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Targeting Stroma in Pancreatic Ductal Adenocarcinoma

Review Article

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the one of most lethal cancer types in the world. Its aggressiveness is due to its usually late presentation and early dissemination, with about 80% of tumors being unresectable by the time of diagnosis. PDAC presents a particular histological feature: the pancreatic cancer cells are surrounded by and abundant desmoplastic reaction, also called stroma, formed by large quantities of extracellular matrix and diverse cells such as pancreatic stellate cells, immune and endothelial cells and growth factors. The stroma establishes an intense crosstalk with pancreatic cancer cells, through diverse mechanisms, establishing a peculiar cancer microenvironment that promotes cancer proliferation, metastatic spread and chemotherapy resistance.

Current unresectable PDAC management is based in chemotherapy agents, which prolong survival for only a few months. There is a great necessity to develop new treatments to improve PDAC's poor prognosis. With the unveiling of its role in PDAC growth, stroma has emerged has a new promising target in this field, with good results in preclinical trials. Clinical trials are ongoing in several stroma targeting modalities, with both hopeful and disappointing results. The modulation of the stroma-tumor crosstalk, rather than its depletion, has the potential to become the next step in PDAC management.

This review intends to summarize the role of stroma in PDAC progression and chemotherapy resistance, and to explore recent discoveries in stroma targeting therapies, analyzing its promises and failures.

Keywords: Pancreatic Ductal Carcinoma, Pancreatic Stellate Cells, Tumor Microenvironment, Molecular Targeted Therapy, Immunotherapy

INTRODUCTION

Pancreatic cancer is one of the most lethal malignancies in the world, and carries a very poor survival. It is the 7th most common cause of death from cancer, and the 12th with the highest incidence. [1] Worldwide, in 2012, pancreatic cancer had an estimated prevalence of 2.4% of all cancers and represented 4.4% of all cancer-related deaths, with a mortality/incidence ratio of 98%. [2] The incidence and mortality of pancreatic cancer are higher in developed countries and increase with age. [3]

In 2017, in United States, there were 53 670 estimated new pancreatic cancer cases and 43 090 deaths, being the 4th most fatal cancer both in males and in females. In the last few years, the five year survival of many cancer types remarkably increased, but that's not the case for pancreatic cancer, which still has an overall five year survival of 8% for all stages, and of 3% when diagnosed with distant metastasis already present. [4] It is estimated that, by 2020, pancreatic cancer will become the 2nd leading cause of cancer-related deaths overall, and that by 2030, pancreatic cancer will be the 3rd leading cause of cancer death in US in each sex, following lung and liver cancers in men and lung and breast cancers in women. [5]

Only a very small percentage of pancreatic cancers, around 9%, are diagnosed in a localized stage; around 29% are detected in a regional stage and 51% in metastatic disease, which prevents the possibility of a curative resection in most cases. [4]

Pancreatic ductal adenocarcinoma (PDAC) accounts for about 85% of pancreatic cancer cases. [3] Several different risk factors, such as smoking, obesity, pancreatitis and heavy alcoholic consumption have been pointed out as contributing components to PDAC progression, but the knowledge of the exact mechanisms involved is yet to be defined. [3, 6]

PDAC's management options are still very limited and offer a very poor prognosis. Only 10 to 20% of the patients diagnosed with PDAC are considered candidates for surgical

resection, the only curative approach available, with a 5 year survival rate of about 30% in lymph node negative cases. [7] Those who present with unresectable locally advanced tumor or with metastatic disease have few chemotherapy regimens options.

Gemcitabine has been considered the gold standard for management of metastatic PDAC since its clinical demonstrations of efficacy in 1997, offering a median survival of about 6 months, but with a significant improvement in quality of life and nutritional status. In recent years some drug combinations have showed some increase in survival, but it is a marginal one at best, such as the combination of gemcitabine with erlotinib. [8, 9] A phase II/III trial with the combination of fluorouracil, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) demonstrated an increase in survival of around 4 months more when compared with gemcitabine alone, but with an exacerbated toxicity, being a therapeutic option for patients with a good performance status. [10] More recently, the addition of nanoparticle albumin-bound (nab)-paclitaxel to gemcitabine demonstrated an increment in survival of 2.1 months, compared to gemcitabine monotherapy. [11]

It's then clear that PDAC presents some characteristic features that make it a challenge to manage, one of the most flagrant ones being the development of a rich stromal response, wich vastly decreases tumor blood perfusion and drug delivery, contributing to drug resistace. [3] However, it is known that the complete depletion of stroma leads to more aggressive tumors, with even poorer outcomes, which points to a much more complex stroma-tumor interaction than the one initially described. [12, 13]

The research of new treatment methods that beat the challenges presented by this highly fatal cancer is of great need. This review pretends to shed some light on the relationship between the stromal components of PDAC and the cancer cells, summarizing which targets

can be used in therapeutics and exploring the current clinical trials, highlighting its promises and failures.

METHODS

This review is the result of the analysis of relevant scientific papers published and referenced on the databases PubMed and Cochrane Library, limiting the results to the publications in English, published online from July of 2002 until July of 2017. The keywords used in the research were "pancreatic ductal adenocarcinoma", "stroma", "stroma targeted therapies", "tumor microenvironment", "pancreatic stellate cells" and then each individual therapy explored in this review (e.g. "Vitamin D", "Sonic Hedgehog inhibitors"). There were excluded studies done in other types of neoplasms that weren't PDAC and studies that centered on other therapeutic techniques other than stroma-targeting ones. For clinical trials' reference it was used the database Clinicaltrials.gov, searching for the specific therapies in study.

This review was based on both original articles and review articles.

THE STROMA IN PDAC

In PDAC the tumor cells are swaddled by a rich stroma, more exuberant than in most tumor types, accounting for up to 80% of the tumor bulk. It is formed both by an acelullar element, the extracellular matrix (ECM), as well as different cell types such as fibroblasts, immune cells (lymphocytes, dendritic cells, neutrophils, mast cells, eosinophils) and soluble factors (chemokines, cytokines, growth factors and pro-angiogenic factors). Also, tissues adjacent to the tumor can be incorporated into the stroma, and these organ-specific cells,

adipose and nervous tissue, among others, can also influence neoplastic and biological processes. [14] It forms an unique and complex tumor microenvironment (TME), that nourishes the cancer cells, facilitating their invasive and metastatic potential. [15]

There are three known precursor neoplastic pancreatic lesions: pancreatic intraepithileal neoplasm (PanIN), mucinous cystic neoplasm, and intraductal papillary mucinous neoplasm. [16] The most known and studied one is PanIN, found in the smaller pancreatic ducts, characterized by a columnar mucinous epithelium with increasing architectural disarray and nuclear atypia, progressing from PanIN-1A to PanIN-3. [16,14] High grade PanINs can ultimately evolve into invasive PDAC, passing the ductal basement membrane.

