaling pathways[156,195–202] concerning PDAC. Moreover, they were exploited for preclinical applications of chemotherapy,[189] targeted therapies,[132,193] and immunotherapy.[203–207] Experimental murine models have also been thoroughly used to study the impact of risk factors for PDAC, such as family history,[208] pancreatitis,[209] smoking,[56,210,211] alcohol,[212] and diabetes,[213] and the evolution of precursor lesions.[190–192]

The exploitation of these and many other GEMMs, which have been thoroughly described in dedicated reviews,[187,214,215] is however accompanied by several limitations, such as I) variability in tumor initiation, progression and metastasis incidence; II) labor intensive and time-consuming breeding of mice colonies; III) fewer mutations and less genetic complexity with respect to humans; IV) the need of crossing three or more lines of mice.

Xenografts derived from human pancreatic cancer cell lines, implanted subcutaneously or orthotopically in athymic and therefore immunodeficient mice, have been extensively used to evaluate and optimize therapeutic approaches, especially targeted therapies. For this purpose, cell lines have been the preferred choice for a long time due to their defined growth kinetics, their easy maintenance at specific culture conditions, their reproducible behavior, and a solid literature background. Common drawbacks to cell-derived xenografts are the absence of the immune system influence, the lack of genetic and phenotypic heterogeneity offered by immortalized cell lines, the absence of tumor stroma, the risk of alterations during in vitro passages, the infrequent metastasis formation. Part of these limitations have been addressed by coimplantation models using CAFs, and more recently by patient-derived xenografts (PDXs), namely fragments of primary tumors derived from surgical resection[216] and implanted subcutaneously, orthotopically, or under the renal capsule[187,178] in mice. The main advantages of this model are the retention of the morphological characteristics of the parental tumor, the preserved metastatic potential, and the genomic/architectural stability of the obtained xenografts, which make them able to respond to therapy as the original tumor.[193] Conversely, critical issues are mainly related to the long growing time, the infiltration of murine stroma after implantation,[217] the absence of host immune system influence, and the propagation of aggressive phenotypes in mouse models.[218]

Many alternative approaches have been suggested and carried out, like I) the coimplementation of stromal cells derived from patients, II) the use of syngeneic/allograft mouse models, consisting of tumor tissues derived from the same genetic strain of mice which do not elicit an immune reaction and therefore allow the use of immunocompetent animals,[219] III) the production of humanized mice able to develop human immune system; their application was hence mainly focused on immunotherapy studies.[220,221]

Overall, the absence of a golden standard able to perfectly mimic PDAC, its microenvironment, and the immune system has led to some discrepancies between therapeutical results obtained in mouse models and the actual response in clinical trials.[202,222] Nevertheless, all these mouse models are still an essential step in preclinical studies and have contributed to considerable advance in the understanding of PDAC progression,

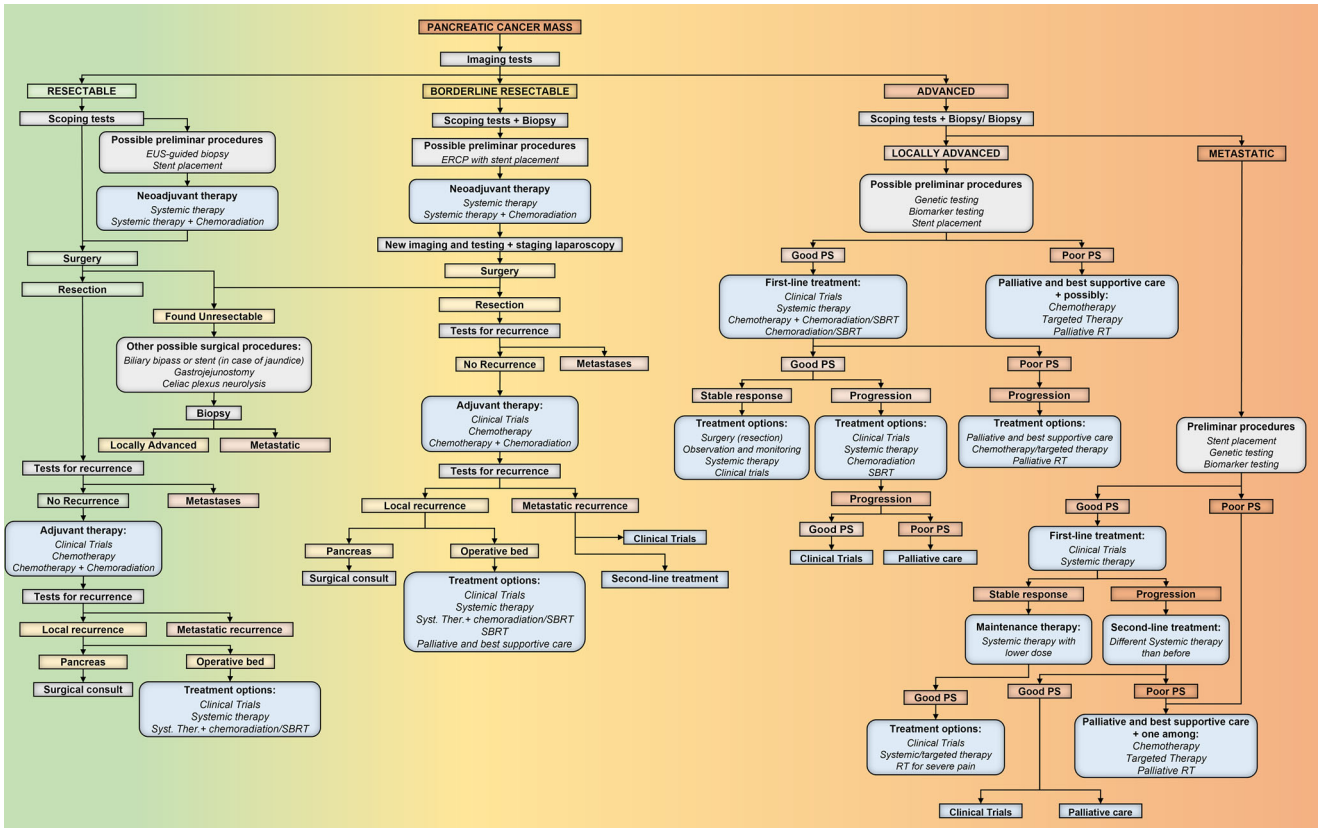

Figure 2. Schematic representation of current recommendations for diagnosis, treatment, and follow-up of patients affected by PDAC, depending on the stage of the tumor.

metastasis, stromal heterogeneity, and response to therapies, continuously evolving, thanks to emerging knowledge.

11. Current Treatments and Guidelines

Currently, the standard therapeutical approach to PDAC is surgery followed by adjuvant multiagent chemotherapy in case of resectable tumors,[21,223] with a recorded median survival of up to 26 months. On the other hand, borderline resectable tumors are generally pretreated with a neoadjuvant therapy before surgery, to allow tumor shrinkage and a better resection outcome.[15] Surgical resection is the only hope for long-term survival, however most of the times diagnosis is given when the disease is already unresectable or metastatic. Nevertheless, advances in surgical techniques and systemic chemotherapy have enabled, after the application of neoadjuvant protocols, the extension of resection to locally advanced tumors[12] (not so long ago generally excluded from surgical options[224]) along with borderline resectable ones. As far as metastatic tumors are concerned, systemic palliative chemotherapy is usually offered as first-line treatment and combinational therapies are nowadays producing promising results in prolonging the median survival of patients. Most pancreatic cancers progress after first-line palliative chemotherapy, leading to the need of a second-line one.[18] This second-cycle chemotherapy must be carefully chosen depending on the first administered therapy, but promising results are today evidence-based. In 2015, European Society for Medical Oncology Clinical Practice Guidelines collected an overview of therapy recommendations, including the application of clinical trials to borderline resectable tumors.[225]

A 2018 extensive review concerning therapeutical developments in pancreatic cancer reported that targeted therapies and antiangiogenic drugs generally failed due to the hypovascular nature of the stroma surrounding cancer cells. In addition to that, the authors did not highlight any breakthrough in immunotherapy applied to PDAC, but concluded that the application of recent understanding regarding its complex molecular mechanisms and the TME could be the key to future clinical improvements and success.[15]

National Comprehensive Cancer Network Clinical Practice Guidelines in Oncology, published in 2021, reported an update of recommendations for diagnosis, evaluation, treatment, and follow-up for patients with pancreatic cancer.[226] **Figure 2** presents a summarizing and simplified scheme based on the main points highlighted by this guideline, merged with information extracted from other aforementioned sources.[15,225]

An extensive dissertation about the main currently available therapies for the treatment of PDAC is reported hereinafter, and the latest findings and publications regarding each discussed therapy are included to go deeper into future outlooks.

11.1. Surgery

PDAC usually takes more than a decade to metastasize, however early stages and precancerous lesions are largely asymptomatic. Consequently, they are difficult to be diagnosed in time for surgery to represent the main route to avoid further spreading of the disease into nearby organs. The tumor stages are generally referred according to the American Joint Committee on Cancer "TNM" classification, namely tumor size (T), spread to nearby lymph nodes (N), and metastasis to distant sites (M). The standard stage classification includes resectable, borderline resectable, and/or locally advanced unresectable and metastatic tumor, while the Eastern cooperative oncology group (ECOG) defined a score indicating performance status (PS), which varies from 0 to 5.[227] Other important prognostic factors are tumor grade (G), which describes the tumor likeliness to normal tissue under a microscope, and extent of resection (R), that indicates whether or not all the tumor is removed after surgery and ranges from R0 (the desirable outcome of any surgical resection) to R2 (visible tumor not removed).[228]

For patients eligible for surgery, some options are available depending on their overall status, extension of the tumor and venous involvement: pancreaticoduodenectomy, distal pancreatectomy, total pancreatectomy, or palliative surgery.[12,229] Complete resection (R0) followed by adjuvant therapy still represents the course of action with the best results over time. In this regard, some recent systematic reviews and meta-analyses argued that the achievement of a radical resection could effectively change the outcome in patients with clinical N0 disease (no lymph nodes involved) by prolonging their overall survival (OS).[230,231] Moreover, these studies linked some specific initial recurrence patterns and clinicopathological factors or surgery outcomes such as R1 resection to recurrence locations, proposing strict postsurgery follow-ups to monitor patients and promptly start systemic chemotherapy.[232] Altogether, it has conclusively been shown that, although undisputed progress in terms of surgical techniques has been made over the last 20 years,[12] systemic recurrence within 2 years from surgery is nonetheless as prominent today[233,234] as it had been previously reported.[235,236]

11.2. Chemotherapy Evolution over Time

Over the years, only few but pivotal improvements have been made in terms of new clinically approved chemotherapeutic drugs for PDAC, and here, a brief summary of their history is reported.

The first anticancer drug used for PDAC treatment was 5-fluorouracil (5-FU), which had been first introduced in 1957 and already applied to tumors such as breast and colorectal cancers.[237] It is an S-phase specific uracil analog whose accumulation in cells results in increased cytotoxicity, eventually leading to cell death. In fact, once inside the cell, it is converted to several active metabolites which disrupt RNA synthesis and the action of the nucleotide synthetic enzyme thymidylate synthase. Its main drawback is the low stability due to the presence of an enzyme called dihydropyrimidine dehydrogenase, abundantly expressed in the liver, and therefore in the last 20 years, important modulation strategies have been developed in order to increase its anticancer activity and decrease its degradation.[238] Another critical aspect that has been increasingly considered is 5-FU's emerging resistance in PC;[239] a recent systematic literature review thoroughly discussed its mechanisms and proposed some novel approaches to overcome chemoresistance such as combination of 5-FU with other therapeutics, DNA repair pathways' targeting, nanoformulated drug delivery, novel MDR modulators.[240]

Gemcitabine (Gem) is a prodrug which undergoes phosphoactivation after cell uptake via nucleoside transporters; the derived active drug metabolites inhibit DNA synthesis.[241] Since 1997, it has been proposed as first-line therapy over 5-FU against pancreatic cancer, due to the undeniable improvements in terms of median overall survival rate and one-year survival rate.[242] Unfortunately, despite the initial sensitivity of PDAC to gemcitabine, drug resistance occurs within several weeks of treatment[243] and metabolic clearance of gemcitabine was observed in vitro in the PDA cell line PANC-1,[244] due to the macrophage-induced cytidine deaminase upregulation, whose effect is gemcitabine inactivation and excretion out of the cells.

While multiple different agents have been proposed over the years in combination with gemcitabine, none of them has ever shown signs of significant survival advantages, although some studies suggested some potential benefits on patients with good PS.

CO-101, a lipid–drug conjugate of gemcitabine, was developed to overcome cancer Gem resistance by entering cells independently of human equilibrative nucleoside transporter-1, but the results of a randomized study carried out in 2013 showed that CO-101 was not superior to gemcitabine in patients with metastatic PDAC.[245]

Gemcitabine was hence combined with capecitabine (GEMCAP), another nucleoside analog with which a synergistic antitumor activity had been previously demonstrated without any overlapping toxicity. On this matter, a phase III trial was carried out almost 20 years ago to compare the efficacy of the GEMCAP combination with respect to Gem alone. The results showed that GEMCAP could be considered as a valuable alternative to Gem if applied to patients with a good PS.[246]

In a study conducted by Heinemann et al. in the same period, the addition of cisplatin to gemcitabine every 2 weeks did not show any statistically significant increase in progression-free survival (PFS) and OS of patients with advanced PDAC.[247]

GEMOX, a combination of gemcitabine and oxaliplatin, was tested in a phase III study which confirmed its safety and efficacy but failed to prove statistically significant improvements of metastatic overall survival,[248] and a following randomized phase III confirmed the lack of an improvement in PFS as well.[249] The same lack of improvements was pointed out in a phase III study concerning gemcitabine plus irinotecan versus gemcitabine monotherapy.[250]

In 2011, a multidrug combination called irinotecan, oxaliplatin, fluorouracil, leucovorin (FOLFIRINOX) was proposed in the PRODIGE 4/ACCORD 11 trial in patients with metastatic pancreatic cancer, and it showed a median overall survival of 11.1 months with respect to the 6.8 months previously achieved with gemcitabine.[251] PFS, one-year survival, and the time to definite deterioration of the quality of life were significantly improved as compared to gemcitabine, but the safety profile of the treatment raised major issues concerning its toxicity.[252] For this

reason, FOLFIRINOX was suggested since then as a first-line option specifically for patients younger than 76 years, with a good PS and without limiting comorbidities. In a following study, FOLFIRINOX regimen was proved biologically active in borderline resectable and locally unresectable PDAC, with a 33% R0 resection rate achieved,[253] and its role in downstaging the tumor and improving resection rates was further confirmed in a recent review on the topic.[230]

Erlotinib, a tyrosine kinase inhibitor, was added to gemcitabine in a phase III trial on patients with advanced pancreatic cancer, which often overexpresses human epidermal growth factor receptor type 1, but the OS was prolonged by only 2 weeks. However, this result was statistically significant and therefore considered clinically meaningful with respect to previous failures.[195]

To enhance the effect of paclitaxel (PTX), an anti-microtubule agent which is insoluble in aqueous medium and whose efficacy in patients with PDAC had previously been disappointing, it was conjugated with human serum albumin to form negatively charged spherical nanoparticles (nab-paclitaxel, Abraxane). Stromal SPARC was suggested as a potential target, although its role was debated and resized over time, but evidences confirmed stroma disruption as an exclusive effect of Abraxane, which could be related to SPARC mediation.[254] Moreover, micropinocytosis was suggested to enhance nab-paclitaxel uptake, thanks to the presence of albumin.[255]

Nab-paclitaxel was administered in combination with gemcitabine, and their synergistic activity resulted in prevention of tumor growth in genetically engineered mice and sometimes even in a regression of the tumor size.[189]

Overall, these multidrug regimens were accompanied by diverse and severe side effects and showed higher toxicity with respect to single-agent gemcitabine, without contributing in an effective way to the improvement of OS in patients unless these showed a good PS in the first place.

All the abovementioned studies support the hypothesis that a better drug intratumoral delivery was often the main explanation behind some of the few improvements observed in the course of the last decade. Consequently, improving the targeting of drug administrations seems the best way toward new therapeutical approaches.

11.3. Palliative and Second-Line Therapies

For patients with locally unresectable PDAC or distant metastases, most of the current available therapies are palliative and hence their aim is to relieve symptoms and to prolong survival as long as possible, preserving an acceptable quality of life.[226] Before the debut of FOLFIRINOX regimen against pancreatic cancer in 2011,[251] gemcitabine had been the only standard care since the trial that assessed its superiority over 5-FU.[242] In 2013, the introduction of the combination therapy including nab-paclitaxel and gemcitabine offered a new treatment option with a survival benefit over gemcitabine monotherapy.[256] However, a careful patient selection is a crucial point to consider, especially for combination therapies, since their administration is accompanied by many toxicity issues.[225]

Moreover, since tumor progression within few months is very common after first-line palliative care, many patients find themselves in need of second-line chemotherapy, whose options are nowadays very limited.[17] Usually, only patients with good PS despite progression of disease on frontline treatment are included in second-line regimens. In this regard, the role of first-line FOLFIRINOX and Gem/nab-paclitaxel in following lines of therapies must be elucidated in view of the conflicting results obtained so far.[19] Nanoliposomal irinotecan and/or 5-FU–folinic acid is by now considered the best second-line option for patients previously treated with Gem therapy,[257] while studies analyzing second-line therapies after the failure of FOLFIRINOX regimens are currently under investigation to confirm the suitability of Gem-based treatments.[17]

11.4. Drug Resistance

As stated above, PDAC MDR is currently one of the major obstacles to treatment. In the last decade, the mechanisms underlying its behavior have been thoroughly analyzed in order to better understand this phenomenon and eventually overcome it.[7,120,239,243] As recent findings pointed out, MDR is driven by many molecular mechanisms such as overexpression of signals responsible for cell survival, DNA damage repair mechanisms, redistribution of the drug from the cell nucleus to cytoplasm, downregulation of apoptotic activity, and the presence of drug efflux pump which alter the drug concentration inside the cancer cells.[8,258,259] Furthermore, another pointed out effect of EMT in addition to invasiveness appears to be the loss of sensibility toward drugs (to gemcitabine in particular), and the resulting release of mediators such as cytokines and transcriptional factors might as well be related to this acquired mechanism.[260]

Moreover, drug resistance is believed to be associated with metabolic aberrations that lead to altered angiogenesis and apoptosis, and it is supported and promoted by the TME.[120] Tumor stroma seems to be one of the main actors involved in gemcitabine resistance,[259] because it prevents chemotherapeutic drugs from reaching the tumor microenvironment and at the same time, it promotes metastasis and PDAC cells' penetration of the surrounding tissues.[261]

Finally, drug resistance is undoubtedly a result of the interaction of the abovementioned factors, since all the main cells of PDAC and the innate immune cell population are responsible for the instigation of a highly hostile environment, and their continuous crosstalk with tumor stroma induces and enhances chemoresistance.[262]

However, further extensive study of the molecular mechanisms of survival and MDR is surely necessary to design new therapies, which are nowadays focusing on addressing TME and stromal components with the aim of improving PDAC response to chemotherapy.[4]

11.5. Targeted Therapies

In order to enhance drug delivery into the tumor microenvironment and to improve the toxicity profile of drug treatments, new strategies have been implemented over the years and resulted in targeted therapies. Their aim is interfering with some of the many dysregulated signaling pathways in PDAC, which result

from the considerable variety of accumulating mutations taking place during carcinogenesis.[29,263] In fact, due to its pivotal role in tumor development, TME has been considered as a target for cancer treatment. The main advantage of TME targeting is the higher genetical stability of nontumoral cells, which results in their reduced predisposition toward the development of drug resistance,[264] but a major related challenge is minimizing toxicity to normal and healthy cells. Moreover, the complexity of pancreatic cancer TME and its stromal interactions have caused the failure of most of the targeted therapies widely used with other types of tumors, since they showed good results in preclinical settings but disappointing ones when it came to phase II/III clinical trial translation.

Among others, targeting the growth factor receptors has been a typically implemented strategy over time. EGFR, overactivated in PDAC patients, was addressed by both antibodies, blocking its activation (cetuximab plus capecitabine[222]), and inhibitors of tyrosine kinase domain of the receptor (gefitinib plus gemcitabine[265]), which however failed to show improvements over standard therapies. Human epidermal growth factor receptor 2 is correlated with poor patient survival, and its targeting by means of capecitabine and trastuzumab[266] was unsuccessful in phase II clinical trials; lapatinib in combination with capecitabine[267] was tested as second-line therapy but the low number of enrolled patients impaired the interpretation of its clinical benefit. Likewise, the targeting of insulin-like growth factor 1 receptor by monoclonal antibodies (ganitumab and cixutumumab[268]) and by a combination of ganitumab and gemcitabine[269] was proved to be unsuccessful.

Inhibition of KRAS pathways by direct targeting was proved ineffective as well, hence upstream effectors of RAS or KRAS downstream signaling molecules such as MAPK (mitogen-activated protein kinases) pathway were targeted with a combination of tipifarnib and gemcitabine[270] and with selumetinib,[271] respectively. Although these approaches were unsuccessful, ERK inhibition is currently explored as a potential PDAC treatment because of preclinical promising results, with combinations of gemcitabine/nab-paclitaxel and Ulixertinib BVD-523.[272]

PI3K (phosphoinositide 3-kinase) signaling is considered a crucial pathway to be addressed for PDAC therapy, and it was the target of studies involving gemcitabine and rigosertib, that however did not show huge improvements in patients' response.[273]

Everolimus, which suggested preliminary promising results in terms of progression-free survival time,[274] was combined with capecitabine, prolonging capecitabine monotherapy's OS to 8.9 months.[275] Nevertheless, due to differences in the studies' design and populations, this last comparative result could be arguable. Notably, a combination of PI3K and MEK inhibitors was suggested to possess a potential synergic activity,[276] as well as that of Notch and JAK–STAT (Janus kinases-signal transducer and activator of transcription protein) pathways. The inhibition of the former by anti-DLL4 (Delta-like ligand-4) antibodies showed a therapeutic potential in possibly reversing chemoresistance,[277] but this outcome was not echoed by following beneficial results; however, new studies have uncovered the use of γ-secretase inhibitors, such as RO4929097.[278] JAK–STAT pathways' inhibition in patients resistant to gemcitabine was achiegved with ruxolitinib and capecitabine and showed improved PS and pain management in those with evidence of systemic inflammation.[279] Finally, poly ADP-ribose (adenosine diphosphate-ribose) pathway[280] and tumor suppressor TP53[281] are currently being studied in clinical trials.

Much of the available literature on targeted therapies deals with the huge heterogeneity and complexity of PDAC, whose crosstalk between molecular and signaling pathways has usually led to the failure of these treatments. Moreover, other typically highlighted issues are the presence of surrounding stromal and inflammatory components and the unselected patient populations. In fact, none of them actually improved patient survival, apart from a combination of gemcitabine and erlotinib that conferred a statistically significant mean survival benefit of 2 weeks over gemcitabine alone.[195] This effect was however marginal and raised many questions concerning its underlying molecular mechanisms.[45]

Undoubtedly, combinations of chemotherapy and multiple targeted molecules have generally reported better results than targeting individual molecules, reducing upregulations and compensations implemented by adjacent pathways, and should be further explored. Nonetheless, future studies should focus on combined therapies involving Abraxane and FOLFIRINOX as chemotherapeutic drugs rather than just gemcitabine,[15] and the eventual determination of predictive biomarkers could definitely help determining in advance possible responding patients.[45]

11.6. Antiangiogenic Therapy

Antiangiogenic approaches to PDAC focus on targeting specific angiogenic pathways, such as VEGF and its receptors, these being the most studied among many others. Such approaches aim at blocking tumor blood vessel increase by reducing proliferation of endothelial cells, oxygen and nutrient supplies and thus inhibiting cancer growth.[167] Nevertheless, despite promising preclinical results, targeting the tumor vasculature was proved unsuccessful in different clinical studies, eventually leading to more invasive tumor phenotypes and metastases spreading.[282] Some abortive attempts in the last decades included the use of bevacizumab combined with gemcitabine,[283] axitinib plus gemcitabine,[284] sorafenib alone or in combination with gemcitabine,[285] and aflibercept plus gemcitabine.[286]

Multiple factors supporting the failure of vascular targeting studies have hence been proposed: I) long-term antiangiogenic therapies sometimes lead to tumor hypoxia, triggering VEGF production and genetic instability in tumor endothelial cells;[287] II) as previously reported, other mechanisms such as vessel co-option[168] and vasculogenic mimicry[169] are implemented by the tumor microenvironment to compensate the antiangiogenic treatments; III) hypoxia in turn negatively affects drug delivery and contributes to the creation of a hostile TME, increasing chemoresistance; IV) these treatments determine a compensatory upregulation of proangiogenic and prometastatic cytokines, some of which recruit immune cells to create new metastatic niches.[170]

Taken together, these results suggested that vasculature normalization should be preferred over antiangiogenic strategies aimed at starving tumor cells:[288] the benefits provided by such approach include a subsequent more effective drug administration, a potential attenuation of tumor-hypoxia-associated EMT,

and a promotion of PDAC immune response through the infiltration of immune effector cells.[289]

This emerging therapeutic strategy appears very promising and should therefore be validated by further studies, to carefully identify the key regulators of angiogenesis and to eventually propose personalized therapies based on the use of molecular biomarkers to select appropriate patient populations prone to responding to vascular normalization. Finally, the emerged critical role of tumor stroma in blood vessel compression suggests that a stromal normalization should be taken into account as well, by targeting its components.[170]

11.7. Stroma Targeting

One of the hallmark pathological features of PDAC is the disproportion between stromal elements, which constitute the majority of the tumor volume, and malignant cells.

This dense stroma was proved to promote tumor progression and metastasis and to impair drug delivery, hence it has been addressed over the years by multiple targeted therapies with the aim of depleting it.[39,289] However, despite the effort made by researchers into studying and understanding the complex tumor-stroma crosstalk, many antistromal therapies resulted in failure during recent clinical trials because they somehow favored tumor aggressiveness.[290]

The abovementioned phase I/II clinical trial involving nab-paclitaxel plus gemcitabine for the treatment of metastatic pancreatic cancer pointed out a significant OS increase in patients with high stromal SPARC expression.[254] Due to this promising result, the randomized phase III MPACT trial was performed with the hope of finding an existing correlation between stromal SPARC levels and overall survival in patients,[291] but no evidence of it was pointed out. Furthermore, another recent study using patient derived PC xenografts suggested that SPARC expression and nab-paclitaxel tumor delivery might not be correlated, and that the main responsible for the drug accumulation might be SPARC expressed by the stroma rather than that one expressed by tumor cells. Finally, the authors did not observe a depletion of tumor stroma or a change in tumor microvascular perfusion due to nab-paclitaxel, concluding that the key to previously reported nab-paclitaxel encouraging results could be mainly due to its improved delivery in PDAC, thanks to the albumin carrier.[292]

Another crucial pathway of PDAC, Hh, was proved to regulate stroma through the signaling between cancer cells and CAFs, but its inhibition showed contradictory results.[293] IPI-926, an inhibitor of the sonic hedgehog (SHH) pathway, caused a reduction of tumor stroma, increased mean vessel density and perfusion, and therefore provoked a higher gemcitabine delivery and a subsequent enhanced therapeutic response in mouse models.[156] This result led scientists to postulate that the therapeutic resistance in PDAC could be caused by the biophysical rigidity of the ECM and its compressing action on blood vessels.[40,294] When translated to clinical trials, however, the combination of IPI-926 and gemcitabine failed to show improved survival over gemcitabine monotherapy.

Another SHH inhibitor, vismodegib, was not able to improve overall outcomes in phase I/II studies when added to chemotherapy.[295,296]

Further preclinical studies focused on relieving vessel compression by addressing some crucial noncellular stroma compartments, with the aim of reducing IFP and enabling the delivery of chemotherapeutic agents. The abundance of hyaluronic acid in PDAC stroma, associated with elevated IFP, was addressed by hyauronidase encapsulated by polyethylene glycol (PEGPH20), combined with gemcitabine. Their effect was a depletion of HA, a decreased intratumoral IFP, and an increase in vessel diameter in mouse models, which in turn provoked a decreased incidence in metastases and an increased survival.[84] A similar experiment was performed by Jacobetz et al., in which PEGPH20 enhanced intratumoral gemcitabine delivery, leading to a doubling of mice survival.[132] Despite promising preclinical results, clinical translations of this treatment were proved unsuccessful: a phase Ib/II trial applying PEGPH20 plus modified FOLFIRINOX in patients unselected for tumor HA status observed an increased toxicity which resulted in decreased treatment duration;[297] likewise, the combination of PEGPH20 and gemcitabine plus nab-paclitaxel, administered to patients whose eligibility criteria included high levels of HA, did not prolong OS and PFS with respect to chemotherapy alone.[298]

These negative findings suggested the inadequacy of targeting desmoplasia alone, therefore current studies are now including the addition of immune checkpoint inhibitors to PEGPH20,[299,300] after promising results obtained in murine models.[301,302]

Connective tissue growth factor, whose overexpression in PDAC results in pancreatic stem cells proliferation, migration, and fibrogenesis mediated by chemokine activation,[303] was addressed by pamrevlumab, a monoclonal antibody, in mouse models as a single agent or with gemcitabine.[206] In clinical trials, its combination with gemcitabine and erlotinib in a phase I study did not show further toxicity,[304] while a phase I/II combining it with gemcitabine/nab-paclitaxel showed higher percentage of surgical resection and improved median survival rate than chemotherapy alone.[305] A phase III trial on locally advanced, unresectable PC treated with pamrevlumab plus gemcitabine/nab-paclitaxel is currently ongoing.[306]

Rho-associated protein kinases, whose expression in PDAC correlates with decreased survival,[307] were addressed in KPC models by small-molecule inhibitor Fasudil. A pretreatment (called priming) of the animals with Fasudil before the administration of gemcitabine or nab-paclitaxel resulted in enhanced disease control, due to increased microvessel density and consequent improved drug delivery. This priming strategy was also proved effective when applied before the administration of a combination of gemcitabine and nab-paclitaxel, which in turn resulted in increased animal survival.[308]

Focal adhesion kinases, whose activation is correlated with the formation of an immunosuppressive TME, were targeted in KPC mouse models by another inhibitor, defacitinib, resulting in reduced tumor metastasis and infiltrated immunosuppressive myeloid populations, and eventually leading to improved survival.[309]

The contradictory results obtained so far have brought the ambivalent effect of stroma depletion into focus: on the one hand, the subsequent relief from interstitial pressure led to undeniable enhancement of therapeutic delivery in many studies, on the other hand, it might support the release of cancer cells and

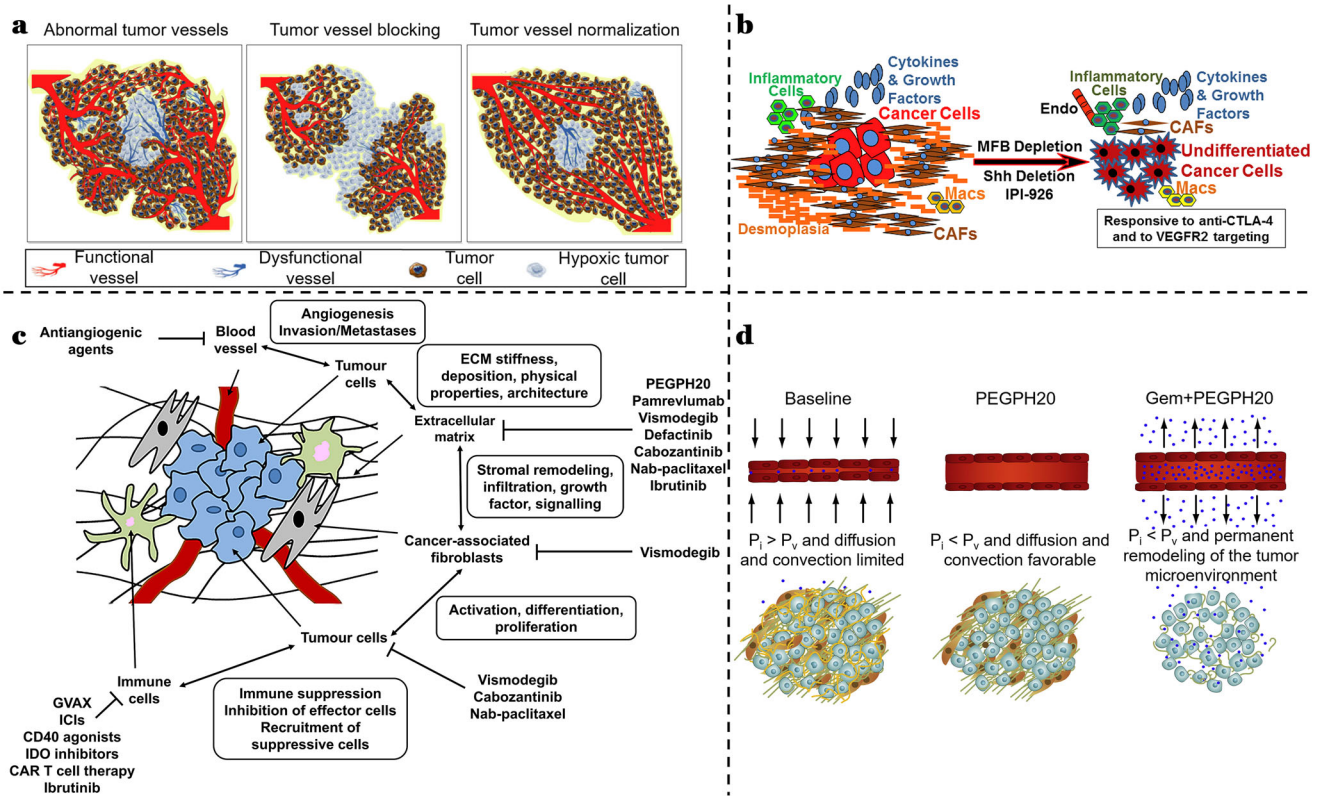

Figure 3. a) Schematic representation of tumor vessel normalization. Reproduced with permission.[288] Copyright 2015, FEBS. b) Unexpected effects of stroma targeting in PDAC, such as enhanced EMT, increased PC cell differentiation, altered immune cell infiltrate profiles. Reproduced with permission.[5] Copyright 2014, Elsevier Inc. c) Scheme of some strategies implemented to address PDAC stroma. Reproduced under terms of the CC-BY license.[311] Copyright 2021, The Authors, published by Frontiers. d) Stroma remodeling by means of enzymatic degradation or combined enzymatic and cytotoxic therapy. Reproduced under terms of the CC-BY license.[84] Copyright 2012, The Authors, published by Elsevier Inc.

thus metastasis formation.[38] Therefore, strong evidence reinforced the emerging approach of tumor stroma reprogramming and normalization as a new and more effective therapy against PDAC.[39]

The effectiveness of the reprogramming technique was illustrated in a 2014 study by Sherman et al., in which the regulation of PSCs via vitamin D receptor activation was successfully achieved. Indeed, activated stellate cells were reversed into a quiescent phenotype, leading to reduced fibrosis. TME was hence reprogrammed to a noninflammatory and physiological state; consequently, tumor delivery of gemcitabine was enhanced and resulted in improved antitumor response in GEMMs.[310]

Nevertheless, it is imperative to take stroma duplicity into careful consideration from now on while designing new combinational targeted therapies addressing PDAC TME.[311] **Figure 3** resumes some of the aforementioned studies regarding targeted and stromal therapies.

11.8. Immunotherapy

Activating T cells against cancer is the cardinal principle of checkpoint inhibitors, a class of recently approved immunotherapy drugs that aim at disrupting the signals sent by cancer cells to evade patrolling T cells.[312] T cells are a type of lymphocyte that play a crucial role in the adaptive immune response: through cytokines as messenger molecules, they send chemical instruction to the immune system in order to elicit its response.

In brief, when a foreign antigen in a peripheral lymphoid organ is displayed on the surface of antigen-presenting cells (APCs, like dendritic cells, macrophages, B cells) by class II major histocompatibility complex (MHC) molecules, naïve helper T cells recognize it and hence activate. As a consequence, they secrete the cytokine IL-2 and make high affinity IL-2 receptors, whose mutual binding causes their proliferation and differentiation into effector T_H1 and T_H2 cells, depending on the cytokines produced by APCs at the site of infection. T_H1 cells secrete interferon-gamma (IFN-γ) and TNF-α, display the costimulatory protein CD40 ligand, migrate to the site of infection, and activate macrophages. On the other hand, effector T_H2 cells remain in the lymphoid organ, secrete interleukins IL-4, IL-5, IL-10, IL-13, and help the activation of B cells to produce antibodies.[313] T_H1 cells can also help activate cytotoxic T cells, which provide protection against intracellular pathogens by making infected target cells kill themselves by apoptosis. Cytotoxic T cells are activated into effector cells by APC, but in this case through class I MHC proteins. This distinction is crucial, since each class of T cells expresses a coreceptor able to recognize an invariant part of the appropriate class of MHC protein, in order not to misdirect the cytotoxic and helper functions. Cytotoxic T cells express CD8

coreceptor, which binds to class I MHC proteins, while helper T cells express CD4, which recognizes class II MHC proteins.[314] Finally, T-cell activation is controlled by negative feedback. A cell surface protein called cytotoxic T-lymphocyte associated protein 4 (CTLA-4), expressed during T-cell activation, binds to B7 proteins on the APCs. This way, it acts as a CD28 antagonist and suppresses one of the two signals necessary to activate the differentiation of helper T cells, thus inhibiting their action.[313]

The major breakthrough of immune checkpoint inhibition (ICI) immunotherapy consists of addressing the immune cells that inhibit cytotoxic T-cell activity rather than cancer cells, by blocking immune checkpoints like CTLA-4, programmed cell death protein 1 (PD-1), or its ligand programmed death-ligand 1 (PD-L1).[314]

This therapeutic approach has so far offered many advantages in terms of treatment reproducibility and stability, since immune checkpoints represent a stationary target while cancer cells possess a mutational status that varies over time and within the single lesion.[315] Furthermore, it has led to a complete view of cancer by highlighting the role of the immune environment or the tumor mutational burden,[316] whose crucial contribution to immunotherapy was from that point taken into account. ICI immunotherapy was proved to induce delayed tumor responses at the cost of an initial increase in size, therefore new guidelines for evaluation criteria in the immune response evaluation criteria in solid tumors (RECIST)[317] evaluation system had to be incorporated;[318] it introduced a whole new spectrum of related adverse events[314] and caused long-term remissions in spite of therapy interruptions in some patients with melanoma.[319]

Despite representing a major step forward in the context of cancer treatment, ICI is still far from being adaptable to all kind of tumors: in fact, PDAC does not respond as well as other cancer types to this therapeutic option. The most plausible hypotheses to this atypical behavior are to be found in cancer-cell-intrinsic mechanisms and in the immunosuppressive role of TME.[320] Another highlighted immunological avoidance mechanism carried out by PDAC is autophagy: selective lysosomal degradation leads to a reduced expression of class I MHC, which finally results in immune evasion.[321]

Indeed, in a study by Clark et al., since early stages of preinvasive lesions, immunosuppressive cells such as TAMs, MDSCs, and regulatory T cells (T_{reg} cells) were found to persist in the TME, while no sign of effector T-cell activation was detected in GEM models. The authors suggested that this premature invasion could undermine tumor immunity from the very beginning of carcinogenesis.[322]

Moreover, PD-1, usually overexpressed in other tumors and whose binding to its ligand PD-L1 prevents autoimmunity, is nearly absent from PCs due to the lack of T-cell infiltration.[323] For this reason, current PD1/PD-L1 treatments were proved rather ineffective in preclinical studies and were not translated into clinical trials.[27]

CTLA-4, a coinhibitory molecule expressed by T_{reg} cells, was targeted by antibody monotherapy but the treatment proved to be ineffective in pancreatic cancer, probably due to another immune checkpoint called V-domain immunoglobulin-containing suppressor of T-cell activation (VISTA).[324] In fact, its expression on TAMs allows them to bypass single-agent anti-CTLA-4 therapy. In view of this, novel anti-VISTA antibodies, now under a terminated clinical trial,[325] might be able to disrupt the established but so far unsuccessful immune checkpoint therapies applied to pancreatic cancer.