Along this PanIN-to-PDAC progression there are an increasing number of gene mutations, often implicating known cancer genes, the earliest of which being the KRAS oncogene activation, present in more than 90% of human PDACs. [17] Pancreas-specific expression of mutant KRAS in mice recapitulates the human PanIN-to-PDA sequence, implying that the KRAS mutations act as an initiating event. [18] Other mutations involved are the inactivation of the p16 tumor suppressor gene (in 95% of the PDAC cases), loss of p53 (present in up to 75% tumors), SMAD4 inactivation (in about 60% of PDACs) and also INK4A/ARF tumor suppressor loss (in 80-95% of sporadic PDACs). [14] These are only a few examples among many others, making pancreatic cancer progression an extraordinarily complex process. These genes' mutations and new proteins production lead to an excessive activation of downstream signaling pathways that improves cancer cell proliferation and suppresses pro-apoptotic pathways. [15]

Along this continuum the TME evolves progressively, accompanied by its desmoplastic response.

Pancreatic Stellate Cells (PSCs)

The major cellular components of PDAC stroma are pancreatic stellate cells (PSCs) and fibroblasts. PSCs were first described in 1982 and first isolated in 1998; these cells are suggested to be derived from mesenchymal, endodermal and neuro-ectodermal origins. They express desmin, glial fibrillary acidic protein, vimentin, nestin and neuroectodermal markers, that allow their discrimination from other fibroblasts. [19]

PSCs are resident cells of the pancreas; in a healthy organ they are in their quiescent state, possessing abundant vitamin A storing lipid droplets in their cytoplasm. When pancreatic injury occurs, PSCs are activated: they lose their vitamin A stores, acquire a myofibroblast-like phenotype and express α-smooth muscle actin (α-SMA), a cytoskeletal protein. [20] This activation is induced by signaling via oxidative stress, as well as growth factors as cytokines, such as pigment epithelium derived factor, platelet derived growth factor (PDGF), trefoil factor 1 (TFF1), endothelin-1 (ET-1), interleukin-6 (IL-6), activin A and insulin-like growth factor 1 (IGF-1).[21-23] Their function is regulated by several molecular pathways, such as STAT3 phosphorilation upon TP53 mutation, that upregulates Sonic Hedgehog (SHh) and suppresses GLI3, a suppressor of stromal formation; and negative regulation by the CD146. [24] Once activated, PSCs secrete excessive ECM components (collagen, laminins and fibronectins) through activating the mitogen-activated protein kinase (MAPk) family enzymes, leading to fibrosis. [25]

In tumors, these cells are abundant around carcinogenous structures, organized around them in a ring shape. They are sparse around the benign tissue and ducts. [26]

Vonlaufen et al. [27] demonstrated that mice injected with both pancreatic cancer cell lines (PaCa2) and human PSCs developed bigger tumors than mice injected only with PaCa2 .

Also, mice injected only with PSCs didn't develop any tumors, which strongly suggest that PSCs by themselves have no carcinogenic potential.

PSCs interact not only with pancreatic cancer cells, in an intense signaling crosstalk, but also with the immune cells of the stroma. PDAC cells recruit to their vicinity PSCs, establishing a growth-supportive environment and leading to a heightened tumor cell proliferation, decreased apoptotic potential and increased invasion and migration. In turn, tumor cells stimulate the proliferation of PSCs, its ECM production and migration. PSCs support tumor cells by secreting several mediators, as transforming growth factor β (TGFβ), PDGF, connective tissue growth factor (CTGF), epidermal growth factor (EGF), kindlin-2, adrenomedulin and galectin-1. [27, 28]

In addition to these autocrine and paracrine influences, cancer proliferation, progression and invasion are also supported by translocation of metabolic substrate through exosomes, from PSCs to PDAC cells, containing lactate, acetate, lipids, aminoacids and tricarboxylic acid cycle intermediates, that inhibit mitochondrial oxidative phosphorylation and upregulate glycolysis and glutamine-dependent reductive carboxylation, promoting tumor growth under nutrient deprivation. [29]

Another key player in the mentioned crosstalk is fibroblast activation protein-α (FAP), a serine protease. It plays a crucial role in ECM synthesis, cell motility, angiogenesis and immune suppression, facilitating the establishment of the permissive TME to cancer growth.

PSC's excessive ECM production leads to a distorted parenchyma that eventually causes vascular compression and consequent hypoxia of the tumor cells, which further activates PSCs, leading to a complex hypoxia-fibrosis cycle. [30]

Erkan et al. [31] found stroma turnover (translated by activated stroma index) to be an independent prognostic marker in PDAC. PDAC patients were evaluated by

immunochemistry using staining of α-SMA (marker of PDAC activity) and of collagen, defining the activated stroma index as the ratio between the α-SMA-stained areas and the collagen-stained areas. There were observed 4 major patterns of collagen deposition, that allowed to conclude that a higher stroma activation with a low collagen deposition is associated with a poor prognosis, whilst low stromal activity with high collagen deposition translate a good prognosis.

Some of the mechanisms of the demoplastic influence on tumor progression and invasion also show significance on metastasis. It is now understood that PSCs have the ability of transendothelial migration, being able to travel to distant mestastatic locations where they facilitate the seeding and growth of PDAC cells; this effect is mediated by PDGF, secreted by the cancer cells. [32]

Aiello et al. [33] used autochthonous models of PDAC to characterize the stroma within metastatic lesions, reaching the conclusion that α-SMA producing fibroblasts appear in these lesions when they are as small as 6/7 cells, and that stromal volume eventually reaches levels comparable to the primary tumor.

Stromal cells also need to be taken into account in chemoresistance. Not only they provide a physical barrier but paracrine crosstalk between PSCs and tumor cells also facilitates the hallmark chemoresistance of this tumor. Wörmann et al. [24] have recently established that the loss of p53, a very common genetic change in PDAC, leads to activation of janus kinase 2 -signal transducer and activator of transcription 3 (JAK2-STAT3) signaling pathway, which boosts stromal modification and resistance to gemcitabine.

Extracellular Matrix (ECM)

As stated, the abundant ECM seen in PDAC is produced by PSCs, and it is formed by a plethora of proteins, such as fibronectin, laminin, tenascin C, hyaluronan, collagen I, collagen III, collagen IV and collagen XIA; and also by polysaccharides and peptides. These stromal elements act not only as structural support to PDAC cells but also partake in differentiation, remodeling and carcinogenesis. [34]

Type I collagen, produced by PSCs, was associated with survival of pancreatic cancer cells, with increasing proliferation and reduced apoptosis, when treated with the chemotherapy agent 5-fluorouracil, which highly suggests that collagen I supports the malignant phenotype of cancer cells. [35]

Collagen IV was also associated with PDAC progression. It is expressed in the tumor cells vicinity, organized in a basement membrane-like fashion around the cell surface. It is mainly produced by tumor cells and is a promoter of proliferation and migration by interacting with integrins in the cancer cell surface. It has also been shown that it inhibits apoptosis of cancerigenous cells. [36]