Alternatively, pancreatic cancer antigens such as CEA, MUC-1, and MUC-4, shared by the majority of pancreatic cancers, could be used as vaccines to activate APC and the consequent immune response.[28,326] In addition to that, whole cancer-cell-based, peptide-based (like those including mutated KRAS peptide products)[327] and multipeptide vaccines administered after surgical resection[328] have shown very encouraging results too and must be further studied in view of combinatory therapies.[26]

GVAX, a whole-cell vaccine aimed at inducing the expression of proinflammatory cytokine granulocyte monocyte-colony stimulating factor in pancreatic cancer cells, was combined with anti-PD-1 therapy and showed a promising synergistic effect in preclinical models of PC.[329] A clinical trial including GVAX and other immunotherapy combinations is currently ongoing on patients with surgically resectable PDAC.[330]

Indeed, due to the therapeutic impermeability provided by pancreatic cancer TME, traditional immunotherapies have been progressively dismissed in favor of combination approaches.[320] Their strategy consists of reprogramming TME first, in order to eventually allow immunotherapy to fulfil its potential.[27]

As a matter of fact, different clinical trials are now exploring these innovative approaches: genetic or pharmacological inhibition of autophagy synergized with anti-PD1 and anti-CTLA-4 antibodies;[321] colony-stimulating factor 1 receptor (CSF-1R) blockade on TAMs and MDSCs to improve antitumor immunity, in combination with ICI;[331,332] CXCL12/CXCR4 targeting with antagonist plerixafor to investigate the tumor-stromal crosstalk, combined with ICI to reverse immunosuppression;[333] CD40 agonist mAb treatment in conjunction with chemotherapy to reduce T_{reg} and increase infiltrated $CD8^+$ T cells.[334]

Notably, agonist CD40 mAbs were proved to enhance the efficacy of cancer vaccines[335] and to be well-suited to combination therapies involving anti-CTLA-4 and anti-PD-1 mAbs[336] in KPC models. For these reasons, they were then proposed in combination with chemotherapy.[337] The sequence and timing of treatment were shown to play a crucial role in determining the outcome of the therapy, as well as the choice of the most appropriate drug; other major issues that emerged throughout preclinical studies were macrophage activation and re-education rather than depletion.[338] The best combination therapy so far involves gemcitabine, nab-paclitaxel, CD40 mAb, PD-1/PD-L1 mAb, and CTLA-4 mAb and was proposed in a 2015 study,[207] which proved that this combo was able to successfully overcome PDAC resistance to PD-1 and CTLA-4 blockade, improving survival in GEMM mice. Being mostly composed by FDA (Food and Drug Administration)-approved reagents, the study was easily translated into clinical trials and offered initial promising results on metastatic PC patients.[339] According to a recent review on the topic, the efficacy of therapies which involve agonist CD40 mAbs is manifested and supported by evidence of immune activation[340,341] and should be further explored, almost exclusively in combination with chemotherapy, radiotherapy, and immunotherapy due to the exceptional synergistic potential emerged throughout the years.[342] Therefore, PDAC represents a particularly promising niche for future CD40 applications and its TME is the ideal testing ground for combined

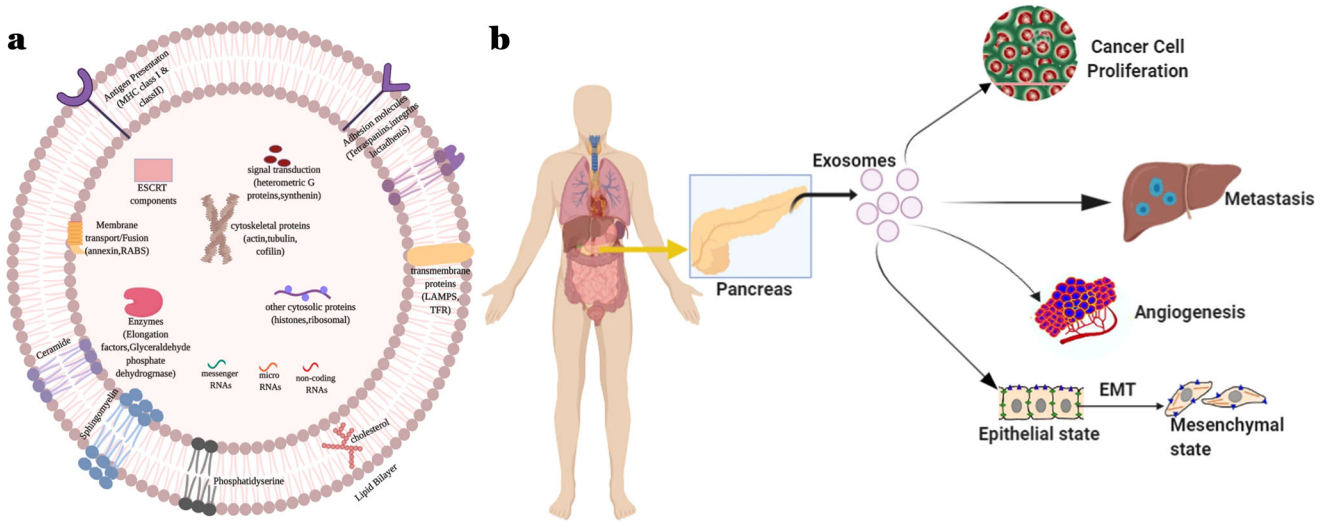

Figure 4. a) Schematic representation of the components of exosomes. b) Roles of exosomes in PDAC progression: cancer cell proliferation, metastasis, angiogenesis, EMT. Reproduced under terms of the CC-BY license.[365] Copyright 2020, The Authors, published by Springer Nature.

therapies aimed at eliciting a robust immune response, otherwise alarmingly absent.

11.9. Exosomes in PDAC

Extracellular vesicles, namely lipid bilayer particles naturally released by cells and unable to replicate,[343] are classified into three main groups[344] among which nanosized exosomes have recently emerged for their roles in cancers. In fact, they act as intermediaries of tumor progression and metastasis formation[345] and as mediators of immune regulation of lymphoid and myeloid cells in cancer.[346] Moreover, they are innate biological carriers which can be exploited as delivery nanosystems, since they are able to cross the main biological barriers while avoiding lysosomal degradation[347] and they possess unique intrinsic targeting activity toward the tumor site.[348]

Their nontoxic and nonimmunogenic nature makes them preferable to synthetic liposome formulations as vehicles for chemotherapy and immunotherapy drugs, which result well protected and shielded from degradation. Another major advantage is that they can be produced using cells derived from the patients, so as to avoid any immune reaction.[349] A recent review highlighted the existing controversy on the most suitable cell types to be used as exosome donors, since those deriving from cancer cells have generally provoked undesired effects and showed inability to stimulate the immune system. Conversely, immune-cell-derived exosomes (and specifically DC-derived ones) have been successfully applied in cancer vaccines and therefore considered as a particular form of immunotherapy.[350] Besides, they were proved to overcome DC-based immunotherapy issues and to possess immunostimulatory abilities which have been extensively explored in different preclinical and clinical studies on exosome-based cancer vaccines. The most common strategy consists of stimulating DCs with cancer peptides, in order to produce exosomes carrying specific antigens able to induce an immune response.[351]

Interesting applications cited in the abovementioned review include I) immune checkpoint blockade therapy combined with exosomes, and more broadly EVs (extracellular vesicles), carrying silencing immunosuppressive genes via siRNA (short interfering RNA); II) cell-free cancer vaccines based on tumor-derived exosomes, containing or functionalized with antigens able to induce a T-cell-dependent antitumor response;[352] III) cancer vaccines based on exosome-pulsed DCs;[353] IV) EV vaccines derived from modified cancer cells.[354]

This last approach was echoed in a study in which mesenchymal stromal donor cells were engineered to produce EVs already loaded with paclitaxel, then used as drug delivery systems to treat a human pancreatic adenocarcinoma cell line.[355] Another similar experiment involved a melanoma cell line, engineered to produce EVs loaded with survivin T34A and gemcitabine, whose synergy produced a strong cytotoxic effect when administered to PDAC cells.[356] DCs were loaded with tumor exosome (TEX) and injected in pancreatic-cancer-bearing mice in another study by Xiao et al. The aim of this work was to prove the improvement made by drugs affecting MDSCs (specifically ATRA, namely all-trans retinoic acid, sunitinib, or gemcitabine) to DC–TEX vaccination. This goal was actually achieved and evinced by a higher activation of T cells in the tumor and a longer survival time.[357]

Exosomes play a crucial role in the PDAC tumor environment (**Figure 4**), participating in tumorigenesis and tumor progression,[358] traveling from the primary tumor location and inducing premetastatic niche formation in distant organs,[359] and favoring cell-to-cell communication mediated by exosomal microRNA (miRNA being a small single-stranded noncoding RNA molecule able to silence or degrade RNA at a post-transcriptional level). Indeed, several studies reported their impact on the TME: I) exosomes derived from pancreatic cancer cells were proved to induce M2 macrophage polarization to promote metastases;[360] II) on the contrary, NK cells were observed to produce exosomes whose miRNA cargo was able to reduce cancer progression in vitro and in vivo by targeting

IL-26;[361] III) CAF-derived exosomal miRNA was shown to contribute to gemcitabine resistance and to be transferred to cancer cells via exosomes, and therefore preventing this transfer and addressing the involved target gene (TP53INP1, tumor protein p53-inducible nuclear protein 1) was suggested to reverse chemoresistance;[362] IV) exosomes derived from PSCs and cultured with Gem-sensitive BxPC-3 and PANC-1 cells could impart them Gem resistance by means of miRNA transfection;[363] V) furthermore, PSC-derived exosomal miRNA was proved to promote EMT in pancreatic cancer cells through the enhancement of different signaling pathways.[364]

In addition to that, exosomes have been considered as potential biomarkers of PDAC.[366] In fact, they can be isolated from different body fluids and their membrane is able to protect diagnostic molecules at their core, thus overcoming the limitations of traditional serum tumor markers.[367,368]

Their employment as drug carriers has also been extensively studied,[369] due to the low toxicity, high biocompatibility, and ability to penetrate the blood–tissue barrier to efficiently deliver cargoes.[370] For these reasons, miRNA and siRNA (a class of double-stranded RNA able to interfere with the expression of specific genes) capable of blocking some of the abnormal signaling pathways in PDAC cells were loaded in engineered exosomes: a 2017 study successfully delivered mesenchymal-stem-cell-derived exosomes loaded with siRNA molecules to immunocompetent mice models, targeting oncogenic KRAS better than previously achieved with liposome carriers and increasing OS by cancer suppression.[371] This promising outcome paved the way for a phase-I clinical trial in patients with $KRAS^{G12D}$ mutations (NCT03608631).[372]

Finally, the application of exosomes to disrupt cell-to-cell communication could be the key to provoking an angiogenic regulation in PDAC, thus overcoming the failures of prior antiangiogenesis therapies.[373] Although encouraging results have been achieved with other types of tumors, the role of exosome-mediated interactions between endothelial cells and other components of pancreatic cancer must be further investigated under hypoxia, and future developments should include strategies to impart more specific targeting abilities to exosomes, in order to better direct them to PDAC tumor cells.[44]

As shown by the reported studies, once exosomes are delivered to the target cells, the carried immunomodulatory agents can either stimulate antitumor responses or block immunosuppression; this latter outcome could be achieved through the inhibition of PDAC T_{reg} cells via exosome-based therapy.[43]

Evidence suggests that the most promising strategy to exploit exosomes' potential in PDAC might be once more a combination therapy involving the use of chemotherapy, to effectively eliminate cancer cells while minimizing side effects, with the final aim of facilitating a subsequent surgical removal.

Nowadays, the main drawbacks to the clinical use of exosomes are related to technological issues rather than safety ones: a thorough study and a following standardization of purification processes is urgently needed to eventually achieve large-scale productions, which however would be still very expensive and subjected to strict controls in order to receive pharmaceutical approval. Nevertheless, the development of re-engineered exosomes able to mimic the pivotal features of the natural ones might represent a challenging alternative route to follow, which could lead to new useful critical insights about these promising tools and their application on PDAC.[343]

11.10. Neoadjuvant Therapy

Since recurrence rates after surgery are very high,[374] it is now well established from a variety of studies that there is an urgent need of preoperative and postoperative therapies to improve long-term survival[375] in patients affected by PDAC.

Preoperative or neoadjuvant therapy, mainly consisting of the aforementioned combination regimens, is a strategy meant to I) improve the likelihood of complete macroscopic and microscopic resection of localized PDAC, II) while treating potential micrometastases not yet visible at the time of diagnosis,[376,377] III) penetrate neoplastic tissues not altered by the inflammation and fibrosis typically induced by surgery,[378] IV) cause tumor shrinking, V) reduce nodal involvement,[379] and VI) result in increased radical resection rates and superior OS in borderline resectable tumors.[380,381]

Examples of postoperative therapies mainly consist of chemoradiation[382] and second-line drug administrations,[223,383] among which the combination gemcitabine–capecitabine emerged as a new possible benchmark therapy over gemcitabine alone,[384] and was recommended by the American Society of Clinical Oncology in the Clinical Practice Guideline for potentially curable PC[385] updated in 2017.

Although it has been shown that neoadjuvant treatments can be safely used preoperatively in PC patients without negative effects on their recovery,[11] several related limitations have been raised. In particular, retrospective studies published on the subject have remarked the need to further address the topic with additional randomized controlled trials, some of which are currently ongoing.[386–389] New evidences suggest that the main role of neoadjuvant therapies has shifted over time from tumor shrinkage to micrometastases control,[390] but fundamental questions in this regard remain unanswered. These limits thus preclude at present the establishment of a gold standard for neoadjuvant treatments.

The present lack of a standardized approach is the underlying issue in a retrospective review of the National Cancer Database, at present the most up-to-date piece of literature regarding total neoadjuvant therapy (TNT) for PDAC.[391] Barrak et al. started defining TNT as the administration of both chemotherapy and chemoradiation before definitive resection. They observed the rates of pathologic complete response (pCR), namely tumor downstaging to a pathological stage 0, in the patients involved in the study.

Drawing on an extensive range of sources through the latest studies on neoadjuvant therapy, the authors sorted the advantages of identifying aggressive tumors, to avoid inappropriate surgery,[16] and of completing multimodal treatments before resection.[392] Furthermore, they reported the improved OS of patients treated with TNT approaches based on FOLFIRINOX and chemoradiation[393,394] and those involving multimodal chemotherapy followed by chemoradiation.[395] Finally, they concluded that total neoadjuvant therapy could be associated with improved pCR and thus selected as the treatment of choice for patients with stage II pancreatic cancer. Nevertheless, they

suggested more detailed investigations on vascular grade and toxicity data in future guidelines.[391]

In view of what has been mentioned so far, the implementation of a personalized treatment to better direct neoadjuvant therapy might represent the keystone to effectively fight PC. This can be achieved by exploiting precision medicine and molecular prognostic biomarkers[396] to create subsets of selected patients who could benefit from tailored therapy and thus improve their survival outcomes.[397]

11.11. Radiation Therapy

Radiotherapy (RT) failed in proving its superiority over chemotherapy in terms of survival, so the two approaches were combined together and as a matter of fact, they showed more encouraging results when proposed as a neoadjuvant therapy before surgery.[398,399] In particular, gemcitabine proved to be the drug whose combination with radiation led to the best results with respect to other chemotherapeutics.[400] This trend was further confirmed by means of a more recent study concerning resectable or borderline resectable PDAC tumors, which claimed that preoperative chemoradiotherapy effect on secondary end points such as disease-free survival (DFS), distant metastasis-free interval, and resection rate was superior to postoperative adjuvant chemotherapy, despite an absence of OS benefits.[10]

A 2017 study was the first to investigate the outcome of chemotherapy and RT treatments (in brief, postoperative radiotherapy (PORT)) administered after microscopically positive margin (R1) resection surgery. Park et al. noted that PORT was associated with a favorable survival outcome, confirming previously reported literature evidences, and that adjuvant chemotherapy was a prognostic factor for OS and DFS,[382] thus confirming the efficacy of the synergistic effect of the postoperative therapy.

In spite of previous discouraging results in terms of survival benefit, neoadjuvant radiation in addition to multiagent chemotherapy also suggested increased rates of tumor downstaging and R0 resection rates.[401,402] This combination was hence explored in a recent study by Vidri et al., who hypothesized a positive effect of preoperative radiotherapy on OS and pathological response in the context of a multimodal treatment. Nevertheless, although increased rate of R0 resection and improved outcomes like downstaging were achieved, no effect on OS was pointed out.[403]

Given the conflicting results concerning radiotherapy, a 2019 review by Hall and Goodman concluded that, in view of the continuous transformations and advances of radiation therapy like real-time MR guidance,[404] this technology is likely to offer enormous advantages to PDAC treatment, especially in the context of neoadjuvant therapy in borderline resectable tumors, provided it is robustly evaluated and proven by future clinical trials.[405]

11.12. Local Ablative Therapies

The most frequently applied local therapies for unresectable pancreatic tumors are radiofrequency ablation (RFA), irreversible electroporation (IRE), stereotactic body radiation therapy (SBRT), and high intensity focused ultrasound (HIFU). However, these local ablative therapies suffer from the lack of randomized controlled trials and the absence of univocally established procedures. Indeed, many studies have been published on the subject throughout the years with heavily biased and overall sparse results.[25]

RFA and IRE belong to the thermal and nonthermal ablation techniques, respectively. The former is based on the generation of high local temperatures through the application of high-frequency alternating current conducted by one or more needle electrode. The needle is introduced inside the tumor under ultrasound (US) or CT guidance with laparotomy, percutaneously or using an endoscopic approach. Its effect is to provoke coagulative necrosis, protein denaturation, and irreversible damage to the cells of the neoplastic tissue.[23]

IRE has been more recently applied to locally advanced pancreatic cancer and is ought to preserve surrounding structures, since it does not produce significant thermal energy.[406] It achieves cell membrane disruption by applying around the tumor tissue at least two needles pulsing short high-voltage direct current. Such current, whose rate is synchronized to the heart rate for safety reasons, causes the irreversible permeabilization of the lipid bilayer with consequent disruption of intracellular homeostasis and eventual activation of apoptotic pathways.[23,407] The whole procedure takes place under general anesthesia and paralytic induction.

The two techniques have generally been applied to stage-III patients not responding to standard systemic treatments (more rarely to stage-IV ones) in combination with systemic chemotherapy with the aim of achieving local tumor control.[408,409]

The most frequently reported drawbacks after RFA are the risk of thermal injuries to adjacent structures (such as vessels and nerves), pancreatic fistulae, gastrointestinal hemorrhages, acute pancreatitis, and duodenum injury.[25,410,411] Notably, after IRE, some common complications such as pancreatitis, pneumothorax, abdominal pain, duodenal leakage, and deep vein thrombosis have been described as well.[23,412]

Another major concern is the type of imaging technique to use for follow-ups and recovery assessments of these ablative therapies. Actually CT, which is the first choice in the case of PC, has sometimes failed in correctly detecting or staging this specific tumor. In addition to that, factual evidence appears to confirm the reduction of CT diagnostic performance after therapy, and especially after these ablative treatments.[413]

RFA and IRE have shown some undeniable advantages like low morbidity, possible percutaneous application and consequent involvement of patients at high risk for surgery, limited damage to surrounding tissues, and low cost.[23] However, the absence of solid clinical studies leaves some important questions open, such as the optimal sequential combination with chemotherapy and chemoradiotherapy within the context of multimodal treatments, the choice of patients, the best technique to access the organ.[407] Moreover, the causes underlying their several adverse consequences give rise to many concerns even nowadays, and they are reported and extensively argued in recent reviews on the topic.[224,412,414]

SBRT consists of delivering high-dose radiation to the pancreas with sub-millimeter precision, in single or multiple sessions. The main challenges related to this application are the

respiratory movement of the target and the nearby presence of sensitive organs at risk. Typically, one week after the implantation of three or four gold seeds (fiducial markers) into or near the pancreatic tumor using endoscopic ultrasound guidance, a 4D CT simulation is performed during free breathing, then the scans are examined, and the motion ranges are considered by applying respiratory tracking or gating schemes. After assessing the planned target volume, the prescribed radiation dose is administered considering the maximal point dose of the surrounding organs.[415]

Over the course of the last decade, SBRT has been considered as a safe local treatment for unresectable PDAC. A 2012 study by Goyal et al. observed an increase in OS in patients involved in the treatment. It pointed out the low adverse events and excellent local control rates from radiotherapy or fiducial marker placement and suggested the use of SBRT as a palliative option for unresectable tumors to prevent or delay local recurrence. Furthermore, the application of this technique was proposed in the context of a multimodal treatment including chemotherapy.[416]

In addition to safety and feasibility encouraging results concerning SBRT, a 2015 systematic review on ablation therapies by Rombouts et al. reported promising quality-of-life outcomes for this technique.[25]

A recent research article confirmed the beneficial effects of SBRT in terms of freedom from local disease progression and OS in patients with local advanced pancreatic cancer, with minimal toxicity related to the treatment. The authors however argued that, since distant metastases represent the major pattern of failure, local control of the tumor by SBRT alone is not sufficient to avoid progression. Therefore, SBRT should be further studied in combination with chemotherapy in order to establish an optimal scheme of treatment.[415]

Overall, as reported by Ruarus et al., the literature on SBRT is heterogeneous with respect to the delivered radiation doses and fraction, making any kind of comparison to other ablative techniques hard to draw. Some major disadvantages such as the risk of complications after more than 3 months from the treatment and the maximum tolerable dose of nearby organs at risk (with consequent reduction of radiation at the borders of the tumor) must be carefully addressed in future studies.[24]

HIFU is an ultrasound-based system which causes thermal tissue destruction and is currently used against a variety of solid malignant tumors.[417] Applied to pancreatic cancer, it increases the local temperature up to 65 °C, killing tumor cells and disrupting the PC stromal barrier, potentially allowing chemotherapy delivery to the tumor.[418] Therefore, in the last 20 years, it has been successfully employed alone or in combination with chemotherapy,[25,407] generally showing promising results in terms of tumor reduction and survival rates. HIFU is now recommended for the treatment of unresectable PDAC.[419] As far as side effects are concerned, they range from absence of complications to pain, transient pancreatitis, skin burn, and subcutaneous and fat necrosis.[420]

Once again, the lack of randomization and potential selective bias of the studies on the topic represent an issue that must be overcome to elucidate the mutual influence between HIFU and chemotherapeutic drugs,[421] with the final aim of formulating new treatment schemes for future applications.

A 2020 systematic review on endoscopy-ultrasound-guided thermal ablation therapy for pancreatic cancer drew the attention on the postnecrotic infiltration or the marginal tumor zone by neutrophils, macrophages, dendritic cells, T and B lymphocytes, and natural killer cells, with consequent enhancement of the antitumor systemic immune response. The suggested reason behind this recruitment was thermal-mediated tissue damage, but the authors cautiously concluded to wait for further studies to validate this high-potential-induced secondary effect.[422] Indeed, the balance between apoptosis and necrosis following thermal ablation therapies is a crucial issue influencing the acquired immune system activation.[423] In fact, the apoptotic bodies are not capable of triggering dendritic cells, thus preventing the cascade of events that results in T-cell activation.[424] On the other hand, cells that undergo necrosis produce necrotic fragments and activate damage-associated molecular patterns (DAMPs) that cause an immune response.[425]

Nevertheless, the immune implications of ablation techniques of locally advanced PC had already been proposed in previous publications.

With respect to RFA, it was demonstrated that the accumulation of immune infiltrates mainly takes place in the peripheral or transitional zone of the tumor, namely those cells that surround the central coagulative necrosis zone and that are subjected to a steep negative temperature gradient; they receive oxygen from the increased blood flow, which in turn enhances the formation of reactive oxygen species (ROS) and favors drug accumulation.[426] Immune cell subsets were also observed in distant and untreated tumors, once again suggesting an overall immune activation provoked by RFA, and the combination of ablative techniques and topic immunotherapy was hence proposed to elicit an antitumor reaction aiming to a systemic immune response.[23]

Later, Giardino et al. observed a general activation of the adaptive response and a reduction on immunosuppression in 10 patients with locally advanced pancreatic cancer treated with RFA; despite being limited by the small sample and the open approach adopted (laparotomy), this study reported the presence of immunogenic molecules after ablation, that improved dendritic cell activation and maturation and cross-priming of T lymphocytes. A systemic reaction to RFA in terms of immunomodulation was witnessed by a marked difference between typical patterns of surgical stress or inflammation and the general detected trend toward a decrease in immunosuppressive chemokines and immune cells (which normally contribute to tumor progression). The authors concluded with the hypothesis of a prolonged immune activity even weeks after the procedure, which however should be further confirmed by studies with larger numbers of patients.[427]

RFA was also proved to reduce the levels of T_{reg} cells,[428] and higher levels of tumor-specific T cells were detected after this ablation therapy, with related improved survival in hepatocellular carcinoma patients.[429] On the other hand, an often-encountered adverse effect of RFA in liver models was distant tumor growth and development of metastases,[430] whose immunologic interpretation is nowadays being elucidated.[424]

Moreover, the first performed clinical trials proved the superior overall response rate of patients treated with IRE and NK cells,[431] the longer PFS and OS in those who received IRE and allogenic Vγ9Vδ2 T-cell infusion,[432] and the higher median OS

in patients treated with cryoablation and immunotherapy.[433] A clinical trial in course, named PANFIRE-III, is currently testing a combination of IRE, systemic anti-PD1, and intratumoral TLR-9 (Toll Like Receptor 9) agonist in metastasized PDAC patients.[434]

The discovery of immune activation following local ablative therapies has opened new horizons for PDAC treatment possibilities, paving the way for new combination therapies which could take advantage of the immunomodulation witnessed after the application of such procedures.

11.13. Light-/Ultrasound-Triggered Minimally Invasive Therapies

Among other ablative therapies based on external stimulation with an energetic source, photodynamic therapy (PDT) and sonodynamic therapy (SDT) are emerging in the context of minimally invasive treatments for cancer applications. For the application on PC, both techniques are still nowadays poorly documented, and for this reason, here reported in detail.

11.13.1. Photodynamic Therapy

An extensive recent review highlighted the salient points of photodynamic therapy reported in literature, with respect to PDAC current and future applications.[35]

PDT is a technique which selectively destroys target tissues by means of a photosensitizer (PS), previously injected to allow its accumulation in malignant tissues by passive or active targeting, exposed to a light at a certain wavelength. The effect of light absorption by the molecular PSs is an excitement from their ground state, which can rapidly drop back by emitting fluorescence or undergo intersystem crossing to an excited triplet state, whose lifetime is very long and cannot return to a ground state. As a result, PS molecules can transfer electrons to form ROS or give rise to singlet oxygen molecules ($^1O_2$) through collisional quenching.[435]

ROS effects in biological environments have been thoroughly investigated over time: they possess high reactivity and are thus responsible for redox modifications of biomolecules.[436,437] They act as intracellular signaling molecules but can also provoke cell death if overly produced. Therefore, a delicate redox homeostasis takes place in healthy cells to balance ROS production and elimination. However, many endogenous factors such as hypoxia can trigger an exaggerated generation of these radical species: their effects are an increased disease incidence, pathological dysfunctions, aging, tumorigenesis, and eventually cell death.[438–440] When generated in the tumor proximity through PDT, ROS can exert their cytotoxic action on cancerous tissues.[441]

The advantages of PDT technique are the preservation of connective tissues, mechanical integrity of critical structures,[24] and the absence of accumulating toxicity derived by ionizing radiation. A main drawback is the limited penetration in tissues of most light wavelengths used, such as red and near-infrared ones, together with possible toxicity of the PS over time. Early preclinical developments of PDT for pancreatic cancer highlighted the importance of PS and light delivery strategy selection. The first clinical trials assessed the use of verteporfin as PS, thanks of its absorption at 690 nm and rapid excretion with respect to the previously used mesotetrahydroxyphenylchlorin.[442]

After the assessment of endoscopic ultrasound (EUS)-guided PDT, disclosed in previous studies, a 2019 phase I clinical study reported on the use of porfimer-sodium-mediated EUS–PDT followed by nab-paclitaxel and gemcitabine chemotherapy in patients with locally advanced PC. The treatment proved to be safe and effective in prolonging progression-free survival rate[443] and was followed shortly after by another trial, in which porfimer sodium was replaced by verteporfin, a short half-life and FDA-approved PS.[444]

Due to the deeply hypoxic tumor microenvironment, PDAC response to PDT is typically poor and should be enhanced by means of new strategies meant to deliver or produce oxygen in situ, like the use of microbubbles[445] or the exploitation of the excessive amount of H_2O_2 produced by cancer cells.

Collectively, much of the current literature on the subject agrees on the considerable potential of PDT[446] and of its combination with chemotherapy as a pretreatment to enhance drug transport.[34,447] Other light-based approaches such as photothermal therapy (PTT)[448,449] and an ultrasound-based one, SDT,[450] have also been encouraged so far together with PDT by promising results.

11.13.2. Sonodynamic Therapy

Sonodynamic therapy is a US-based technique which consists of the simultaneous combination of low-intensity US, molecular oxygen, and a sonosensitizer, able to produce ROS.

The main advantage with respect to PDT is the greater penetration of ultrasound, which enables deep tumor treatment while avoiding tissue sensitization and skin phototoxicity.[418,451] Moreover, US is also a widely accepted safe technology in the context of clinical imaging due to its low cost, high sensitivity, and absence of ionizing radiation.[452]

When a biological medium is exposed to US, it undergoes thermal and mechanical effects. Thermal effects are those exploited with HIFU, as reported above, while the main mechanical ones are sonoporation and cavitation.

Sonoporation is namely the formation of pores in cell membranes which are usually exploited to enhance drugs' penetration, gene delivery, or nanoparticle (NP) uptake[453] inside cells.

Cavitation consists of the nucleation and growth of gaseous bubbles from gases dissolved in liquid media when exposed to ultrasound pressure waves. Typically, such gas bubbles can either rapidly collapse (inertial cavitation) or maintain their oscillatory motion (stable cavitation). The violent collapse due to inertial cavitation can produce acoustic emissions, microstreaming, jetting, and shockwaves, all leading to mechanical damages. Alternatively, it can mark the beginning of chemical reactions like ROS production from water molecules, including singlet oxygen ($^1O_2$), hydroxyl radicals ($\bullet O_2$), and superoxide anions ($O_2^{\bullet -}$).[452,454] Since their generation rate is limited and their spatial distribution is highly heterogeneous, the amount of produced ROS is typically not therapeutically effective. Therefore, the presence of sonosensitizers is needed and by now generally approved in combination with ultrasound to achieve an effective SDT.[455]

The currently available and applied sonosensitizers are either organic or inorganic. Examples of the most commonly used organic sonosensitizers are porphyrins and protoporphyrins

(such as PpIX),[456,457] hematoporphyrins, hematoporphyrin monomethyl ether, Rose Bengal (RB),[458] indocyanine green, drugs such as doxorubicin,[459] IR-780 iodide.[460] They possess excellent catalytic performances and broad-ranging optoelectronic features.[461] However, they show poor water solubility and are subjected to enzymatic degradation, therefore they are rapidly eliminated from the blood circulation with the result of not providing an adequate concentration in the tumor site.[454,462]

On the other hand, titania (TiO_2) NPs are the most employed inorganic sonosensitizers.[463] They possess chemical stability and reduced phototoxicity, which however are counterbalanced by a low ROS quantum yield and a fast recombination of electrons on their surface.

Since hypoxia is one of the hallmarks of pancreatic cancer, particular attention has been given to efficient O_2 tumor delivery with the aim of enhancing the efficacy of chemotherapy, PDT, and SDT, by direct oxygen delivery in the TME or by triggering the transformation of the overly expressed H_2O_2 in O_2 in situ.

In 2016, a combination of ultrasound, microbubble, and chemotherapy was proposed by Dimcevski et al. in a clinical trial. The treatment consisted of gemcitabine infusion followed by an ultrasound treatment during the injection of SonoVue (a contrast agent made of microbubbles stabilized by phospholipids and containing sulfur hexafluoride). The consequences of this combination therapy were an enhancement of the tolerated gemcitabine cycles, an increase of the median survival, and a decrease of the maximum tumor diameter in some of the patients involved.[464]

In the last decade, data from several studies suggest that a multimodal approach involving a combination of SDT with different techniques such as chemotherapy, starvation therapy, PTT, PDT, or immunotherapy is the most promising strategy to pursue, in the light of the documented synergistic cancer effects achieved so far. Although many applications of SDT-based combination therapies on cancers have generally provided promising results,[34,418,461,462,465–467] literature concerning pancreatic cancer is still poor and mostly limited to in vitro studies.[468,469]

12. Nanoparticle-Based Medicine and Theranostic Approaches

12.1. Nanomedicine and Nanoparticles in Cancer Treatment

As repeatedly highlighted above, the major drawbacks and limitations of currently applied treatments prevent them from being the ultimate PDAC solution, or from being applicable indistinctly to all patients. Nevertheless, promising findings in the field of nanomedicine are allowing scientists to develop more personalized therapies, with the final challenging aim of being able to meet the specific needs of each tumor in question. Nanomedicine has been described as the application of nanotechnology for medical purposes, exploiting nanomaterials for diagnosis, monitoring, and treatment of diseases.[470]

Nanomaterials, typically defined as engineered materials with at least 1D in the nanoscale range (1–100 nm),[471] possess physiochemical characteristics which differ from those of their bulk counterparts and confer them remarkable properties.[472–475] Among the plethora of currently available nanomaterials, nanoparticles have emerged, thanks to their countless therapeutic uses in cancer therapy, mainly reported for imaging and diagnostic purposes and for drug delivery applications.[31,476–478]

In this last case, their use offers various advantages: I) they are able to protect the eventual cargo from biodegradation, prolonging the circulation time and thus maximizing the chances of reaching the tumor without being cleared from the body; II) they improve the therapeutic window by providing a sustained release over time;[479] III) they can accumulate in the tumor site by both passive or active targeting; IV) they can be internalized by cancer cells to release their payload without interference of drug efflux pumps, thus reducing toxicity to other cells; V) they can simultaneously transport more than a single drug, enabling combination and multimodal therapies; V) their surface can be modified with coatings in order to avoid immune surveillance, or with ligands to actively target the tumor in question.[480–483] Finally, their ability to combine diagnosis and therapy in a single construct, referred to as theranostic, is nowadays the most promising investigated application of nanomedicine in the context of cancer treatment.[49,123]

The most commonly used and approved formulations of NPs are lipid-based, polymeric, and inorganic nanoparticles,[484–486] chosen depending on the nature of the cargoes to be delivered and to the therapeutical application they are destined to.[487] Size, shape, and surface properties of NPs are all factors hugely influencing their behavior in biological media and the subsequent reaction of the body to their presence,[488,489] and therefore must be carefully tuned and optimized.[490–492]

Different camouflage strategies, such as PEG grafting on the NP surface (which has however recently raised some concerns with respect to its immunogenicity[493]), the addition of self-markers like CD47 peptides and the coating with phospholipidic or cell-derived membranes to inhibit their phagocytosis[494–496] have hence been proposed to avoid the capture of NPs by macrophages and other phagocytes.

Once in the bloodstream, the key point of an efficient delivery is the localization into the tumor site.[481] Although most of the existing literature concerning nanoparticles considers passive targeting, and hence the EPR (Enhanced Permeability and Retention) effect,[497] as the main responsible for their accumulation in tumors, a series of experiments depicted in a 2020 study by Sindhwani et al. were carried out in order to prove the real extent of EPR in solid tumors and proved that an active transport through endothelial cells via transendothelial pathways was the main tumor accumulation mechanism shown by NPs in mouse and human models.[480]

In addition to that, passive targeting is strongly dependent on the leaky tumor vasculature adjacent to the tumor, which is limited in some types of cancers. In regards, a review by Liu et al. raised an important issue connected to drug delivery in PDAC, in which the presence of thick tumor stroma has the effect of hindering the access to the tumor site. The authors hence suggested transcytosis as the major mechanism for PDAC drug delivery.[498,499] Moreover, as previously reported by the same authors, the presence of iRGD (internalizing Arg-Gly-Asp) peptides was proved to further promote the penetration of irinotecan-loaded silicasome-based carriers in patient-derived xenograft.[500] Taken together, these findings pointed out the role of specific ligands and tumor-penetrating peptides in NP selec-

Table 1. Selected alternative nanomedicine approaches recently applied to PDAC.

Conventional treatment	Major limitations and drawbacks	Possible nanomedicine solutions
Surgery	• Surgeon- and hospital-dependent[505] • Late diagnosis, mostly when PDAC is unresectable[9]	• Modern combination therapies and neoadjuvant therapy to downstage tumors and extend surgery[12,506] • NP-based contrast agents to reduce positive resection margins[507–512] • Novel biomarkers to identify at risk populations[396,513,514]
Chemotherapy	• Limited tumor accumulation, high toxicity, significant side effects[4] • Poor selectivity toward cancer cells[515]	• Nanoparticle-based drug delivery[482] • Nanoformulations of hydrophobic[189,255] and hydrophilic[257,516] chemotherapeutic drugs • Stimuli-responsive NPs to trigger drug release[517,518] • Targeted theranostic NPs[519] • Gemcitabine combination nanotherapies[520]
Radiation therapy	• Radioresistance and damages to nearby normal tissues[521]	• NPs to radiosensitize PC cells or to protect healthy ones[522–525]
Targeted/stromal therapies	• Difficulties in successfully penetrating the stromal barrier with drugs[290] • Conflicting preclinical/clinical results[5] • Stroma depletion can also enhance the tumor[38]	• NPs to improve siRNA and miRNA distribution[526–529] • Stimuli-responsive nanoconstructs[530,531] • Active targeted drug delivery with modified specific ligands[530,531]
EVs and exosomes	• Scaling and technological issues[345] • PDAC applications limited to cells or animal models in normoxic conditions[44]	• Biomimetic nanoengineered EVs[343] • Exosomes' engineering strategies[532] • Nanotechnologies exploiting exosomes as diagnostic biomarkers[521,533] • Exosome-based nanovehicles[534,535]
Immunotherapy	• Immunosuppressive TME[536] • Poor response to ICI[320] • Early immune infiltration[537]	• NP to deliver innate immune agonists[538] or to induce immunogenic cell death[539,540] • NPs to disrupt tumor–pancreatic stem cells interplay[541,542] • NPs to target macrophages[543] • NPs to target MDSCs and T_{reg} cells[544]
Local ablative therapies	• No standard procedures[25] • Injuries to nearby systems[24] • Inadequate imaging for follow-up[413]	• Intracellular hyperthermia through NPs[545–547] • Dynamic monitoring of PC through MRI nanoprobes[548]
PDT	• Poor penetration of photosensitizers[446]	• Nanovehicles to better deliver photosensitizers[549–552] • Nanovehicles delivering oxygen to alleviate hypoxia[553,554]
SDT	• Limits of organic and inorganic sonosensitizers[451,462,555]	• NPs to protect organic sonosensitizers[451] • Surface functionalizations of inorganic sonosensitizers[556] • Gas-generating nanosystems[557]

tive accumulation, and the evidence-based importance of relying on their use by means of NP surface functionalization for efficient active targeting,[501,502] especially in stroma-rich tumors like PDAC.[503]

12.2. Pancreatic Cancer Nanomedicine Applications

In the last decades, nanotechnology has been profusely applied to both pancreatic cancer diagnosis and therapy.[504] Thanks to the use of nanoparticle-based medicine, some of the major drawbacks of conventional therapies have been addressed, especially in the context of drug delivery. Nevertheless, chemotherapy is not the only field that could definitely benefit from nanotechnology applications. **Table 1** reports some selected nanomedicine approaches, discussed hereinafter in detail, which have already been applied to PDAC in the last decade to overcome certain limitations and drawbacks shown by conventional treatments.

The next paragraph will discuss the application of nanotechnological tools to different conventional therapeutic treatments.

12.2.1. Surgery

As far as PDAC surgery is concerned, for example, it has extensively been proved that clean resection margins are correlated with better outcomes in patients which undergo surgery, whereas late diagnoses are usually made when the tumor is not localized anymore and thus not easily resectable.[9] Thus, improvements in terms of correct staging, tumor visualization, and recurrence detection could be the key to enhance tumor response to surgery. Neoadjuvant therapy, and more generally systemic therapies administered before resection could benefit from the use of nanotechnology: I) a more efficient delivery of drugs could help tumor shrinking and favor downstaging, enabling the extension of surgery indications to a larger slice of PDAC stages;[12,506] II) moreover, NP-based contrast agents could help defining tumor margins with higher precision via medical imaging, improving the chances of achieving clean resection margins;[507–512,558] III) finally, the discovery of novel biomarkers targetable by means of nanoconstructs could help identifying at risk population for mass screening purposes, patients' stratification, and more personalized therapeutic approaches.[396,513]

12.2.2. Chemotherapy

Limited accumulation of chemotherapeutic drugs in PDAC TME, their high systemic toxicity, and their poor selectivity toward cancer cells have been overcome by nanoparticle-based drug delivery[482] and the implementation of nanocarriers able to incorporate hydrophilic[257,516] and hydrophobic[189,255] drugs, improving their circulation in the bloodstream. Moreover, their surface functionalization with targeting peptides can confer them tumor homing abilities,[519] while the use of stimuli-responsive NPs can trigger drug release once the nanocarrier is located inside the tumor,[517,518] thus providing a selective and precise delivering to cancer cells.