Hyaluronan (HA) is a glycosamingoglycan found in abundance in the ECM. It is connected with the processes of angiogenesis, epithelial-mesenchymal transition and chemoresistance in PDAC. Deposition of HA compromises vascular patency by increasing the interstitial fluid pressure in the tumor, decreasing the efficacy of chemotherapy agents. [37]

ECM breakdown is a fundamental step in the process of tumor invasion and metastasis. [34] Matrix metalloproteinases (MMPs) are a family of ECM-degrading enzymes, and MMP2, that breaks down collagen IV and laminins, is connected to tumor progression by enabling the degradation of the basement membrane. MMP2 is a pro-enzyme, mainly

produced by PSCs, activated by membrane-type matrix metalloproteinase, a protein expressed on the surface of the tumor cells. It is upregulated in PDAC and higher levels of epithelial MMP2 were correlated with worse prognosis. [38] However, in a later tudy, MMP2 expression, albeit being higher in PDAC, displayed no relation with overall survival. [39] MMP activity is regulated by tissue inhibitors of metalloproteinases (TIMPs). [34]

Immune Cells

The immune system in PDAC progression plays a double paradoxical role of anti-tumor and pro-tumor activities. While it performs its function of immunosurvaillance, preventing tumor growth and eliminating cancer cells, it can be "high-jacked" through tumor cells' influence, and contribute to cancer proliferation, invasion and metastasis.

In PDAC tissues significantly elevated levels of immunomodulatory and chemotatic factors such as IL-6, TGFβ, Indoleamine 2, 3-dioxygenasase (IDO), cyclooxygenase-2 (COX-2), C-C motif chemokine ligand 2 (CCL2), and CCL20 and immune cells like macrophages, myeloid, and plasmacytoid dendritic cells were detected, when compared to healthy pancreatic tissue. [40]

As the tumor progresses, changes along the tumor infiltrate accompany it alongside. Even in the earliest stages of PDAC there's a strong response of immunosuppressive cells such as tumor-associated macrophages (TAMs), myeloid derived suppressor cells (MDSCs) and regulatory T cells (Treg), that persist through to invasive cancer. These suppressive cells of the host immune system precede and outweigh antitumor cellular immunity, contributing to cancer progression. [41]

As previously mentioned, the release of cytokines such IL-6 and IL-1 is part of the complex crosstalk between PSCs and cancer cells that promotes cancer progression. Furthermore, high levels of IL-6 have a strong correlation with tumor stage, cachexia and

decreased survival in PDAC patients. The IL-1 family also fosters several agents connected to PDAC: increased IL-1α levels in tumor tissue are associated with decresed survival; Il-1β gene and promoter polymorphisms are related to worsened disease outcome; and on the other hand, elevated serum IL-1 receptor antagonist (IL-1ra) levels have been linked to increased survival. IL-10 has also been involved into this matter, its levels in tissue and serum being upregulated in PDAC patients. [42]

Tumor Associated Macrophages (TAMs)

TAMs are the main cell type seen in PDAC immune infiltrate; they come either from recruited blood monocytes or resident tissue macrophages. [42]

Usual macrophage activation, done by T helper 1 (Th1) cytokines such as interferon-γ (IFN-γ) and IL-1β is designate the "classical" activation, while the one done by Th2 cells is often recalled as the "alternative" activation. These two types of macrophages are then designated as M1 polarized macrophages or M2 polarized macrophages. M1 macrophages produce high amounts of tumor necrosis factor-α (TNFα) and nitric oxide being efficient effector cells that destroy microorganisms and tumor cells. M2 macrophages, in turn, promote angiogenesis, tissue remodeling and repair, and express CD163. [43] TAMs are majorly formed by M2-polarized macrophages. However, contradictory studies have come out on this matter. Tjomsland at al. [40] found that patients with higher levels of CD163-expressing phenotype macrophages have the best clinical outcome. On the other hand, Kurahara et al. [43] reported that high levels of CD163 macrophages correlated with lymphatic spread and therefore worst prognosis. These inconsistencies point to the fact that the M1/M2 division of macrophages might not be enough where it is concerned with its role on PDAC promotion.

TAMs have an important role on PDAC progression via production of IL-6, that works as an activator of the STAT3 pathway, initiating a feed-forward mechanism in the TME to stimulate PanIN progression and, ultimately, PDAC development. [44]

Myeloid-derived Suppressor Cells

MDSCs are described as a heterogeneous group of cells that include the precursors of macrophages, dendritic cells and granulocytes at earlier stages of differentiation, and that potentiate tumor progression and invasion through several pathways such as inducible nitric oxide synthase, Treg recruitment and reactive oxygen species upregulation. They are increased in PDAC, not only in the pancreas but also in bone marrow, spleen and metastatic sites. They inhibit lymphocyte activation, slackening anti-tumor immunity response. [42, 45] MDSCs recruitment has been attributed to tumor-derived granulocyte-macrophage colony stimulating factor (GM-CSF), secreted by PDAC cells. The *in vivo* blocking of GM-CSF halted the recruitment of MDSCs to the TME, and inhibited tumor development, through CD8+ T cell activation. [45]

Tumor Associated Neutrophils (TANs)

Traditionally, neutrophils have been seen as a casual observer in PDAC. However, recent discoveries found they also have a TME-modifying ability, which enables them to support tumor growth. In PDAC there is an upregulation of polymorphonuclear lymphocyte-chemotactic substances, that assures constant replenishment of the short-lived TANs. Cancer cells themselves secrete several chemokines, such as IL-8 that mediate neutrophil recruitment. [42]

Neutrophils' functions were evaluated in several types of cancer. They produce numerous chemokines, such as IL-1β, TNF-α, Il-6 and IL-12 that contribute to inflammatory cell recruitment; they also liberate reactive oxygen species that are severely genotoxic and

contribute to tumor initiation; there's also production of neutrophil elastase that shows differential effects on tumor development on a contraction-dependant fashion, but that can lead to tumor cell proliferation by promoting the phosphoinositide 3-kinase (PI3K) pathway. [46] There's also release of serine proteases and MMPs (MMP8 e MMP9) that also add to the immunossupressive environment by promoting cell motility and matrix turnover. [47]

Similarly to macrophages, two types of polarization have been suggested in TANs, one that favors tumor growth (N2) and one that opposes it (N1). Most remarkably, Fridlender et al. [48] described two populations of TANs, which affected the tumor growth in two confronting ways, one present normally within the tumor and the other revealed when TGF-β was blocked. TGF-β blockade resulted into the recruitment of TANs with an anti-tumor phenotype, which produced higher levels of cytokines than the "usual" TANs and were cytotoxic to tumor cells. The depletion of these N1 neutrophils led to impaired CD8+ activation and a consequent increased tumor burden.