Inspired by Abraxane coadministration with gemcitabine, Meng et al. designed a lipid-coated mesoporous silica NP codelivering both paclitaxel and gemcitabine via intravenous (IV) injection in mice carrying subcutaneous PANC-1 xenografts. The obtained nanocarriers achieved an effective inhibition of primary tumor growth and eliminated metastatic foci outperforming the free drug counterparts, while allowing a precise ratiometric drug loading and cargo protection, thanks to the uniform surface coating provided by the lipid bilayer.[559] Liu et al. designed a mesoporous silica NP platform loaded with high-dose irinotecan and coated by a lipid bilayer (LB–MSNP), whose administration to an orthotopic KRAS-derived PDAC model in immunocompetent mice resulted in enhanced primary tumor killing and metastases reduction, along with controlled release and toxicity decrease with respect to liposomes.[560] Multifunctionalized iron oxide NPs including anti-CD47 antibody and gemcitabine were successfully tested on various primary cell cultures and PC cell lines, showing a selective drug release and an efficient induction of apoptosis with respect to the free antibody in another recent study.[561]

To overcome gemcitabine rapid clearance and resistance, various nanotechnologies have been applied to shield it from metabolic inactivation; one of these strategies, as previously mentioned, involved the combination of gemcitabine with the paclitaxel nanoformulation Abraxane.[189] Previous works regarding the design and construction of gemcitabine prodrug nanoparticles have provided superior antitumor activity against PDAC, such as stereocomplex prodrugs of oligo(lactic acid)*n*-gemcitabine in poly(ethylene glycol)-*block*-poly(D,L-lactic acid) micelles,[562] whose improved physical stability resulted in enhanced antitumor efficacy on PANC-1 pancreatic cancer cells, and self-assembled gemcitabine prodrug nanoparticles coated with PEG and decorated with a target peptide,[563] whose administration in xenograft models of human PDAC inhibited tumor progression. In other studies, gemcitabine or its derivatives were directly entrapped in nanoparticles, achieving both high and prolonged accumulation in the tumor tissue compared with the free drug. Various gemcitabine combination nanotherapies applied to PDAC exploiting micelles, liposomes, metal-based nanoparticles, and hydrogels have already been reported in detail in a 2019 review;[520] hereinafter, some additional applications, focused on multimodal approaches, are briefly described. Das et al. used lipid coated calcium phosphate NPs to entrap gemcitabine monophosphate and triggered a strong antitumor response by delivering them to a treatment refractory PDAC model, bypassing the typical hallmarks of gemcitabine chemoresistance;[564] Chen et al. coloaded phosphorylated gemcitabine with paclitaxel in a micelle (2-propionic-3-methylmaleic anhydride, CDM) coated with PEG and decorated with a stroma targeting peptide (AE105), which could take advantage of the distinctive low pH of PDAC TME by means of a pH-triggered disintegration and the resulting dual drug release[565] (**Figure 5**a); Han et al. developed gemcitabine nanovectors based on CdSe/ZnS quantum dots conjugated with MMP-9 (matrix metallopeptidase 9) detachable PEG and a targeting ligand (cycloRGD), hence able to achieve prolonged blood circulation and enhanced tumor internalization[566] (Figure 5c). A study by Zhao et al. reported the employment of biocompatible lipid–polymer hybrid NPs for the codelivery of hypoxia-inducible factor 1α (HIFα)siRNA and gemcitabine, further coated by a lipid bilayer to avoid aggregation as well as Gem leakage, tested on subcutaneous and orthotopic tumor models; results showed a suppression of HIFα expression and the inhibition of tumor metastases, proving the efficacy of the combination therapy strategy[526] (Figure 5b). Uz et al. designed temperature and pH responsive polymeric nanoscale devices to codeliver microRNA (miR-345) and gemcitabine to PC cells and to mice carrying xenograft tumors; their documented effect was a downregulation of SHH signaling, which in turn enhanced Gem perfusion, and a significant decrease in metastasis.[567] Khan et al. reported a multifunctional super-paramagnetic iron oxide nanoparticle formulation of curcumin combined with gemcitabine administration, applied to HPAF-II and PANC-1 cells and to an orthotopic mouse model. They actively targeted the TME and facilitated gemcitabine uptake by inhibiting the activation of SHH signaling, reducing tumor growth and metastasis, and inducing changes in cell stiffness.[568] Gemcitabine loaded, PEGylated gold nanoparticles were employed by Elechalawar et al. as delivery systems to specifically inhibit PC cells and PSC proliferation in vitro, with the use of the anti-EGFR antibody cetuximab (C225/C) as a targeting agent.[569]

12.2.3. Radiation Therapy

In order to avoid the huge side effects related to RT, radiation protectants for normal tissues and radiation sensitizers to enhance the damage induced in cancer cells have been developed.[570] Cerium oxide nanoparticles (CONPs) have typically been employed as adjuvants in RT due to their antioxidant properties, but their pro-oxidant behavior has recently been highlighted as well. In this regard, a study by Wason et al. reported the effect of CONP treatment prior to RT in potentiating pancreatic cancer cell apoptosis, while protecting normal tissues depending on the environmental acidity.[522] Other types of NPs have successively been employed for the same purpose: titanium peroxide nanoparticles were shown to be promising agents for ROS production upon X-ray irradiation in a mouse model using engrafted human PC cells,[524] while gold nanoparticles (Au NPs) were incorporated into microgels to create stealth constructs acting as radiosensitizers in a mouse model of PC, as described in a 2019 study.[523]

12.2.4. Targeted/Stromal Therapies

As previously mentioned, the abnormal vascular structure of this kind of tumors is characterized by tortuous, saccular chaotically

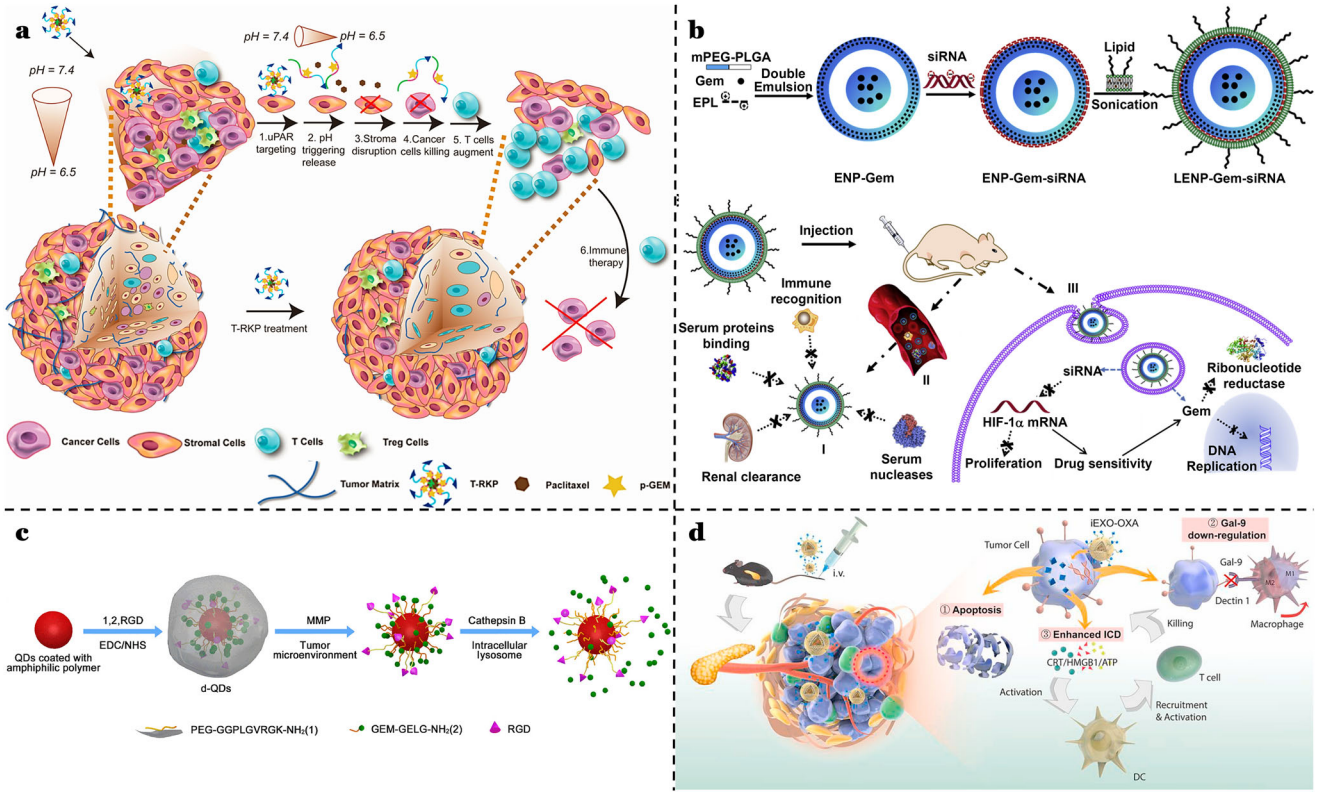

Figure 5. a) Mechanism of TME targeting strategy and pH-triggered micelle disintegration, with consequent dual drug release. Reproduced with permission.[565] Copyright 2019, American Chemical Society. b) Schematic illustration of the fabrication and the tumor cell uptake of lipid–polymer hybrid NPs codelivering (HIFα)siRNA and gemcitabine. Reproduced with permission.[526] Copyright 2014, Elsevier Ltd. c) Scheme of the preparation of dual enzymatic reaction-assisted Gem nanovectors, able to achieve multistage tumor targeting and drug release. Reproduced with permission.[566] Copyright 2017, American Chemical Society. d) Mechanism of immunotherapy enhancement by PC-targeting exosomes, resulting in reversal of immunosuppressive M2-TAMs. Reproduced with permission.[532] Copyright 2020, Elsevier Ltd.

organized vessels, which lead to heterogeneous blood perfusion, high IFP, vascular compression, and hypoxia. Taken together, these conditions hinder an effective drug penetration into PDAC, even in the presence of stromal and targeted therapies.[290] Stroma targeting via nanoconstructs relies on different delivery mechanisms than those used by conventional nonencapsulated drugs, and hence has been proposed to improve PDAC penetration and treatment.[38] For this purpose, strategies as specific ligands to enhance active targeting and smart nanoparticles designed to respond to environmental or external stimuli have been implemented.[530,531] Another novel approach consists of the simultaneous targeting of stromal and tumor compartments, by means of multifunctional nanoconstructs,[571] in order to alleviate TME stiffness and favor the local delivery of chemotherapeutic drugs. Acting on tumor vasculature was also proved effective in a study by Meng et al., which exploited a MSNP targeting a molecular pathway involved in pericyte recruitment as the first step of an engineered approach meant to enhance the penetrance of a nanoformulation of gemcitabine.[572]

As recently pointed out by an up-to-date review on the topic, CAFs still represent an underexplored target for PDAC treatment, although their central role in the multistep processes of tumor initiation, progression, invasion, and metastases has repeatedly been pointed out.[573] Nevertheless, in the context of stromal modulation approaches, some nanomedicine applications targeting CAFs have emerged over the last decade and their resulting tumor tissue normalization has led to promising improvements in PDAC, especially in terms of tumor progression,[574] growth,[575] and immune response.[571,576] These studies had the double effect of improving PDAC therapy by enhancing drug delivery into the tumor site while elucidating the complex and dual role of CAFs in desmoplasia.[577] They involved, among many other mechanisms,[503] the use of polymeric micelle-based nanoformulations to inhibit SHH pathway,[576] nab-paclitaxel to target SPARC glycoprotein,[578] miRNA inhibitors to reprogram CAFs,[579] and anti-microRNA as part of peptide-based nanocomplexes aimed at inhibiting PSC differentiation into CAFs.[580]

PSCs, as stated above, are the main responsible for the increased ECM production which ultimately provokes reduced intratumoral perfusion and nanotherapeutic delivery.[581] To overcome the physical barrier provided by the desmoplastic tissue, nanocarriers targeting the stroma have been proposed to enhance drug penetration.[582] As an example, a two-step sequential delivery strategy employing at first liposomes loaded with a nitric oxide donor (aimed at inhibiting the production of the dense stroma) and then gemcitabine-loaded liposomes resulted in enhanced Gem delivery and tumor growth inhibition.[583] Another recent study applied to orthotopic xenograft mouse models of

PDAC involved an antistromal pretreatment with chloroquine-loaded poly(lactic-*co*-glycolic acid) NPs targeting PSCs prior to gemcitabine administration, whose effect was the restraint of tumor progression and a reduction of PSC activation.[584]

These works strongly support the idea of an efficient stroma modulation as a necessary first step to perform in order to normalize the TME and therefore allow a better intratumoral therapy administration. Furthermore, they highlight the advantages of adopting novel nanomedicine approaches to perform multistep and targeted therapies, able to preliminarily improve the TME before any further curative treatment.

12.2.5. Exosomes

Exosomes' nanoengineering has been recently proposed as a promising way to obtain biomimetic as well as highly tunable vehicles for cancer therapies, and some attempts have already been applied to pancreatic cancer therapy in the context of diagnosis, drug delivery, and immunotherapy.

A 2021 study by Choi et al.[533] proposed a technology exploiting lectin-conjugated Janus nanoparticles, able to detect pancreatic-cancer-cell-derived exosomes with high affinity in a microfluidic device, thanks to the lectin–glycan interaction; Pu et al.[521] used a tethered cation lipoplex nanoparticle biochip to examine the level of exosomal microRNA-21 as a biomarker for PC. Both these approaches were noninvasive ones, based on blood and plasma samples and meant for diagnostic purposes. The incorporation of galectin-9 siRNA by means of electroporation and the surface modification with oxaliplatin of bone marrow mesenchymal stem cell exosomes was proposed by Zhou et al.[532] to target PC and to elicit antitumor immunity through immunosuppressive reversal of M2-like tumor associated macrophages (Figure 5d). Exosomes' engineering by membrane fusion with liposomes using the freeze–thaw method was reported in a study by Sato et al. to obtain hybrid tunable nanocarriers for drug delivery.[585] Finally, an immunotherapy nanoparticle-based approach was proposed to deliver plasmid DNA to pancreatic cancer cells via hyaluronic acid–poly(ethylene imine)/hyaluronic acid–poly(ethylene glycol) self-assembling nanoparticle-based nonviral vectors, in order to modulate their exosomal cargo to achieve a macrophage reprogramming.[586] Exosomes are usually the object rather than the active subject of the aforementioned pancreatic cancer nanomedicine approaches, which instead are mainly focused on influencing their composition by engineering the cells of origin or on targeting them with nanoparticles to improve diagnosis. A 2016 study on murine hepatoma reported dual-functional exosome-based super-paramagnetic nanoparticle cluster used as drug delivery systems.[534] Some years later, Liu et al. designed a functionalized smart nano-sonosensitizer by loading a porphyrin sensitizer, with therapeutic and imaging functions, on both the surface and in the core of homotypic tumor-cell-derived exosomes, for US-responsive controlled release and enhanced SDT tested on various tumor models;[535] nevertheless, literature regarding pancreatic cancer applications is currently missing. A promising multimodal therapy could involve exosomes as biomimetic coatings of inorganic nanoparticles, to enhance their biostability while imparting them specific tumor homing capabilities, thanks to the surface proteins exposed by their membranes.[587]

12.2.6. Photodynamic Therapy

The use of nanoparticles has been proposed to overcome some limitations of photodynamic and sonodynamic therapies when applied to pancreatic cancer. The delivery of photosensitizers to the TME could be facilitated by encapsulating them into nanocarriers.[549,550] Even more simplified, semiconductor nanoparticles able to be photoexcited by light to produce ROS can be used to carry PDT, taking care to coat such inorganic particles with a biomimetic lipid bilayer to promote stability in biological media and rapid cell internalization.[588] Furthermore, nanoparticles or nanorods could be exploited to produce oxygen in situ, thus alleviating hypoxia.[553,554] A recent review reported current clinical studies concerning PDT against PC, highlighting the main advantages of its use: better tumor targeting, improved quantum yield, and hypoxia relief.[551] Herein, those involving the use of photosensitizer nanoparticles in the context of multimodal treatments are briefly described. In a 2016 study, the use of nanophotoactivable liposomes codelivering the photocytotoxic chromophore benzoporphyrin derivative monoacid A and the anti-VEGF monoclonal antibody bevacizumab was proved successful when applied to ASPC-1 cells and to a subcutaneous mouse model of PDAC, since their combination enhanced cytotoxicity and tumor reduction, thanks to the simultaneous spatiotemporal delivery of both agents.[589] A multi-inhibitor nanoliposome was then proposed by Spring et al. to impart light-induced phototoxicity combined with a spatiotemporal-synchronized release of a multimolecular inhibitor with antiangiogenic activity, in order to avoid treatment escape signaling pathways while suppressing tumor regrowth in two mouse models of PDAC.[590] A year later, a gold-nanocluster-based platform for PTT/PDT composed by a PTT-carrier gold nanocluster, a targeting peptide, a PDT therapy prodrug, and an imaging agent was proposed as an innovative strategy to enhance PDAC treatment (**Figure 6**a), showing high tumor uptake and accumulation.[449] A chemo-photodynamic combination therapy was applied in a study by Zhang et al. to alleviate PDAC hypoxia by using Fe(III)-complexed porous coordination network encapsulating PTX NPs. The obtained nanoconstruct, tested on both cell lines and animal models of PC, was able to release drug in response to laser irradiation and pH changes and to convert H_2O_2 in the tumor site to O_2, regulating hypoxia; moreover, it was suitable as MRI contrast agent.[591] Another recent multimodal strategy included oxygen-delivering polyfluorocarbon nanovehicles loaded with photodynamic $DiIC_{18}$(5)-DS (DiD, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine-5,5'-Disulfonic Acid) and chemo-immunomodulatory gemcitabine prodrug (Figure 6b), which exhibited preferential tumor accumulation and ROS production upon laser irradiation, with consequent antitumor immune responses in a PANC-02-induced pancreatic cancer model.[592]

12.2.7. Sonodynamic Therapy

Due to the nature of its TME, PDAC poses many obstacles to the effective delivery of sonosensitizers and to a successful

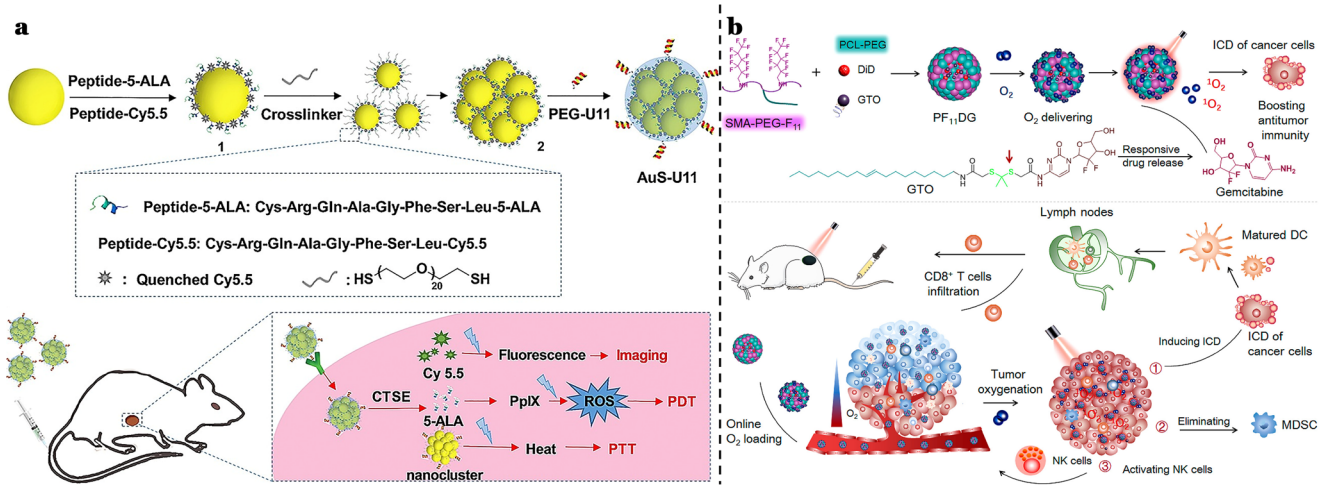

Figure 6. a) Preparation and mechanism of action of gold-nanocluster-based platforms for synergistic PTT/PDT. Reproduced with permission.[449] Copyright 2017, Elsevier Ltd. b) Schematization of oxygen-delivering polyfluorocarbon nanovehicles preparation and their ROS production and Gem release upon laser irradiation. Reproduced with permission.[592] Copyright 2021, American Chemical Society.

application of SDT. The limitations of organic sonosensitizers can be mitigated by their conjugation with nanosized particles able to deliver them to the target regions, thus improving their accumulation and efficacy;[451] on the other hand, the drawbacks of inorganic sonosensitizers have been addressed in the course of the years with different surface modification strategies[556] such as electrostatic adsorption using grafted copolymers exploiting exposed —OH groups,[593] the creation of oxygen-deficient layers on the surface of the NPs,[594] or noble metal coupling.[555] Other inorganic sonosensitizers are silicon NPs,[595,596] polyhydroxy fullerene,[597] composite nanosensitizers consisting of graphene oxide nanosheets coated by mesoporous silica and decorated with RB–PEG-conjugated iron oxide NPs,[598] Au NPs conjugated with PpIX,[599] and other nanocomposites made up of metal-coordinated porphyrins.[461,600]

A novel technique that is currently capturing increasing attention is the so-called sonosensitizer-free SDT: its aim is the generation of ROS species to kill cancer cells by means of an indirect US trigger to boost the initial reaction and enhance inertial cavitation, without using any traditional sonosensitizers.[601–604] To achieve this goal, solid state semiconductor nanoparticles like titania or zinc oxide have been proposed, combined with ultrasonic shock waves, achieving relevant cell damage and a so-called nanoscalpel effect, leading to cell death.[605,606] Most recently, gas-generating nanosystems have emerged as theranostic nanoplatforms that can be activated by either exogenous or endogenous triggers, enhancing the presence of specific gases in the TME with consequent cytotoxic or therapeutic effects.[557]

An example of CO_2 delivery to pancreatic tumor was proposed in a study by Zhang et al., in which a carrier consisting of hollow mesoporous silica nanoparticles (HMSNs) and L-arginine (LA) was triggered by low intensity US, generating and releasing a large amount of CO_2 bubbles (**Figure 7**). According to the authors, necrosis in PANC-1 cells in vitro and in vivo was due to a combination of the acidic environment (endogenous trigger) and the US stimulus (exogenous trigger).[607]

A 2015 study applied oxygen-carrying lipid-stabilized microbubbles (MBs) decorated with a Rose Bengal sensitizer (MB–RB) on pancreatic cancer models (BxPC-3) in vitro and in vivo. Their effects were a higher cytotoxicity in cells cultured under hypoxic conditions and treated with US and a reduction of the tumor volume in mice. Overall, these results confirmed the efficacy of oxygen delivery in hypoxic tumors and its valuable contribution to SDT enhancement,[445] and echoed similar studies proposed by the same author, which exploited polymeric microbubbles as delivery vehicles for sensitizers[608] and a novel combination of chemotherapy and sonodynamic therapy using gemcitabine-loaded/oxygen carrying microbubbles.[609]

Another example of multimodal therapy involving SDT was proposed in 2017 by Sheng et al.: their study designed magnetically responsive microbubbles consisting of an oxygen core and a phospholipid coating functionalized with RB and/or 5-FU, for the combination of antimetabolite and sonodynamic therapy on PC, which produced promising results.[610] A similar study by Nesbitt et al. proposed the use of gemcitabine-loaded microbubbles for a targeted chemo-sonodynamic combination therapy of PC.[611] Although the size of these lastly mentioned particles was at the microscale, excluding them from the category of purely nanomedicine approaches, these studies are of pivotal importance to understand the role of multimodal strategies to better address PDAC treatment and hence have been here briefly reported (**Figure 8**).

A nanoplatform able to self-produce oxygen in hypoxic PANC-1 pancreatic cancer was proposed by Chen et al. (**Figure 9**) and consisted of hollow mesoporous organosilica NP carriers loaded with IR-780 iodide sonosensitizer and coated in modified fluorocarbon (FC) chains that provided binding sites for oxygen (the final nanoplatform was called FHMONs). In vitro applications of FHMONs proved that US could enhance their intracellular uptake via sonoporation; once internalized, they reduced hypoxia providing oxygen supply, which in turn helped the production of ROS and enhanced the efficacy of SDT. In vivo studies showed that US application fulfilled a triple function: I) it provoked the breaching of tumor barriers, allowing the accumulation of the nanoconstructs into the solid tumor; II) it generated O_2 bubbles, whose release broke stroma barriers and reduced hypoxia; III) it

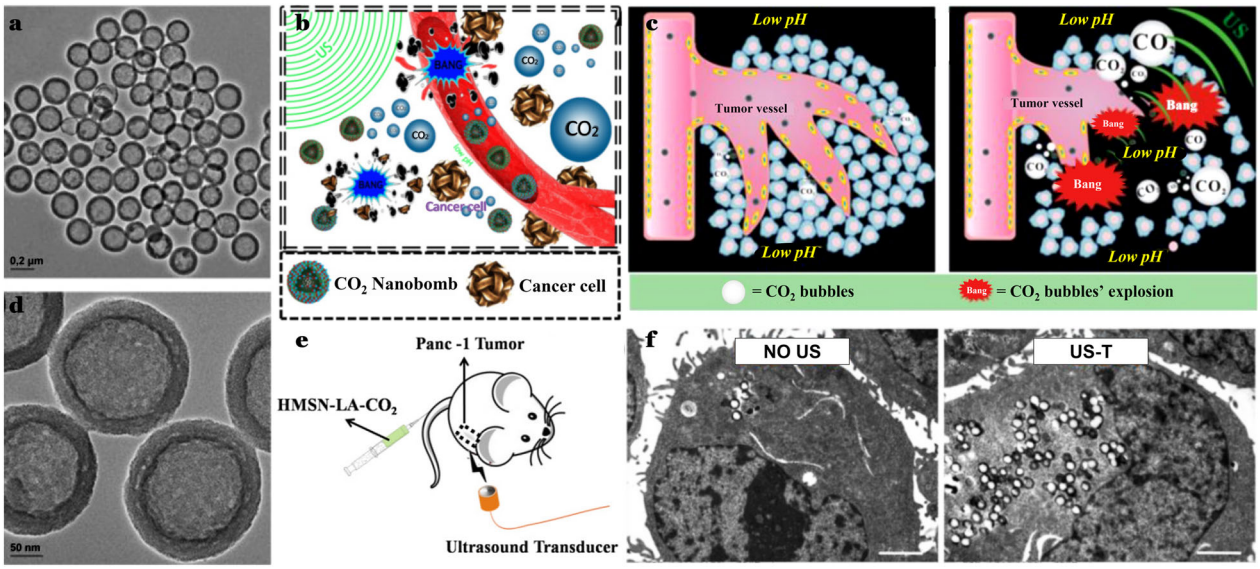

Figure 7. a,d) Transmission Electron Microscopy (TEM) images of HMSN–LA–CO_2. b) Schematization of CO_2 nanobombs' therapeutic mechanism; c) CO_2 bubbles' explosion (inertial cavitation) triggered by US radiation (right), compared to suppressed inertial cavitation taking place in absence of US stimulation (left); e) therapeutic procedure of HMSN–LA–CO_2. f) Bio-TEM images of PANC-1 cells treated with HMSN–LA–CO_2, showing internalization with (right) and without (left) US. Reproduced under terms of the CC-BY-NC license.[607] Copyright 2015, The Authors,published by Ivyspring International Publisher.

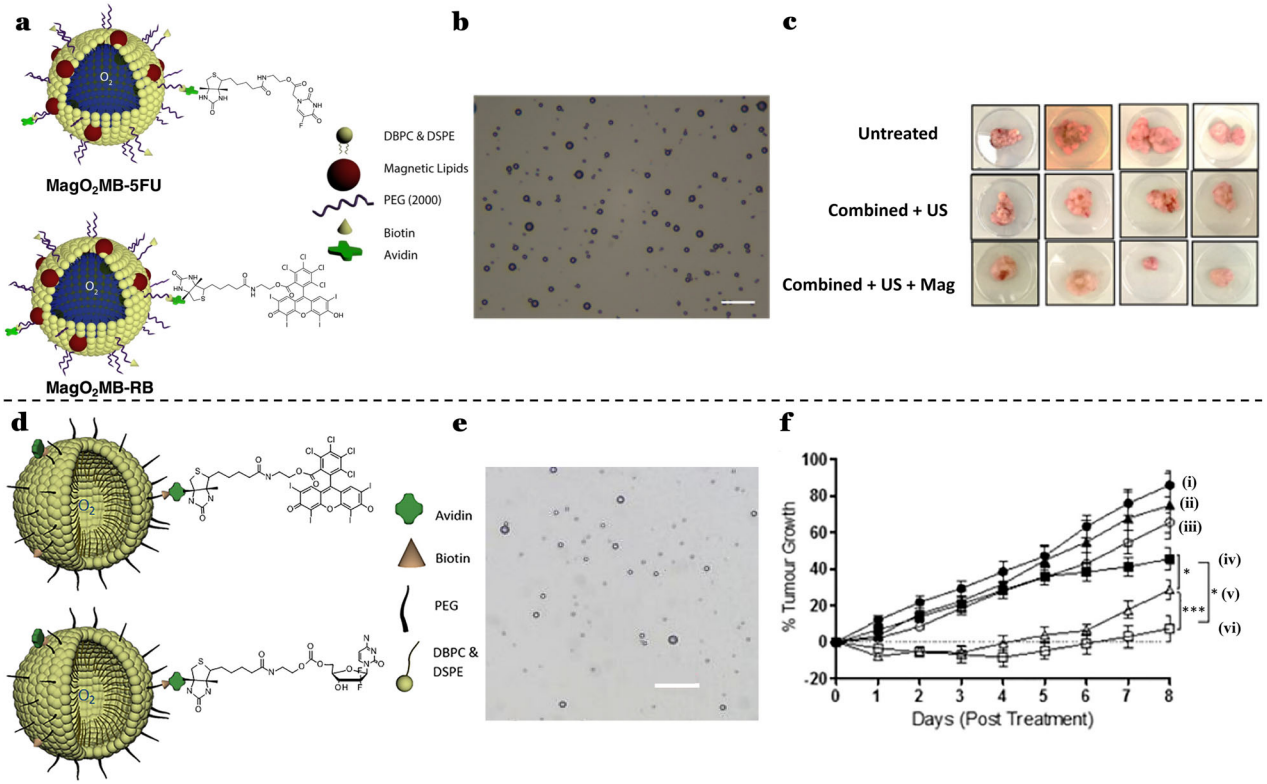

Figure 8. a) Scheme of the $MagO_2MB$–RB and $MagO_2MB$–5-FU conjugates. b) Optical microscopic images, scale bar 20 μm. c) Photos of removed orthotopic BxPC-3 Luc tumors untreated (top), treated with the two combined conjugates $MagO_2MB$–RB and $MagO_2MB$–5-FU + US (center), treated with the two combined conjugates + US and magnet (bottom) Reproduced under terms of the CC-BY license.[610] Copyright 2017, The Authors, published by Elsevier B.V. d) Scheme of the O_2MB–RB and O_2MB–Gem conjugates. e) Brightfield images of a suspension of the two conjugates, scale bar 20 μm. f) Tumor growth in mice models with i) no treatment, ii) O_2MBGem/O_2MB–RB on Day 0 and Day 3 – ultrasound, iii) ultrasound only, iv) Gem IP at 120 mg kg^{-1}, v) O_2MB–Gem/O_2MB–RB on Day 0 + ultrasound, vi) O_2MB–Gem/O_2MB–RB on Day 0 and Day 3 + ultrasound. Reproduced with permission.[611] Copyright 2018, Elsevier B.V.

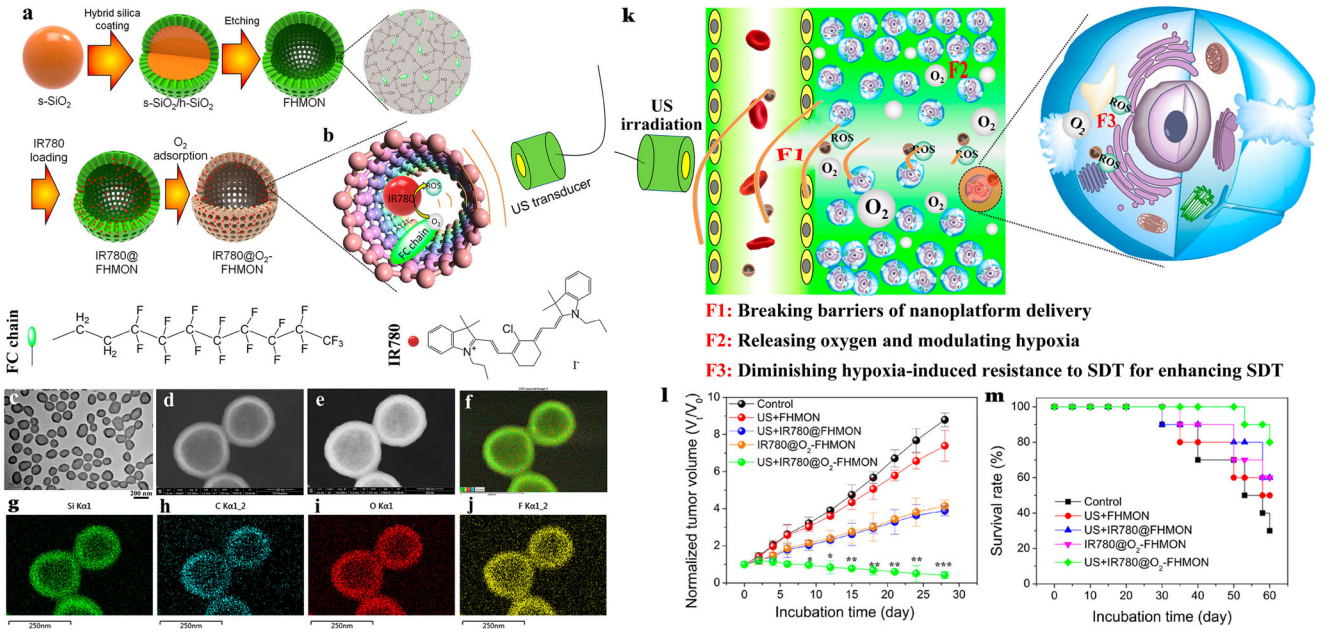

Figure 9. a–j) Scheme of the synthesis process, mechanism of action, and characterizations of IR780@O_2–FHMONs. k) In vivo triple effect of IR780@O_2-FHMON nanoplatforms. l) Tumor volume variation of PANC-1 solid tumors after the reported treatments. m) Survival rate of solid tumor-bearing mice after treatments. Reproduced with permission.[612] Copyright 2017, American Chemical Society.

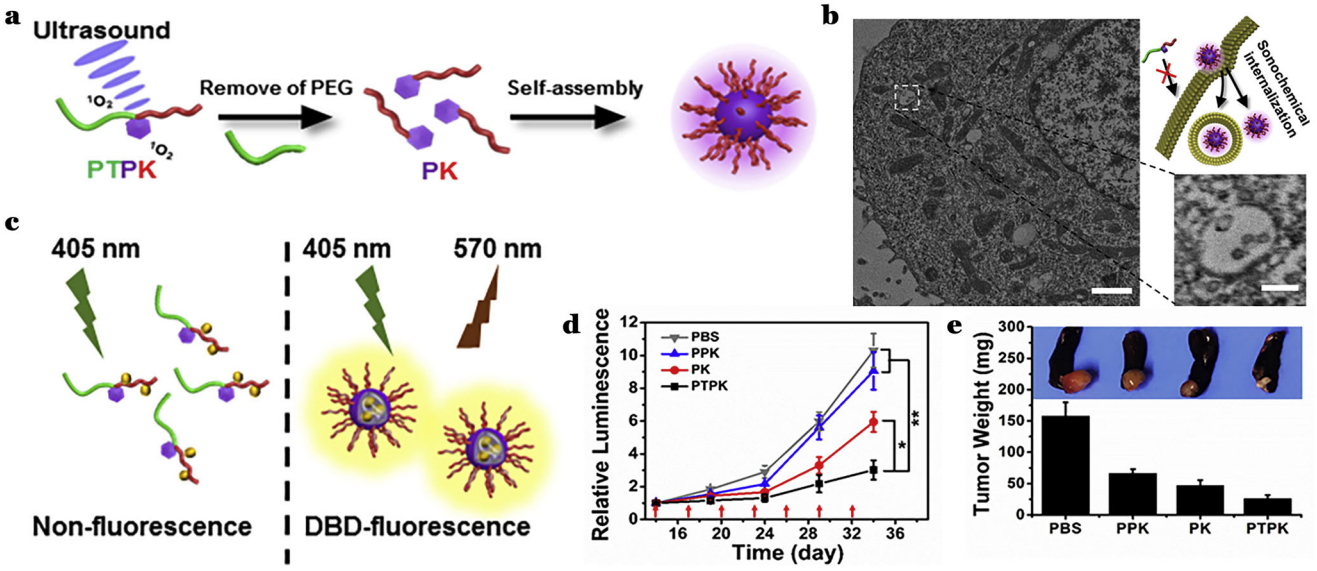

Figure 10. a) Scheme of the proposed cascade effect triggered by US to self-assemble polymer–peptide conjugates into nanoparticles once inside a pancreatic cancer orthotopic model. b) Bio-TEM images of PANC-1 cells incubated with PTPK after US treatment, scale bar 1 µm (left), 400 nm (right). c) Fluorescence detection to verify the self-assembly under US irradiation. d) Tumor volume changes in mice after different reported treatments and US irradiation. e) Average tumor weight and photos. Reproduced with permission.[613] Copyright 2020, Elsevier.

reinforced the SDT effect by diminishing hypoxia-induced resistance to SDT.[612]

Finally, a recent study proposed a pioneering pancreatic cancer therapeutical approach based on SDT: a US trigger of a cascade process (**Figure 10**). Thanks to this, polymer–peptide conjugates self-assembled into nanoparticles once inside a pancreatic cancer orthotopic model. In brief, the complete nanoconstructs (called PTPK) comprised a sonosensitizer (purpurin 18) decorated by a cytotoxic peptide (KLAK, Lys-Leu-Ala-Lys), linked via thioketal bond to a mPEG (methoxypoly(ethylene glycol)); being hydrophilic, the PTPK could dissolve as a single chain in blood circulation and penetrate the tumor, where a following focused US led to the formation of $^1O_2$. The effects of this radical were the thioketal bond cleavage and the consequent acquisition of hydrophobicity. This in turn resulted in a self-assembly in the tumor site, where the nanoparticles proved to induce

enhanced tumor inhibition through apoptosis by mitochondrial disruption.[613]

The studies presented so far provide evidence that micro-/nanocarriers play a crucial role in reinforcing the effect of organic sonosensitizers or gas molecules, by protecting them in biological media and by ensuring their correct delivery and accumulation in the tumor sites. Moreover, they offer the possibility of incorporating multiple agents as payloads, thus obtaining highly tunable nanoconstructs whose US activation can be exploited not only for the SDT enhancement due to the presence of the sonosensitizers, but also for complementary therapeutic applications.

However, important issues concerning nanocarriers have been raised with respect to their biostability and biodegradability, which must be ensured to avoid cytotoxic effects. Furthermore, the possibility of potentially introducing chemical modifications to their cargoes must be carefully considered, since the cargo pharmacodynamics, pharmacokinetics, and therefore potency could consequently be compromised. Biosafety becomes particularly relevant when the previously reported inorganic materials such as TiO_2,[593] silicon NPs,[555] and HMONs[612] are used as sonosensitizers or in addition to them to enhance SDT. On the contrary, liposomes are mainly employed as organic sonosensitizer carriers and are supported by an extensive literature about their use as drug delivery systems.[312] They are typically composed by FDA-approved chemical species and consequently they arouse less biocompatibility concerns.

12.2.8. Immunotherapy

Due to promising results reported with other tumor types,[614,615] the employment of NPs has been proposed to potentiate antitumor immune response in PDAC too, given the typically limited success achieved by means of conventional immunotherapy.

Xie et al. proposed intraperitoneal (IP) administration of cholesterol-modified polymeric CXCR4 antagonist NPs for the codelivery of anti-miR-210 and siKRAS^{G12D} to an orthotopic syngeneic pancreatic tumor (**Figure 11**a). The nanoconstruct in question possessed a triple therapeutic effect, namely blocking cancer–stroma interaction, inactivating PSCs and killing PC cancer cells. This combined therapy resulted in stroma depletion, reduction of immunosuppression, inhibition of metastases, and prolonged mice survival.[542]

Lu et al. reported the design of an IV injectable nanocarrier composed of a LB-coated MSNP platform incorporating oxaliplatin in the porous interior and IND-PL (phospholipid-conjugated indoximod prodrug, an IDO (idoleamine-(2,3)-dioxygenase) inhibitor) contained in the LB. The novelty of the study was the generation of a synergistic immune response by codelivering an immunogenic cell death stimulus through oxaliplatin (OX), while interfering in immune suppression, thanks to the IDO inhibitor[540] (Figure 11b).

Paclitaxel-loaded 3-aminophenylboronic-acid-modified low molecular weight heparin–D-α-tocopheryl succinate micellar nanoparticles were designed and injected by tail vein in orthotopic PANC-02 pancreatic mouse models to investigate their effect on both primary tumor and metastases, and showed an inhibition of tumor growth as well as a reduction of distant metastases, due to the improvement of the immune microenvironment of PDAC[544] (Figure 11c).

Lorkowski et al. developed an immune-stimulatory nanoparticle (immuno-NP) codelivering two immune agonists (cyclic diguanylate monophosphate and monophosphoryl lipid A) aimed at inducing the production of type I IFNs, which in turn are known to promote the recruitment of APCs and activate T-cell priming. Results showed that the systemic administration of immuno-NPs in an orthotopic murine PANC-02 model of PDAC and their subsequent accumulation in the perivascular region resulted in an effective uptake by APCs, that eventually led to an innate immunity boosting.[538]

Finally, Li et al. developed M2 TAM targeting nanomicelles decorated with a targeting peptide (M2pep) to codeliver PI3K-γ inhibitor NVP-BEZ 235 and CSF-1R–siRNA both in vitro and in vivo. This coadministration resulted in the activation of antitumor immune response and in the remodeling of the tumor immune microenvironment.[543]

Considering the proven immunomodulatory capacities of ablation techniques such as RFA, IRE, microwave ablation, and cryotherapy, their combination with local immunotherapy was recently proposed for the treatment of locally advanced pancreatic cancer. This multimodal approach showed a marked synergistic effect with respect to the single monotherapies.[616] The most promising results were achieved with SBRT combined with IL-12 microsphere injection in immunocompetent mice,[220] IRE and systemic anti-PD1 treatment,[617] and IRE combined with systemic anti-PD1 and intratumoral TLR-7 agonist[618] in mouse models.