Tumor Infiltrating T cells

In PDAC there had been observed a clear abundance of CD4+ T cells and a scarceness of CD8+ cells. [41]

CD8+ cells, or cytotoxic T lymphocytes are an element of tumor-specific cellular adaptive immunity. They recognize and attack tumor cells that present tumor antigen peptides, through histocompatibility complex class I. They eliminate tumor cells through IFN-γ and through induction of macrophage's tumor-eradication ability. [42]

CD4+ cells, or T helper cells, have several subtypes that are remarkable in PDAC, such as Th1, Th2, Treg and Th17 cells. They perform a central role in regulating the immune response, as they can regulate the functions of other immune cells. [42, 49]

Th17 cells are IL-17 secreting CD4+ T cells that are present in higher levels both in tumor tissue and in peripheral blood in patients with PDAC. Studies have shown that higher levels of IL-17 expressing cells are associated with increasing TNM stage, which suggests that IL-17 expression is connected with higher invasive and metastatic potential. The same study asserted that Th17 distribution was closely related to micro vessel density, implying that Th17 might be connected to angiogenesis and therefore promoting tumor proliferation and metastasis. [49]

Wu et al. [50] further stated that IL-17B/IL-17receptor B signaling pathway is a key point for pancreatic cancer malignancy. It enhances the tumor aggressiveness by two different mechanisms: one via transcription factor nuclear factor κB and activator protein-1, which activate IL-8 expression and promotes endothelial cells recruitment, with angiogenic properties; the second by enhancing the pancreatic cell recruitment of macrophages through upregulation of chemokine (C-C motif) ligand 20 (CCL20), chemokine (C-X-Cmotif) ligand 1 (CXCL1) and TFF1 expression. Additionaly, IL-17A was associtated with the induction of acinar ductal metaplasia and PanIns, inducing tumor intiation and progression.

However, some evidence has come to light that blatantly contradicts this tumor-promoting role of Th17. Induction of Th17 and subsequent alteration of the Treg/Th17 balance led to an increased overall survival, with delayed tumor growth. [51]

Th2 are allegedly connected to poorer outcomes in PDAC, with the ratio of Th2/Th1 infiltrating lymphoid cells being claimed as an independent predictive marker of survival. [52] This might be explained due to Th2 release of certain cytokines, such as IL-13 and IL-5 that seem to increase ECM deposition, enhancing proliferation and attenuating Th1-mediated immune responses [42].

Tregs are also correlated with a lower patient survival. [42]

Dendritic Cells (DCs)

DCs are antigen-presenting cells with a very important role in immunity. In PDAC, though, this ability is suppressed through compromised recruitment, maturation and survival. To this suppression contributes greatly the activation of STAT3, as well as high levels of TGF-β, IL-10 and GM-CSF. [53]

The number of tumor-associated DC cells is much reduced in PDAC patients and, when present, they are located outside the margin of the tumor. DC cells were also found to be decreased in PDAC patients' blood, and higher levels of blood DC cells correlated with higher survival. [54]

STROMA TARGETING THERAPIES: PROMISES AND MISSTEPS

Since the beginning of the exploration of the role of stroma in PDAC several of its components have been gauged as potential therapeutic targets. Many have reached the clinical phase but, however, several have revealed to be failures, while others brought some hope, in the shape of increased overall survival.

Hyaluronidases

HA, as already mentioned, is a component of the ECM that largely contributes to its growth and to the increase of interstitial fluid pressure in the stroma, which dampens the delivery of systemic therapies to the tumor. Furthermore, HA functions as a cast in itself, in which matrix proteoglycans gather and agglomerate. These proteoglycans are largely hydrophilic, and the whole system forms a viscoelastic gel-like matrix, becoming a mean of retaining growth factors, cytokines and chemokines in the TME, highly supportive of tumor growth. [55]

An important role in tumor progression as also been attributed to the receptor for hyaluronan-mediated motility (RHAMM). It is an intracellular molecule that can also be recruited to the cell surface, binding with the HA receptor CD44. RHAMM is overexpressed in several cancer tissues, including PDAC, and it acts as a modulator of several cell functions, such as cell motility, wound healing, invasion and modification of signaling transduction of the KRAS cascade. RHAMM expression is usually downregulated by the tumor suppressor p53, which is mutated in a vast percentage of PDAC patients. The binding of this receptor with HA activates signaling pathways that lead to cell invasion, proliferation and survival. [56]

Based on these actions on cancer progression, HA has become a promising therapeutic target. Bovine hyaluronidases were used first and had promising results when it comes to reducing interstitial fluid pressure but it presented very limited systemic delivery. This problem was overcome by PEGylation of recombinant human hyaluronidase PH20 (PEGPH20), which lengthens the circulatory half-life from minutes to over 20 hours, allowing sustained enzymatic breakdown of HA. [37, 57]. Jacobetz et al.[37] used genetically engineered mouse models of PDAC and depleted HA using PEGPH20, and it was demonstrated it contributed to a hyper permeability-associated phenotype of the cancer vasculature. Provenzano et al. [58] also showed that the enzymatic degradation of HA by PEGPH20 resulted in relief of vascular collapse and, consequently, in an increased gemcitabine tumor cytotoxicity, when compared to the use of gemcitabine alone, with a significant impact in overall survival.

A Phase Ib clinical trial using the combination of gemcitabine with PEGPH20 in patients with untreated stage IV PDAC (NCT01453153) was completed and had promising results. 28 patients were enrolled and received gemcitabine with PEGPH20 in different concentrations (1.0, 1.6 and 3.0 μg/kg). The most common adverse events found were

musculoskeletal pain/spasms, periphereal edema, fatigue and thrombocytopenia. However, the most worrying one was the high incidence (29%) of thromboembolic events. Progression-free survival (PFS) was of 5.0 months in average and overall survival (OS) was of 6.6 months. The subjects' pretreatment levels of HA were evaluated and those with high HA levels had better PFS (7.2 to 3.5 months) and overall survival (13 months to 5.7) when compared to those with lower HA levels, which makes the combination of gemcitabine with PEGPH20 a benefic one, especially to those patients with previous high HA levels. This good response led to further testing of PEGPH20 with gemcitabine and nab-paclitaxel in a randomized phase II clinical trial. [57,47]

Currently, there are two active clinical trials using PEGPH20: a phase III one, following the previously exposed study, with 570 patients that is testing the combination of PEGPH20 with gemcitabine and nab-paclitaxel versus placebo in patients with high HA pretreatment levels (NCT02715804) and a phase I/II trial, with 172 participants, with PEGPH20 and FOLFIRINOX (NCT01959139). In the first one the interim analysis showed a high incidence of thromboembolic events in the arm receiving gemcitabine, nab-paclitaxel and PGPH20, which led to a protocol amendment, introducing prophylactic enoxaparin.

Sonic Hedgehog (SHh) Inhibitors

Aberrant SHh activation is connected to a various amount of cancers, PDAC among them. It is known to stimulate PSCs to modulate stromal production. The Hh ligand is produced by PDAC cells and it binds to PSC's Patched2 receptor. This leads to a signaling pathway that clears the inhibitory effects of smoothened (Smo) and enhables GLI1 transcription factor translocation and production of ECM proteins. [24, 59]

Olive et. al [59] used KPC mice (genetic engineered mouse models of PDAC), to assess whether drug delivery could be increased using SHh inhibitors through stroma disruption and

vascular network modification. They used IPI-926, also known as saridegib, a semi synthetic derivative of cyclopasmine that inhibits Smo, resulting in a very significant decrease in the expression of GLI1, a transcriptional target of the Hh pathway. They further administered mice either gemcitabine or IPI-926 alone or a combination of the two drugs (gem-IPI), and observed that mice treated with gem-IPI had severe decrease of desmoplastic stroma and of collagen I content, resulting in densely packed tumor cells. They also found this combination had a great effect on tumor vessels, with a marked increased of tumor vessel density and of CD31 positive cells, compatible with active development of endothelial precursors. More importantly, the concentration of gemcitabine metabolites was increased by 60% after 10 days of the treatment, results that meet the hypothesis that the depletion of PDAC stroma spurs angiogenesis and enhances drug delivery. The median survival of KPC mice was extended from 11 days (with gemcitabine alone) to 25 days (gem-IPI), with significant reduction of the incidence of liver metastasis. Such promising pre-clinical results were far from anticipating the failure that the clinical trials would prove to be.

In April 2010 a phase Ib/II trial (NCT01130142) started to assess the safety profile and the efficacy of gemcitabine+IPI-926; in phase II it would compare the administration of gemcitabine+IPI-926 versus gemcitabine+placebo but it was cancelled as the preliminary data showed a difference in survival favoring the gemcitabine+placebo arm.

Another study using IPI-926, this time with FOLFIRINOX (NCT01383538), was ongoing at the time, and closed early when these results were released. The available results of the short trial showed, however, that patients receiving an IPI-926 maintenance dose presented a marked decline in CA 19-9, a pancreatic tumor marker, even after FOLFIRINOX discontinuation. Treatment did not, nonetheless, result in consistent increments in tumor perfusion. [60]

A study with another SHh inhibitor produced equally disappointing results. This pilot trial studied GDC-0449 (vismodegib) and gemcitabine in treating patients with advanced PDAC (NCT01195415), reaching the conclusions that GDC-0449 leads to downregulation of GLI1, but GDC-0449 plus gemcitabine was not superior to gemcitabine alone in PDAC treatment, with no improvement in fibrosis and survival. [61]

After these trials some studies have come to shed light on this matter, suggesting that PDAC stroma action is not entirely a pro-carcinogenic one.

Rhim et al [12] used a mouse model with deleted SHh to gauge its effects on cancer initiation and progression. Not only tumorogenesis was not impaired in the mice with the deleted SHh, but also a higher frequency of acinar to ductal metaplasia and PanIN of all grades was observed. As expected, there was reduced stromal content, but the tumors in those mice were more aggressive, with undifferentiated histology, highly increased vascularity and heightened proliferation. Median survival of mice with the deleted SHh was of 3.61±1.97 months, compared to the 6.17±2.65 months in the control group. These same results were recapitulated when a Smo inhibitor (IPI-926) was used. It was made clear that SHh somehow restrains tumor aggressiveness.

In the same train of thought, Özdemir et al. [13] used transgenic mice with deletion of α-SMA myofibroblasts. Starting at PanIN, this led to very aggressive, invasive and undifferentiated tumors. There was decrease of type I collagen and alterations in the PDAC matrix, but there was also a clear suppression of angiogenesis, higher tumor hypoxia, enhanced endothelial-mesenchymal transition properties and promotion of a cancer-stem cell phenotype, that led to a decrease in survival. They also stated that fibrosis minimization does not increase the efficacy of gemcitabine, urging caution on targeting stroma (and PSCs specifically) in PDAC.

There have been some explanations to this discrepancies, such as there could be different effects thanks to genetic knockout instead of the acute blockade of stromal cells (by SHh inhibitors), that studying the initiation phase versus studying later malignant phases gives birth to different results and that could be some dose-dependent and off-target effects. [62]

Transforming Growth Factor β (TGF-β)

TGF-β is a cytokine that plays essential role in the normal cellular development. In a healthy state, it assumes a role in embryogenesis, differentiation and apoptosis. Furthermore, it is one of the key inhibitors of cell proliferation. However, it also takes part in the progression of several cancers, when its ligand is overexpressed by cancer cells. It's now evident that TGF-β performs a paradoxical role, acting as a tumor suppressor in normal cells and in tumorigenesis early stages and as a promoter in later cancer states, enhancing the proliferation ability of tumor cells. [63] Mutations in the TGF-β receptor are very commonly present in PDAC, in more than half of the patients, likely contributing to this change of functions. High levels of TGF-β expression are connected with tumor cell survival and motility, endothelial-mesenchymal transition, immunosuppression, activation of fibroblasts, collagen deposition and vascular formation. [64]

The TGF-β receptor signaling pathway is a complex one and not entirely clear. It is assumed that when a ligand binds to a type II TGF-β receptor it promotes the phosphorilation of a type I TGF-β receptor, leading to phosphorilation of two proteins, SMAD2 and SMAD3, which form heterodymeric complexes with SMAD4. These activated SMAD complexes then translocate to the nucleus where they regulate transcription on several genes. SMAD6 and SMAD7 play an inhibitory role on SMAD3 signaling. [65]

It was demonstrated that in PDAC the activation of the TGF-β receptor signaling pathway leads to an increased activation of SMAD3 and cell growth inhibition. However, the

same group showed that, at the same time, it also activated SMAD7, which in turn led to β-catenin (part of the Wnt signaling pathway, which promotes cell growth) retention, attenuating the SMAD3 effects. There is also activation of vascular endothelial growth factor A (VEGF-A), which promotes vascularization of PDAC cells, thus enhancing their growth and invasiveness. [65]

Ostapoff et al. [64] blocked stromal TGF-β receptor II through an antibody, which strongly promoted epithelial differentiation, and led to a significant decrease in collagen deposition, fibroblast activation and metastasis. It also supported the M1 (pro-inflammatory) phenotype of macrophages. This established the potential of the TGF-β cascade as a therapeutic target.

Other group used a transgenic mouse model with specific pancreatic SMAD-7 expression and with cerulein-induced pancreatic fibrosis, verifying it substantially decreased collagen I and fibronectin deposition and PSCs activation, reducing the fibrosis. [66]

There was a phase I/II clinical study (NCT00844064), which evaluated the potential use of Trabedersen in pancreatic cancer (37 patients), melanoma (19 patients) and colorectal carcinoma (5 patients). Trabederson is an antisense phosphorothioate oligodeoxynucleotide that was designed for specific inhibiton of TGF-β2 synthesis, which had very promising results in preclinical studies. This trial also had good results, with an overall median survival for the PDAC patients of 13.4 months and with the interesting nuance that one of the patients had a complete response of liver metastasis, being still alive after 75 months. A randomized, active-controlled study in stage IV PDAC patients is in preparation. [67]

Another TGF-β signaling inhibitor that has undergone clinical trials is Galunisertib (LY2157299), a small molecule that inhibits TGF-β receptor1 serine/threonine kinase, suppressing the SMAD signaling pathway, and that also showed potential in treatment of

PDAC. There was a Phase I study (NCT01722825) that ensured Galunisertib has good tolerability and safety profile. 10 out 12 patients had evaluable tumor response. [68] There's also been a Phase Ib study (NCT02154646) that evaluated this same inhibitor in combination with gemcitabine, showing favorable tolerability and safety profiles.[68] Phase II trials are needed to further establish efficacy.