Likewise, stimuli-responsive treatments such as PDT were shown to enhance antitumor immune response by releasing antigens and immunogenic factors such as DAMPs from dying cells,[619–621] and these findings paved the way for their combination with immunotherapy. As an example, a study included the use of a nanoplatform codelivering a bromodomain-containing protein 4 inhibitor (BRD4i) aimed at blocking PD-1/PD-L1 pathway and a photosensitizer to enhance PDT; their synergy increased immunogenicity and promoted intratumoral activation and infiltration of cytotoxic T cells in a pancreatic cancer mouse model[622] (Figure 11d).

Since SDT was proved to induce apoptosis and necrosis as well, and to elicit inflammatory immune response as a consequence of tumor cell debris release and necrosis,[623] a mechanism of immunomodulation similar to that triggered by PDT was suggested. In particular, a 2017 study demonstrated that high levels of IL-2 and low levels of IL-10 were observed in mice treated with sonosensitizer-assisted SDT up to 10 days after treatment, denoting an activation of immune response, inflammation, and a switch from T_H2 to T_H1 cells in hepatocellular subcutaneous and artificially engineered metastatic tumor models.[465] Although not applied to pancreatic cancer, this result was very encouraging since it suggested that a similar strategy could be employed in combination with immune adjuvant therapy to provide a systemic treatment modality (as later effectively demonstrated on murine hepatoma cells and subcutaneous hepa 1-6 tumor models[624]) and to deep-seated tumors such as PDAC, thanks to US penetration.

In fact, a very recent study applied SDT during systemic administration of MB–RB, combined with PD-L1 ICI treatment in

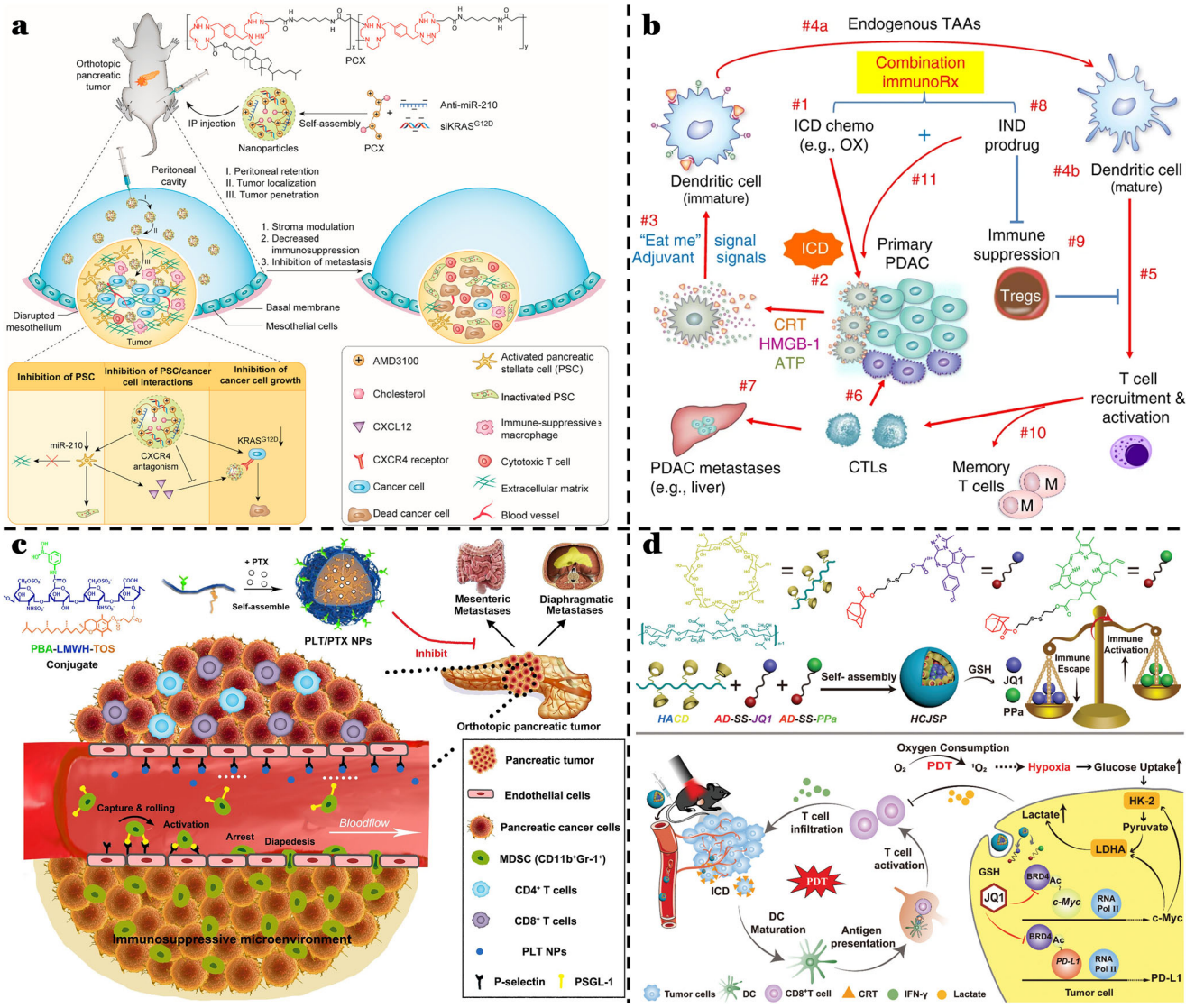

Figure 11. a) Mechanism of EPR-independent delivery of intraperitoneal injected triple miRNA/siRNA nanotherapy and consequent stromal modulation, decrease of immunosuppression, and metastases inhibition. Reproduced with permission.[542] Copyright 2020, American Chemical Society. b) Proposed mechanism of PDAC immune response to the synergistic administration of a chemotherapeutic agent (OX) and an IDO inhibitor (IND), resulting in enhanced induced cell death (ICD) and T-cell recruitment. Reproduced under terms of the CC-BY license.[540] Copyright 2017, The Authors, published by Springer Nature. c) Mechanism of micellar NPs' self-delivery to both an orthotopic PC and to its spontaneous metastases, with consequent remodulation of the immune microenvironment. Reproduced with permission.[544] Copyright 2021, Elsevier B.V. d) Prodrug NP preparation via self-assembly and proposed mechanism of combinatory immunotherapy, promoting T-cell activation and infiltration and overcoming adaptive immune resistance. Reproduced under terms of the CC-BY license.[622] Copyright 2021, The Authors, published by Wiley-VCH GmbH.

a mouse model of pancreatic cancer. The mechanism proposed by the authors was a production of DAMPs due to SDT, which in turn provoked DC maturation and migration to lymph nodes. Once there, they could present antigens to CD4$^+$ and CD8$^+$ T cells, that therefore were activated and returned to the circulation to infiltrate the tumor. Moreover, PD-1/PD-L1 blockade by means of antibodies avoided the inhibition of T-cell activation and enhanced the therapeutic effect, indicating a strong systemic immunogenic response.[625]

Finally, chimeric antigen receptor T (CAR-T) cell therapy is rapidly emerging as a promising new cancer treatment.[626] It consists of the modification of autologous T cells, engineered to expose specific receptors (CARs) which then can specifically direct them toward tumor-associated antigens in a MHC-independent manner, eventually leading to the elimination of the tumor in question. Although being currently studied in view of pancreatic cancer applications,[627,628] its effective application is typically hindered by the TME strong antagonism to T cells exhibited by PDAC.[262,320]

A recent review reported in detail all the currently ongoing clinical trials regarding PDAC application of CAR-T-cell therapy, and highlighted the importance of a multidisciplinary approach, involving immunotherapy or chemotherapy administrations, aimed at preconditioning and sensitizing the TME to

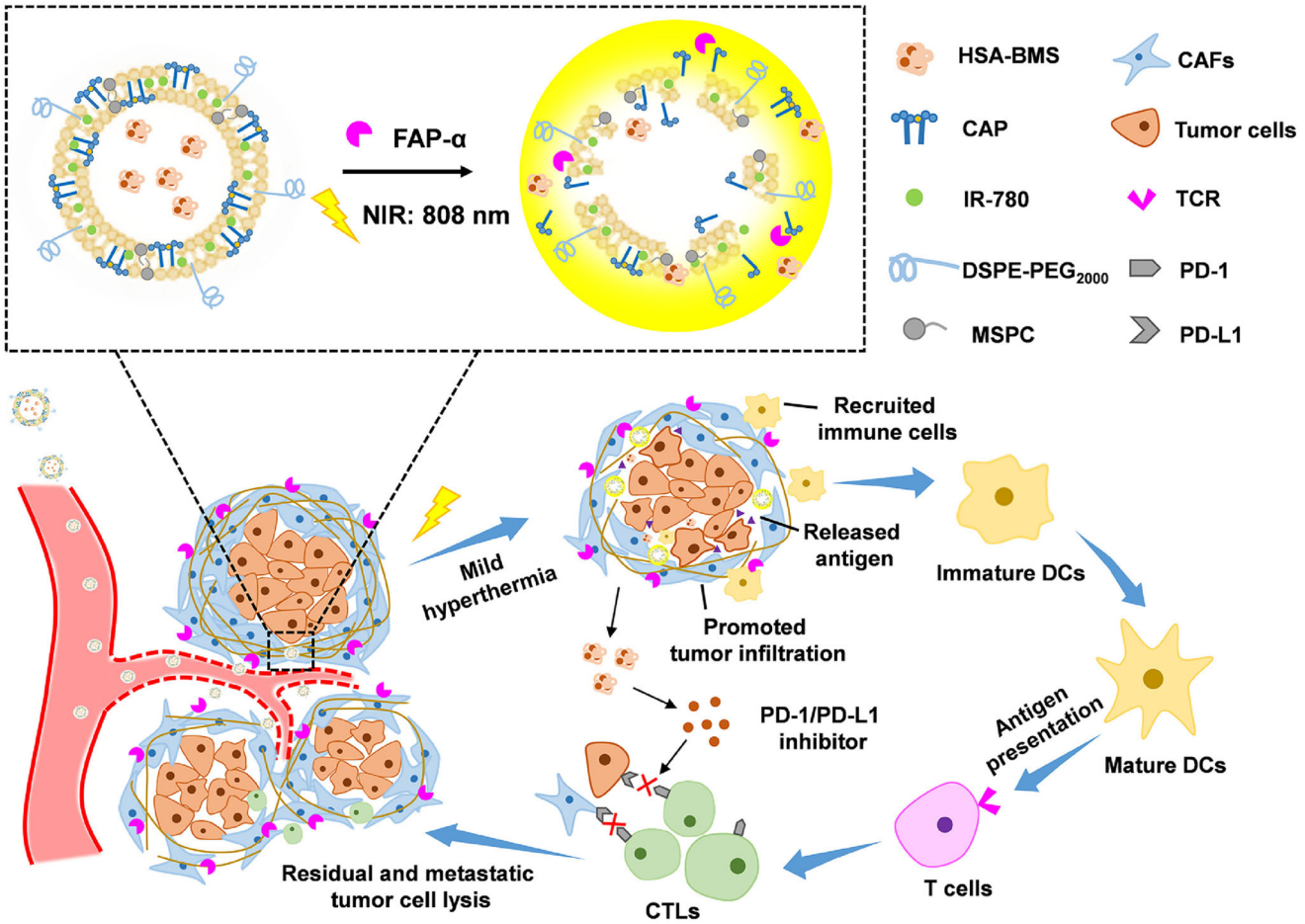

Figure 12. Effect of mild hyperthermia induced by PTT and combined with ICI therapy in pancreatic cancer, enhanced by the presence of size-adjustable and thermosensitive lipid–albumin NPs: reduced tumor hypoxia, enhanced blood perfusion, promotion of tumor infiltration by immune cells. Reproduced with permission.[632] Copyright 2021, Elsevier Ltd.

achieve better therapeutic outcomes.[629] Therefore, the use of nanoconstructs and nanocarriers able to specifically target the tumor zone could be exploited to enhance these cell therapies in various ways. In this regard, a study by Zhang et al. proved that the administration of lipidic nanoparticles decorated with an iRGD peptide and loaded with a PI3K inhibitor and an α-GalCer agonist could create a therapeutic window of TME immune stimulation, exploitable for T-cell therapy, in different solid tumor mouse models.[630] A mild hyperthermia elicited by photothermal therapy was observed to promote solid tumor infiltration and antitumor activity of CAR-T cells,[631] and the use of nanoparticles could potentially further increase this effect: indeed, a recent work by Yu et al. reported the enhancement of ICI-based immunotherapy against metastatic pancreatic cancer when photothermal therapy was applied in the presence of dual responsive lipid–albumin NPs[632] (**Figure 12**). Another study reported the administration of polymeric nanoparticles, loaded with a DNA payload coding for CAR (specifically leukemia CAR genes), to circulating T cells to impart them with long-lasting tumor-recognizing capabilities in mouse models.[633] Current research in the context of PDAC immunotherapy is also focusing on the search of further cancer-associated antigens as possible targets for CAR-T therapy, and promising data have emerged with respect to the use of anti-CD40 antibodies for immune modulation; therefore, the use of antibody-decorated nanoparticles could help enhancing the efficacy of these two applications.[3]

12.2.9. Multimodal Treatments

Taken together, these findings suggest that the most promising therapeutic options in order to undermine the intricate panorama of dysregulated signaling pathways, chemoresistance, intrinsic and TME-regulated impaired immunogenicity characterizing PDAC should include a multimodal therapy. **Table 2** summarizes the salient multimodal nanomedicine-based PDAC treatments reported so far. In particular, combining local treatments with immunotherapy was proven a promising strategy in the above-reported studies.[220,617,618]

Although many combinations have already been established and tested on different tumors in the course of the last decades, little improvements have been achieved with respect to PDAC. Nevertheless, a thorough characterization and understanding of the specific tumors in question could certainly lead to more

Table 2. Innovative multimodal therapies against PDAC reported in this work.

Multimodal treatment	Mechanism of action	Preclinical model	Results	Ref.
• Lipid-coated mesoporous silica nanoparticle platform codelivering gemcitabine and paclitaxel	• Combination therapy inspired by Abraxane's enhancement of gemcitabine activity • PEG-containing lipid-film-coating procedure to seal the pores and entrap drugs	• Mice carrying subcutaneous PANC-1 xenografts	• Effective tumor shrinkage with respect to free Abraxane • Inhibition of cancer growth • Elimination of metastatic foci	[559]
• Multifunctionalized iron oxide magnetic NPs for selective targeting of PC cells	• Anti-CD47 antibody and gemcitabine included in a single formulation	• Primary cell cultures and PC cell lines, (PANC-1, BxPC-3)	• Drug release occurring under reducing intracellular conditions • Efficient induction of apoptosis compared to the free antibody	[561]
• Stimuli-responsive micelle platforms codelivering paclitaxel and phosphorylated gemcitabine	• PEG coating to enhance biocompatibility • Stroma targeting peptide (AE105) • pH-triggered micelle disintegration for drug release	• MiaPaCa-2 and PANC-02 cells • Balb/c orthotopic PC tumor model (PANC-02)	• Disruption of the central stroma • Maintenance of external stroma to prevent metastases • Increase in the number of cytotoxic T cells	[565]
• Dual-enzyme-sensitive gemcitabine nanovectors	• CdSe/ZnS quantum dots • Conjugation with MMP-9 detachable PEG • Targeting ligand (cycloRGD)	• BxPC-3 cells • BxPC-3 xenografts	• Prolonged blood circulation • Enhanced tumor internalization • Effective and specific drug release • Reduced gemcitabine deactivation in blood	[566]
• Codelivery of HIFαsiRNA and gemcitabine via lipid–polymer NPs	• Lipid coating to prevent aggregation and gemcitabine leakage • Combination therapy strategy with siRNA and chemotherapy	• PANC-1 cells • Subcutaneous and orthotopic Balb/c nude mouse models	• Prolonged life in bloodstream • Synergistic antitumor effects • Inhibition of tumor metastasis in mice	[526]
• Polymeric dual delivery nanosystem for miR-345 and gemcitabine delivery	• Temperature- and pH-responsive copolymer • Tunable miR-345 and gemcitabine release • Sustained corelease	• Capan-1 and CD18/HPAF PC cells • Mice carrying xenograft tumors	• Sonic hedgehog signaling downregulation • Improved gemcitabine perfusion • Reduced tumor growth • Downregulation of desmoplastic reaction	[567]
• Super-paramagnetic iron oxide NPs of curcumin (SP-CUR) enhancing gemcitabine efficacy	• Suppression of aberrant SHH expression in PC • Targeted and sustained curcumin delivery into tumor • Possible application in MRI	• HPAF-II and PANC-1 cells • HPAF-II cells orthotopically injected into mice	• Effective delivery of curcumin in pancreatic cancer • Combination effect of gemcitabine and SP-CUR • Reduced tumor growth and metastasis • Improved survival	[568]
• Polymeric micelle formulation codelivering cyclopamine and paclitaxel	• Extravasation due to small size • Cargo protection in bloodstream • Combination therapy of Hedgehog inhibitor and cytotoxic chemotherapy drug	• Orthotopic PDX mouse models • KPC-Luc transgenic mouse models	• Synergistic attack on both tumor and stromal components • ECM remodeling • Increase of microvessel density • Hypoxia attenuation • Disruption of tumor cell–CAF communication	[571]
• Two-step engineered approach to enhance gemcitabine penetrance	• First-wave nanocarrier based on copolymer-coated mesoporous silica carrying a TGF-β inhibitor • Second-wave PEGylated gemcitabine-carrying liposome	• BxPC-3 cells • BxPC-3 tumor xenograft model	• Decreased pericyte coverage of the vasculature • Facilitated systemic biodistribution and retention at the tumor site • Rapid tumor entry of liposomes • Shrinkage of tumor xenografts	[572]
• Nano-photoactivatable liposomes codelivering cytotoxic (benzoporphyrin derivative, BPD) and biologic (bevacizumab) therapeutics (nanoPAL)	• nanoPAL–PDT treatment • Simultaneous spatiotemporal delivery of bevacizumab • Neutralization of VEGF burst following PDT	• AsPC-1 cells • Subcutaneous mouse model of PDAC using AsPC-1 cells	• Photocytotoxicity enhancement • Enhanced cytotoxicity in vitro • Tumor reduction in vivo	[589]

(*Continued*)

Table 2. (Continued).

Multimodal treatment	Mechanism of action	Preclinical model	Results	Ref.
• Photoactivable multi-inhibitor nanoliposome (PMIL) to suppress tumor regrowth and treatment escape pathways	• XL184-loaded NPs encapsulated in nanoliposomes carrying a photoactivable chromophore BPD in the lipid bilayer • PMIL intravenous administration • Near-infrared tumor irradiation	• AsPC-1 cells • Xenograft tumors from AsPC-1 cells (implantation in mice) • Metastatic mouse model by PDAC cells (implantation in the pancreas)	• Photodynamic damage of tumor cells and microvessel • XL184 intratumoral delivery • Prolonged tumor reduction • Suppression of metastatic escape	[590]
• NPs for MRI-guided chemo-photodynamic therapy alleviating tumor hypoxia	• Paclitaxel encapsulation in Fe(III)-complexed porous coordination network • Combination of PDT and chemotherapy • Fenton-like reaction to convert H_2O_2 to O_2 • MRI imaging for therapy monitoring	• PANC-1 cells • Nude mice implanted with PANC-1 cells	• Drug release in response to laser irradiation • Drug release in response to pH changes • Hypoxia regulation • ROS generation in vivo	[591]
• Oxygen-delivering polyfluorocarbon nanovehicles	• Photodynamic DiD and chemo-immunomodulatory gemcitabine prodrug loading • Laser irradiation	• PANC-02 pancreatic cancer model	• Hypoxia-relieving capacity (tenfold enhancement of tumor oxygenation) • ROS production • Responsive drug release • Delay of tumor growth • Boost in antitumor immunity	[592]
• Oxygen-self-produced sonodynamic therapy nanoplatforms (IR780@O_2–FHMONs)	• Mesoporous organosilica nanoparticle carriers • Fluorocarbon (FC) chains offer binding sites for oxygen and IR780 storage • Ultrasound radiation (SDT)	• Hypoxic PANC-1 cells • Nude mice bearing hypoxic PANC-1 solid tumor	• In vitro oxygen supply from IR780@O_2–FHMONs • In vitro ROS generation • Accumulation in hypoxic tumor • In vivo permanent hypoxia relief • Reduction of SDT resistance	[612]
• US-activated self-assembled polymer–peptide nanoparticles (PTPK)	• Deep tissue penetrating polymer–peptide conjugate • Self-assembly due to US irradiation • Departure of hydrophilic PEG from PTPK, resulting in hydrophobic interaction	• PANC-1 cells • PANC-1 subcutaneous xenograft mouse models	• Remarkable solid tumor penetrability and spatial precision • US-assisted membrane permeabilization and enhanced cellular internalization • Effective inhibition of tumor growth	[613]
• Local administration of triple miRNA/siRNA nanotherapy for stromal modulation	• Cholesterol-modified polymeric CXCR4 antagonist nanoparticles (blocking of cancer–stroma interactions) • Codelivery of anti-miR-210 (PSC inactivation) and siKRASG12D (PC cell killing)	• Primary tumor cell line KPC8060 • Orthotopic KPC-derived PC model	• Modulation of desmoplastic TME • Inactivation of PSCs • Promotion of T cells' infiltration • Delayed tumor growth • Stroma depletion • Inhibition of metastasis	[542]
• Supramolecular prodrug nanoplatform for combinatory photo-immunotherapy of PC	• Codelivery of a photosensitizer and a prodrug of BRD4i • HA-based nanosystem addressing CD44 receptor	• PANC-02 cells • Subcutaneous PANC-02 model	• Prolonged retention and deep tumor penetration • Promotion of T lymphocyte intratumoral infiltration • Inhibition of tumor growth	[622]
• Combination of sonodynamic therapy and PD-L1 immune checkpoint inhibitor	• Microbubble (MB)-mediated SDT • Lipid-stabilized MBs loaded with Rose Bengal (MB–RB) • IV injection of O_2MB–RB, anti-PD-L1 treatment, SDT	• Bilateral tumor model of PC generated using T110299 cell line	• Decrease in tumor volume • DAMP production due to SDT and resulting T-cell recruitment • Infiltration of CD4+ and CD8+ T lymphocytes • Elicited immune response, potentiated by anti-PD-L1 ICI	[625]

(*Continued*)

Table 2. (Continued).