Angiogenesis Blockers

Angiogenesis, the formation of new vasculature, is one of the hallmarks of every solid tumor, being a necessary step for tumor growth and metastization. In PDAC, hypoxia is one of the main stimulators of angiogenesis, and the new vessels are tortuous and compressed by the large quantities of ECM. [30,69]

The Vascular Endothelial Growth Factor (VEGF) is one of the main players in this vessel formation. VEGF-A, the most studied member of this family, binds to VEGF receptor 2 (VEGFR-2) on endothelial cells, signaling that is enhanced by neurophilin-1. High levels of VEGF in PDAC are connected to increased liver metastasis and poor survival. The therapeutic possibility of using VEGF inhibitors in this type of cancer is based on their ability to normalize tumor vasculature, thus increasing drug delivery. [69]

To date, several clinical trials using VEGF inhibitors have been done. Bevacizumab is a recombinant humanized monoclonal antibody against VEGF-A which had very good results in other solid malignancies, such as colorectal cancer, but provided poor results in PDAC. There was a double-blind, placebo-controlled, randomized phase III study (NCT00088894) of the combination of gemcitabine with bevacizumab versus gemcitabine with placebo in advanced PDAC. It was documented that the trial combination didn't improve survival, despite the promising results in Phase II studies, which had translated in a median OS of 8.8 months. In Phase III, survival was of 4.99 months in the gemcitabine+bevacizumab arm and

of 5.45 months in the gemcitabine+placebo arm. This disparity in results between the two phases has been attributed to patient selection. [70]

The other antiangiogenic agents that have been submitted to phase III trials have had similar dreary results. It has been the case of sorafenib and ziv-aflibercept. [69]

Vatalanib is a tyrosine kinase inhibitor with high receptor binding affinity for VEGF and PDGF receptor-tyrosine kinases, and had promising results after a Phase II trial, with a 6 months survival, but it is important to point out that it was a single-arm trial and that other receptor tyrosine kinsase inhibitors have had previous seemingly encouraging phase II results, to then flop on the phase III trials. [71]

Due to the failure of anti-VEGF therapies in PDAC, new molecules have been researched. Evofosfamide is an hypoxia-activated prodrug, whose reductive metabolism forms an alkylating species that causes DNA damage in quiescent as well as in dividing cells. Experience with this agent is still limited, but there are already results of clinical trials. A phase II trial that compared the combination of evofosfamide+gemcitabine with gemcitabine alone (NCT01144455) was conceived, and reached encouraging results. PFS, OS at 6 and 12 months, objective tumor response rate and CA-19.9 response rate were favorable to the evofosfamide+gemcitabine combination. This led to the phase III MAESTRO trial (NCT01746979), with 693 patients. However, the primary analysis of this study reveals that this combination failed to prolong survival. [72] The combination of evofosfamide+gemcitabine+nab-paclitaxel was also being studied (NCT02047500), but the trial was halted following the company decision to discontinue the clinical development of evofosfamide.

Another target that has been explored is the hepatocyte growth factor (HGF)–c-MET pathway. PSCs are HGF producers, and this molecule binds to cMET on PDAC cells, leading

to the phosphorilation of the cMET receptor and downstream signaling, supporting tumor growth and cell migration. [47] *In vitro* and *in vivo* experiments showed that HGF blockade using AMG102, an antibody, demonstrated similar efficacy to gemcitabine in containing tumor growth, but was much more effective in reducing angiogenesis and metastasis. Curiously, the antimetastatic effect was lost when AMG103 was combined with gemcitabine, which might be explained by gemcitabine's ability in selecting a subpopulation of cancer cells with higher epithelial-mesenchymal transition and stem-cell characteristics. These studies are encouraging to the forging of clinical trials using HGF pathway blockade. [73]

Angiotensin II (AngII) Inhibitors

Angiotensin II is a peptide hormone that has also been involved in PDAC progression. It is one of the main players in the renin-angiotensin system that regulates blood pressure and renal excretion. There are two main receptor subtypes for angiotensin II: angII type1 receptor (AT1R) and angII type 2 receptor (AT2R). When AT1R is activated it stimulates several pathways that lead to the production of inositol, 1,4,5 triphosphate and activation of protein kinase C. Higher levels of AT1R have been detected in premalignant and invasive PDAC lesions. [74]

After observing that the use of angiotensin converting enzyme inhibitors (ACEi) attenuated pancreatic fibrosis *in vivo*, the hypothesis that AngII is involved in fibrosis was established, and its mechanisms have been unveiled. AngII, through AT1R, stimulates PSC proliferation through EGF transactivation- anti-phospo-extracellular regulated kinase (ERK) activation pathway. [75] Ang II, also through AT1R and activation of ERK1/2 pathway induces production of VEGF, which leads to increased vascularity and subsequently enhanced invasiveness and metastasis potential of PDAC cells, effect that was reduced by the use of an

AT1R antagonist. [76] This not only established the role of angiotensin in PDAC progression but also brought to light its possible uses as a therapeutic target.

Nakai et al [77] published an interesting study in which they concluded that inhibiting the renin-angiotensin system in patients with advanced PDAC cancer receiving gemcitabine correlates with a better prognosis. They analyzed the data of 155 patients undergoing gemcitabine therapy that were divided in three groups: one receiving either angiotensin-converting-enzyme inhibitors (ACEi) or AT1R blockers (ARB) for hypertension (HT), one with hypertension but not receiving any medication and a final one without hypertension and thus not medicated. The group receiving ACEi or AT1R blockers had a PFS of 8.7 months and median OS of 15.1 months. The PFS of the other groups was of 4.5 in the HT non-medicated group and 3.6 months in the non-HT group. OS was 8.9 and 9.5 respectively.

Losartan is an AT1R blocker largely used in hypertension therapy. A group established that losartan dose-dependently decreased cell survival, by stimulating the proapototic signaling pathways, such as p53. It was also shown that it induces caspase-3 activation, a key player in the execution phase of apoptosis. [74] Furthermore, its use was associated with decrease expression of several profibrotic signals downstream of AT1R signaling, like TGF-β, reducing stromal collagen and hyaluronan production. Ultimately this causes vascular decompression and improves drug delivery. [78] In preclinical studies with orthopic pancreatic cancer models the combination of gemcitabine and losartan significantly improved survival by suppressing VEGF production and cancer cell proliferation. [79]

Clinical trials have been occurring using another ARB, candesartan, but had disappointing results. Overall response rate was of 11.4%, PFS of 4.3 months and OS 9.1 months. This combination therapy was tolerable but failed to demonstrate efficacy in advanced PDAC. [80]

There is currently an active phase II trial (NCT01821729) that tests the efficacy of the combination of FOLFIRINOX and losartan before proton radiation therapy in controlling tumor growth.

Interestingly, AT2R has demonstrated an anti-tumor effect. It has been verified that levels of this receptor have shown a negative correlation with overall survival but the use of a sythethysed ATR2 agonist in low concentrations significantly attenuated the growth of PDAC and increased apoptosis. [81] Clinical studies are needed to establish the safety and efficacy profile in humans.

Connective Tissue Growth Factor (CTGF) Targeting

CTGF is another element found in abundance in the stroma of PDAC. CTGF expression is mediated by chemokine (C-X-C-motif) signaling via CXC receptors, which in turn bind to various growth factors and integrins and modulate fibrotic material deposition and tumor growth and progression. [47]

Elevated levels of CTGF are present in clinical models of PDAC, with a 40 to 60 fold increment when compared to normal pancreatic tissue; its values are well connected to the extent of the desmoplastic reaction in the tumor, and are located mainly in the cancer fibroblasts, including PSCs. CTGF production seems to be stimulated both by TGF-β signaling and by the MEK/ERK pathway, and it results in an complex and intense crosstalk between both the PSCs and the tumor cells, regulated by CXC receptor-dependent chemokines. It was also found out that, in hypoxic conditions, there's a higher expression of CTGF by the PSCs, and that leads to increased aggressiveness of the cancer. [82]

Studies that used a human monoclonal CTGF-specific antibody, FG-3019, came to confirm the role of CTGF in PDAC progression. In an orthopic mouse model, tumor growth, metastasis and angiogenesis were inhibited, without attenuating gemcitabine effects. [83]

More recent studies supported the hypothesis that the blockade of CTGF-CXC axis is a promising therapeutic target. Ijichi [84] et al. used CXCR2 inhibitors and verified significantly decreased CTGF expression and angiogenesis, which lead to extended survival. Neesse et al. [85] stated that CTGF is an important agent in chemoresistance. They used, as in previous studies, FG-3019 but reached the conclusion that alone it has no anti-tumor activity *per se*, and that it didn't affect the vessel density. When combined with gemcitabine, however, it prolonged OS, suggesting that CTGF gives survival cues to cancer cells that counteract the cytotoxic response to gemcitabine. This result is distinct from increased gemcitabine delivery. Based on these discoveries, a Phase I trial (NCT01181245) was launched to evaluate the safety and tolerability of the combination of gemcitabine, erlotinib and FG-3019 in advanced stage PDAC. Results showed improved PFS and OS with high exposure (15 days minimum) to this combination and low baseline plasma level of CTGF.

There's currently another phase I/II trial (NCT02210559) that is probing the use of FG-3019 in combination with both gemcitabine and nab-paclitaxel.

Immunotherapy

Immune and inflammatory cells play a central part in developing and maintaining PDAC's special TME. Immunotherapy has been resurfacing as promising method in cancer treatment, shifting the therapeutic paradigm in several malignancies, such as malignant melanoma and lung cancer. In PDAC, immune therapeutic targets have been deeply explored, as well, with promising results in some cases, and some failures. Immunotherapy in PDAC is a vast field, with numerous ongoing trials; this review, however, will focus solely on three aspects of it.

Cancer Vaccine

Cancer vaccines increase the exposure of cancer-associated antigens to the immune system, stimulating the production of tumor-specific cytotoxic T-cells. In PDAC, the most studied and the vaccine with the best results has been GVAX.

GVAX is a vaccine created from pancreatic tumor cell lines, altered to express GM-CSF, that has had good results in prolonging the OS and PFS, boosting the production of anti-tumor CD8+ T cells in the peripheral lymphocytes. [86]

PDAC is considered a non-immunogenic cancer, given its lack of T cell infiltrates, which makes it less responsive to immunotherapy. An adjuvant clinical trial was developed, using low dose cyclophosphamide with GVAX to deplete Tregs. The infiltration of T cells was substantially increased, which improved survival. [87]

Later on, a chimeric vaccine was developed, adding to GVAX another component, CRS-207, live attenuated *Listeria monocytogenes*-expressing mesothelin. This GVAXCRS-207 vaccine, given with low-dose cyclophosphamide yielded satisfactory results, extending survival for PDAC patients with minimal toxicity. [88]

CD40 Agonists

CD40 is a cell surface molecule that is a part of the TNF super family and constitutes an important step in antitumor immunity. Its effect was thought to be exclusively through T cell regulation, but more recently it was established that its tumor-inhibiting effects were largely due to macrophage activation. CD40 promote the macrophage tumor infiltration and those activated macrophages become tumoricidal and facilitate depletion of tumor stroma. [89]

There has been a phase I study with 22 patients (NCT00711191) that analyzed the combination of a CD40 monoclonal agonist (CP-870,893) with gemcitabine. The results were

promising, since this combination was well tolerated and showed response on tumor growth. The CD40 agonist provoked immune activation, gauged by an increase in inflammatory cytokines and B-cell expression of co-stimulating molecules. It also showed some response in metastatic lesions, but these were very heterogeneous. Although the small experimental group, this trial hinted to a clear therapeutic benefit. Phase II studies are warranted. [90]

Programmed Cell Death 1 Receptor (PD-1) and Cytotoxic T Lymphocyte Antigen 4 (CTLA-4)

Programmed cell death (PD-1) is a cell surface receptor that binds to PD ligands (PDL1 and PDL2) and negatively regulates T-cell activity, aiding cancer cells to escape immune checkpoints. Both ligands are expressed in PDAC. PDL1 positive PDAC tumors are connected with poor prognosis, being associated with low CD8+ infiltration. [91]

PDL1 blockade was found to promote CD8+ T cell infiltration and induce a local immunologic reaction, establishing PD-1 as a critical regulator of tumor expansion in PDAC. Furthermore, it was attested that the combination of a PD-1 monoclonal antibody and gemcitabine has a significant synergistic effect that induces full response without great toxicity. [91]

In a phase I trial, an anti-PDL1 antibody was used alone and had effect on several malignancies, such as melanoma and renal-cell cancer, but not on PDAC tumors. [92]

Cytotoxic T lymphocyte antigen 4 (CTLA4) is a cell surface molecule expressed in T lymphocytes that, similarly to PD-1, negatively regulates T-cell activation. Although preclinical trials were encouraging, a phase II clinical trial using single agent ipilimumab, an anti-CTLA-4 antibody, didn't achieve tumor response. [93]

Despite the discouraging results both these therapies upheld in single agent trials, their combination with other therapeutic agents seems to be a promising technique. Combination of a PD-1 antibody with GVAX was shown to improve survival when compared to therapy with each agent on its own. PD-1 inhibition led to higher CD8+ T lymphocytes levels and tumor-specific IFN-γ production of CD8+ T cells in the stroma. [94] There is currently a large amount of clinical trials that are testing this combination with other therapies, such as a phase II study (NCT03190265) that has just started and evaluates the efficacy of cyclophosphamide, CRS-207, Nivolumab (the PD-1 monoclonal antibody), and Ipilimumab with or without GVAX; and another phase II one (NCT03161379) that is probing the combination of cyclophosphamide, Nivolumab, GVAX, and stereotatic body radiation.