Multimodal treatment	Mechanism of action	Preclinical model	Results	Ref.
• Exosome-based dual delivery biosystem (iEXO-OXA) to enhance PC immunotherapy and reprogram tumor microenvironment	• Galectin-9 siRNA loaded by electroporation • Surface modification with oxaliplatin (OX) prodrug as an ICD trigger • Reversal of immunosuppression of M2-like tumor associated macrophages (M2-TAMs)	• PANC-02 cells • Orthotopic PANC-02 PC tumor model	• Exosome-mediated enhancement of tumor targeting • Induction of ICD stimulus • Interference in immunosuppression • Improved DC maturation • Increase of T lymphocyte infiltration	[532]
• PTX-loaded self-delivery micellar nanoparticles able to target PC and its spontaneous metastases	• Immune microenvironment regulation mechanism • Synergistic PTX cytotoxicity • Phenylboronic acid modification improves tumor targeting, thanks to sialic acid residues in PC cells	• PANC-02 cells • Orthotopic PANC-02 pancreatic tumor-bearing mouse models	• Inhibition of MSDC recruitment to PC tissues • Inhibition of spontaneous metastases • Increase in the activity and infiltration of effector T cells (CD4+ and CD8+)	[544]
• Dual immune agonist-loaded immunostimulatory NPs to induce a proinflammatory immune microenvironment	• Precise ratio control of the immune modulators • Systemic administration • Synergistic effect of STING (stimulator of interferon gene) and TLR-4 agonists to expand APCs and increase local IFN-β secretion	• Orthotopic murine PANC-02 model of PDAC	• Deposition in the perivascular regions of the tumor • Significant uptake by dendritic cells and expansion of APCs • Increase of T lymphocyte tumor infiltration	[538]
• Dual delivery nanocarrier for immunogenic cell death (ICD) induction and immunosuppression interference	• Lipid-bilayer-coated mesoporous silica NPs incorporating an immunosuppressive IDO pathway inhibitor (IDN) and an ICD-inducing agent (Oxiplatin) • IV administration	• Orthotopic KPC model	• Induction of effective innate and adaptive anti-PDAC immunity • Recruitment of cytotoxic T lymphocytes in the tumor • Significant tumor reduction • Increase in animal survival	[540]

personalized combined therapies,[634] with the final aim of reducing and controlling metastases currently evading the immune response in all those cases in which complete regression of the primary tumor is not achievable.

12.2.10. Current Nanomedicine-Based Clinical Trials

As stated above, only a small percentage of nanomedicine-based preclinical trials are effectively translated to clinical ones. In fact, there are still several obstacles to nanomedicine systematic application, and failures in clinical trials dramatically increase when it comes to phases II and III. Huge heterogeneity of tumor biology, incomplete understanding of nanoparticles' interactions with biological components, safety issues, difficulties in scaling and production, poor pharmacokinetics, low tumor accumulation, and the lack of fully adequate animal models are just some of the reasons behind delays in clinical translation, which in turn has to face issues concerning patient selection and the choice of the best combination therapy to maximize its therapeutic efficacy.[635] **Table 3** reports some selected pancreatic cancer nanomedicines which are currently undergoing phase II and III clinical trials.

As far as clinically approved nanomedicines are concerned, two products recognized by both Food and Drug Administration (FDA) and European Medicines Agency (EMA) for PDAC treatment after completing phase III studies are the previously mentioned Abraxane (nab-PTX, Abraxis BioScience, CA, USA), and Onivyde (Merrimack Pharmaceuticals, Inc., MA, USA, also known as MM-398 or PEP02).

Abraxane was approved by FDA for the treatment of patients with metastatic PDAC in combination with gemcitabine as a first-line treatment in 2013,[256] while Onivyde, namely nanoliposomal irinotecan, was found to be effective in extending the survival of patients with metastatic PDAC previously treated with gemcitabine and was combined with 5-FU and folinic acid in a phase III study (NAPOLI-1).[257,516]

12.3. Novel Preclinical Models

Although the contribution of mouse models to major advancements in the understanding of PDAC is undeniable, many current limitations hinder a proper reproduction of its actual microenvironment. Recent advances such as 3D organoids, 3D bioprinting, and organs-on-chip aim at better mimicking the intricate tumor/stroma interactions, the influence of the immune system, and all the morphological features that contribute to the complexity characterizing PDAC but not provided by other current preclinical platforms.[641]

12.3.1. 3D Organoids

Tissue-derived embryonic or adult stem cells embedded into a 3D matrix are able to grow and self-organize in structures called organoids.[642] They reproduce more closely the morphology of the in vivo original tissues and are mainly used in cancer research for xenotransplantation, drug screening and discovery,

Table 3. Nanomedicine-based selected pancreatic cancer clinical trials.

Product	Nanocarrier	Payload	Current application	Trial phase	Status
SGT-53	Cationic liposome nanoconstruct	Human wild-type p53 DNA	Combination with gemcitabine/nab-PTX	Phase II study	Ongoing[281] (NCT02340117)
Genexol-PM	Polymeric micelle	Paclitaxel	Combination with gemcitabine	Phase II study	Ongoing[636] (NCT02739633)
NC-6004 (Nanoplatin)	Micellar formulation	Cisplatin	Combination with gemcitabine	Phase III study	Completed[637] (NCT02043288)
Atu027	Cationic liposomal formulation	AtuRNAi	Combination with gemcitabine	Phase I/II study	Completed[638] (NCT01808638)
Nano-SMART	Gadolinium-based NPs	–	Activation and guidance of Irradiation X (AGuIX) combined with MR-guided SBRT	Phase I/II study	Recruiting[639] (NCT04789486)
NBTXR3	Hafnium oxide-containing NPs	–	Activation by radiation therapy	Phase I study	Recruiting[640] (NCT04484909)

stromal cell cocultures, immuno-oncology and analyses of mutational signatures, gene expression patterns or proteomics.[643]

With the aim of better identifying genes and pathways involved in pancreatic tumorigenesis, a 2015 study[644] modified some existing approaches already applied to other tumors to generate organoids from normal and neoplastic murine and human pancreas tissues. These pancreatic organoids were then used to investigate PDAC pathogenesis since, after orthotopically transplantation into immune-deficient mice, they generated lesions similar to PanIN that were able to progress to locally invasive and metastatic carcinomas.

Meanwhile, Huang et al. established a procedure to generate pancreatic progenitor organoids from human pluripotent stem cells and from freshly resected PDAC. In contrast with the formerly mentioned study, their culture conditions promoted histostasis, namely the preservation of the differentiation status observed in the original primary tumor. Moreover, they pointed out the short time required by their protocol to establish organoid cultures from the time of surgery (21–45 days), which could minimize genetic drifts. Therefore, they suggested that the resulting organoids, better representing the primary tumor than cell lines and whose realization was relatively fast, could be used to personalize cancer treatments.[645]

As previously mentioned, it is now well established that distinct populations of CAFs with different phenotypes exist in mouse and human PDAC tissues. This finding emerged in a 2017 study, in which a coculture of murine pancreatic stellate cells and PDAC organoids revealed the presence of a subpopulation of CAFs located distantly from neoplastic cells, later named iCAFs, and activated by paracrine factors secreted from cancer cells. The authors highlighted the importance of this study in partly accounting for the conflicting results emerged in the context of stroma targeting therapies, which had not taken into account the heterogeneity of CAF populations and behaviors until then.[103]

A coculture protocol of organoids composed by PDAC cells and CAFs derived from the same patient was proposed by Seino et al. to investigate the role of stem cell niche factor dependency during tumor progression. In fact, after establishing a library of 39 PDAC organoid lines, they noticed that various Wnt-niche dependencies existed (Wnt being a molecular pathway involved in initiation and progression of PDAC[646]). They concluded that CAFs could transmit a protumorigenic niche signal to PDAC through the production of stromal Wnt ligands, and proposed Wnt-targeting therapeutic strategies as a possible future application, exploiting organoid-centered screenings.[647]

The importance of patient-derived organoids (PDOs) resides in their ability to recapitulate the disease of the original tumor and to allow personalized drug screenings: Driehuis et al. compared the molecular characteristics of 30 tumor organoids and then exposed them to therapeutic agents to reveal their drug sensitivity. Therapy responses differed among the PDOs, suggesting that a personalized approach could be the key for future effective treatments and that organoids might be used to guide therapeutic decisions, as previously reported,[648] after further validations.[649]

Some limitations of organoids, like the lack of some important components of the in vivo TME such as blood vessels and immune cells, must nevertheless be addressed.[650] A recent study by Tsai et al. was the first to report a coculture of pancreatic cancer organoids, CAFs, and T cells, and observed promising results such as activation of myofibroblast-like CAFs and tumor-dependent lymphocyte infiltration, which however require further mechanistic studies to be validated.[651]

Emerging innovative techniques therefore aim at improving the poor representation of the TME by simulating its architecture and vascularization.[652]

To improve the formation of PDAC cell spheroids realized with the assessed hanging drop technique, Ware et al. modified this method by adding methylcellulose polymer. This study was an early attempt of incorporating biopolymers into 3D cell cultures, and the authors observed uniform spheroid formation of 5 different cancer cell lines, namely PANC-1, BxPC-3, AsPC-1, MiaPaCa-2, and Capan-1, with distinct hallmarks of solid tumors such as the presence of a necrotic core, hypoxia, and apoptotic regions. Their robustness and mechanical properties, enhanced by the use of methylcellulose, made them resistant to manipulations and thus applicable as study platforms.[653]

12.3.2. 3D Bioprinting

3D bioprinting consists of the precise deposition of multiple layers of various cell types and biomaterials to generate 3D bioengineered tissues. The main 3D bioprinting techniques are laser-assisted bioprinting, microextrusion, and inkjet. Cells are suspended in a biocompatible gel-like material (bioink) able to retain their viability and functionality in terms of growth, proliferation, and signaling.[654] Cancer applications of 3D bioprinting involve the realization of tumor models based on a computer-assisted design, which eventually contain patient-derived cancer and stromal cells, bioink, ECM proteins, growth factors, and genetic material and thus accurately reflect the heterogeneity of real tumors. By mimicking the cell-to-cell and cell–matrix interactions of the TME and the 3D heterogeneity of real tumors, they provide an excellent in vitro support for the study of cancer behavior in drug screenings and personalized therapies.[655,656]

This technique has already been applied to PDAC in some pioneering studies. 3D organoids, produced in flat-bottom well plates with a cell-repellent surface employing a bioprinting technology incorporating a magnetic force, were applied for high throughput screening purposes. Briefly, two pancreatic cancer cell lines, hT1 and hM1, and two types of CAFs, hT1–CAFs and hM1–CAFs, were employed to create these organoids, which were tested with more than 3000 approved drugs in a large-scale screening and better reflected the in vivo tumor architecture and drug resistance by comparison to 2D models.[657]

In another study, the authors incorporated multiple cell types into bioprinted pancreatic tumor tissues and observed self-organization capabilities, secretion of ECM factors, and abilities to respond to extrinsic signals (in the present case GFs). The first part of the study involved the use of a pancreatic cell line, HPAF-II, bioprinted in stromal bioink of pancreatic stellate cells and human umbilical vein endothelial cells (HUVECs). The resulting tissue was then treated with gemcitabine, showing dose-dependent response. Then, a trial with primary patient-derived tissue, enzymatically disassociated and bioprinted in stromal bioink to compensate the lack of stromal tissue typical of PDXs, was performed. Notably, similar morphology to the PDX model but also to the primary tumor was observed, and the spatial organization was replicated, thanks to 3D organization of cells. Finally, the bioprinted tissue derived from PDX tissue showed resistance to gemcitabine and therefore was suggested to be used as a test platform for therapeutic sensitivity.[658]

Finally, a 2020 study executed laser-assisted bioprinting of spheroid arrays from exocrine acinar and ductal pancreatic cells on a gelatin methacrylate substrate to study the initial stages of PDAC development. The evolution of the bioprinted spheroids was explored over time, and cell-to-cell communication by heterotypic signaling between acinar and ductal cells was proved to be implicated in the proliferation/survival of these last.[659]

3D-printing technologies offer great control over geometry, cell deposition, and composition; moreover, cells can be manipulated prior to printing and cell composition in bioinks is highly customizable. Therefore, stromal cells can be incorporated to better mimic the TME, and they can be tuned in order to match with the composition observed in patients, thus allowing personalized therapies. Future improvements should include the addition of other main components of the TME, such as well-established vascular networks and cellular secretions to study paracrine signaling intrinsic of each studied tumor. Nevertheless, the lack of standardized protocols is the main obstacle in terms of clinical translation of these bioprinted tissue models.[656]

A preliminary study concerning 3D scaffold[660] applications to pancreatic cancer was reported in 2015. Ricci et al. analyzed the interaction between PDAC cells and three polymeric scaffolds, which offered different pore topographies and architectures. Their results suggested that a sponge-like scaffold was able to support the generation of aggressive pancreatic tumor models.[661]

A work by Totti et al. was the first to fabricate 3D highly porous polyurethane (PU) scaffolds coated in fibronectin to support the proliferation of pancreatic tumor cells; the resulting system was close to in vivo models in terms of cell proliferation, collagen production, formation of hypoxic regions, and heterogeneity of biomarker spatial distribution.[662]

Recently, Gupta et al. improved the previously mentioned work by developing a multicellular model involving cancer cells, endothelial cells, and stellate cells cultured on a PU scaffold. Moreover, specific ECM-protein-coated zones were implemented to mimic in vivo different cell distributions and induce selective cell adhesion. With respect to prior works, this hybrid model of the PDAC niche successfully supported proliferation and migration for longer time.[663]

Scaffold-based cultures are undoubtedly showing very promising results, but they must be further improved to incorporate crucial PDAC elements such as blood vessels and immune cells. Future applications should also involve the implementation of perfusion systems to provide these models with more physiological culture conditions.

12.3.3. Organs-on-Chip

Organs-on-chip (OOC) are microfluidic devices made of plastic, glass, or polymers (mostly polydimethylsiloxane) with hollow microchannels containing viable cells, which are nourished with controlled flowing culture medium and thus provided with nutrient and oxygen supplies. Cancer applications include I) the incorporation of multiple cell types, like those typically present in the TME, to allow the study of the interactions between cancer cells and surrounding tissues, II) the modeling of microvascular networks to assess antiangiogenic drugs and study tumor vascular perfusion; III) the study of cancer cells' extravasation and migration to induce metastasis formation.[664]

Due to their ability in recapitulating the microenvironments of in vivo tissues, OOC models have been applied to pancreatic cancer with the final aim of providing a complex multicellular model of human PDAC on a chip, designed for drugs and therapeutics testing. One of the first applications included an in vitro model consisting of pancreatic stellate cells cocultured with PDAC cells in an accessible 3D construct with a spatially controlled architecture, which was proposed as an alternative platform for drug evaluation.[665]

Later on, Beer et al. cultured PDAC cells into a cyclic olefin polymer microfluidic chamber enriched in collagen, which offered an optimal surface for cell attachment and proliferation. In fact, cells showed morphological appearance and growth

characteristics resembling grown 3D spheroid models and responded to cisplatin treatment, perfused through the chip, bearing higher doses than classical in vitro 2D and 3D cultures.[666]

A 2019 study reported an OOC that emulated tumor–blood vessel interactions and vascular invasion in PDAC. A biomimetic ductal channel containing PDAC cells was juxtaposed to a rudimentary blood vessel consisting of a perfusable endothelial lumen. Endothelial ablation and PDAC cell invasion into the vessel lumen were observed, and these behaviors were consistent with poorly vascularized tumor tissues noticed in histological studies and the high rate of circulating tumor cells and metastases formation in PDAC.[667]

A recent work focused on the study of EMT and local invasion using a microfluidic platform called ductal tumor-microenvironment-on-chip, in which murine pancreatic cancer cells isolated from GEMMs were embedded in a perfused collagen matrix and cocultured, forming a biomimetic duct. The integration of cancer cells whose genetics and molecular characteristics were carefully engineered ensured a close imitation of the intratumoral heterogeneity; nevertheless, future developments could involve the culture of stromal cells derived by patients.[668]

Finally, a 2020 study combined patient organoids and an OOC platform mimicking a perfusable vascularized vessel to accurately recapitulate a dynamic TME. Fibroblasts and endothelial cells (HUVECs) were included in the culture, and the crosstalk between the organoid and stromal fibroblasts resulted in their activation into myofibroblasts and in an increased proliferation of the resulting coculture. Moreover, collagen secretion by fibroblasts contributed to gemcitabine resistance when the drug was perfused through the vasculature.[669]

The high degree of control and flexibility is certainly one of the main advantages of bioengineered 3D approaches, and their enormous potential in implementing PDAC biomimetic platforms will certainly be the key to future patient-specific therapies.

13. Conclusions and Future Outlooks

Despite major scientific and medical progress, pancreatic cancer is one of the most lethal malignancies nowadays, and its diagnosis and treatment are hindered by the bewildering complexity and resistance displayed by its microenvironment. In fact, the plethora of interlinked molecular and signaling pathways, the highly hypoxic TME, the innate and acquired drug resistance, and the impaired immune response are all factors that must be taken into careful consideration while designing new PDAC treatments. Moreover, the options which go beyond conventional therapies are still very limited and must face many difficulties related to their application to clinic. A thorough understanding of PDAC pathology, carcinogenesis, altered molecular pathways, tumor biology, and current therapeutic limitations is an imperative requirement in order to successfully design and implement new strategies to ultimately overcome this malignancy. The purpose of this review was therefore to provide an in-depth and updated dissertation on the topic first, before reporting major advances in current treatments and focusing on their possible future evolution.

The studies presented thus far provide evidence that multimodal approaches might be the most promising way forward ultimate PDAC treatment, and that nanomedicine advances will continue to boost the efficacy of emerging treatment options. Nanoparticles nowadays constitute an impressive arsenal of highly customizable weapons against tumors, however enormous challenges need to be faced to treat advanced and metastatic PDAC, and current research on personalized therapies is still under intensive investigation. In fact, cell cultures and animal models are not able to recapitulate the EPR effect in humans and are therefore inadequate to accurately mimic drug distribution and more broadly PDAC heterogeneity and response. Therefore, exploiting new bionanotechnological insights to establish new preclinical models, presently at their infancy, is urgently required to guarantee a more robust reproducibility of PDAC TME; furthermore, these engineered models might provide more effective and precise testing platforms for novel promising nanomedicine-based approaches.

We firmly believe that a multimodal and highly interdisciplinary approach, combining conventional and novel therapies and applying nanomedicine and nanotechnological advances to our continuously evolving PDAC knowledge, will eventually lead to robust patient and tumor specific treatments. Finally, we suggest that a necessary convergence of local and systemic therapies and their consequent coadministrations, according to precise and evidence-based ratios and time intervals, could make the best of both approaches. The final aim will be developing high precision and personalized treatments, eventually able to dramatically improve PDAC patients' survival rates in the foreseeable future.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

nanoparticles, pancreatic cancer, personalized therapy, theranostic treatment, tumor microenvironment pathology

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