The combination of a PD-L1 blocker and CD40 agonist also resulted in encouraging results. In preclinical studies, it was found that the use of a CD40 agonist increases systemic PD-L1 expression and the combination of it with a PD-1 blocker leads to substantially improved anti-tumor immunity and increased overall survival when compared to either monotherapy. [95]

A phase I trial of tremelimumab (CP-675,206), a CTLA4 monoclonal antibody in combination with gemcitabine (NCT00556023) came to an end with a good tolerability and safety profile, opening doors for more robust trials.

Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) Blockers

The JAK/STAT pathway is a signaling route with important implications in immunity, proliferation, differentiation and apoptosis. JAK constitutes a family of kinases that includes JAK1, JAK2, JAK3 and TYK2; these kinases are activated by cytokines. JAK activation recruits the transcription factors STAT and transcription of regulator genes ensues. [96]

A myriad of preclinical evidence has connected the JAK/STAT pathway with PDAC progression. STAT3, particularly, seems to be overexpressed in PDAC cells, but not in ductal cells in chronic pancreatitis tissues. Functional inactivation of this agent led to significant restrain of proliferation and tumor growth *in vitro*; and blocking of JAK2, STA3's activator, by tyrphostin also provided similar results. [97] In particular, STAT3 seems to be a required agent for KRAS induced PDAC initiation. [96]

More recently, STAT3 knockdown in nude mouse xenografts of PDAC demonstrated that tumor growth in the STAT3-silent mice was considerably decreased, with suppressed tumor invasion into the muscle and vessels, when compared to controls. It was also demonstrated that MMP-7 expression was reduced in STAT3-silent models. [98]

Ruxolitinib, a JAK1/JAK2 inhibitor, was already submitted to clinical trials. There has been a randomized, double-blind, phase II trial (NCT01423604), with 127 patients with advanced PDAC whose treatment with gemcitabine had failed, that tested the efficacy of the combination of ruxolitinib+capecitabine versus placebo+capecitabine. This trial didn't reach its primary end point for overall survival; however, the subgroup analysis of patients with high inflammation markers, showed a considerable improve on OS, from 1,8 months in the placebo group, to 2,7 months in the ruxolitinib group. [99] This endorsed two phase III trials (the JANUS1 - NCT02117479- and JANUS2 - NCT02119663), that would test ruxolitinib or placebo, plus capecitabine, in patients with advanced or metastatic PDAC who have failed or were intolerant to first-line chemotherapy. This study was, however, terminated early after a planned interim analysis of JANUS1 showed meager efficacy levels.

Another molecule that had promising results in preclinical trials was ganetespib, a heat shock protein 90 inhibitor that interferes with multiple pathways, such has the JAK/STAT one. It was shown to markedly decrease proliferation in PDAC cell lines, downregulating

JAK2 and therefore diminishing the activation of STAT3. It elicited similar results in mouse models and potentiated the effects of 5-fluouracil+oxaliplatin and gemcitabine+paclitaxel. [100] There was a phase II study (NCT01227018) that evaluated the effects of ganetespib as a second or third line treatment for PDAC, but that was terminated when an interim analysis found it to be ineffective.

Focal Adhesion Kinase (FAK) Blockade

Focal adhesion kinase (FAK) is a group of non receptor protein tyrosine kinases, which include FAK1 and FAK2, expressed by the protein tyrosine kinase 2 gene. They play a role in normal embryogenic development and it is known to be overexpressed in several malignancies, and associated with faster disease progression and poor outcomes. FAK1, particularly, has been implicated in tumor progression events, such as cancer cell migration, proliferation and survival. [101]

FAK has been established as an important element of the crosstalk between the tumor stroma and the PDAC cells, promoting tumor evasion by inducing an immunosuppressive microenvironment. It supports transcription of chemokines that, in turn, promote recruitment of Tregs that will stall cytotoxic CD8+ T cells effect, enabling tumor growth. FAK activity is elevated in PDAC tissues and it is connected with higher levels of fibrosis. [102]

Jian et al. [102] used a FAK inhibitor, VS-4718, on KPC and KPPC mouse models and drew several crucial conclusions. FAK inhibition prolonged survival via inducing tumor stasis, rather than regression of the tumor mass. It significantly decreased levels of fibrosis, by diminishing collagen deposition and the PSC activation. It also provoked a hefty decrease of immunosuppressive cells in the TME; it inhibited both pro-inflammatory and fibrotic cytokine secretion, and impaired the PDAC cells ability to induce monocyte and granulocyte

migration. It was also concluded, with a great potential clinical benefit, that FAK inhibition makes previously unresponsive tumor cells sensitive to chemotherapy and immunotherapy.

Furthermore, FAK inhibition also works as a sensitizer for other agents. In a study where FAK inhibitor PF573228 was used in combination with a pro-apoptotic death receptor-5 agonist, lexatumumab, it reversed the previously found insensitivity to lexatumumab therapy alone in human pancreatic cell lines and in mouse models. [103]

A phase I dose escalation clinical trial (NCT00666926) of PF-00562271, a FAK inhibitor, was done with the participation of 99 patients with solid malignancies (head and neck, prostate or pancreatic neoplasms). The maximum tolerated dose and the phase II recommended dose were established, and further trials with this agent were encouraged [104]; a trial that studies its efficacy in PDAC patients alone would be recommended.

Several FAK-targeting therapies are currently in a clinical trial phase. There's an active phase I/II study (NCT02758587) that is observing the safety, tolerability and efficacy of the combination of a FAK inhibitor, VS-6063/defactinib, with a PD-1 inhibitor, pembrolizumab. There is also another ongoing study (NCT02546531), still in phase I that explores this previous combination with added gemcitabine. Another combination being assessed is FAK1 inhibitor GSK2256098 with a MEK blocker, Trametinib (NCT02428270). VS-4718, another FAK inhibitor, was being tested in a Phase I trial in combination with gemcitabine and nab-paclitaxel (NCT02651727), but this study was terminated by company's decision. The results of these studies are awaited, to deepen the exploration of this therapeutic target in PDAC with efficacy trials.

Vitamin D

Vitamin D is a group of fat-soluble secosteroids with a long recognized effect on calcium metabolism. However, more recently, studies point to an involvement of these agents

in regulation of cell growth, differentiation, apoptosis and angiogenesis. Vitamin D3 (cholecalciferol) is produced in the skin upon absorption of ultraviolet b photons by 7-dehidrocholesterol. It can also be ingested. It is converted firstly in the liver to 25-hydroxivitamin D (25D) and then in the kidneys or target tissues to 1,25-dehydroxivitamin D (1,25,D), also referred as calcitriol. The 25D blood levels accurately reflect the amount of vitamin D3 produced in the skin or ingested, being used as a marker of vitamin D status. Vitamin D binds to vitamin D receptor (VDR) that is present in the nuclei of target cells. [105]

Vitamin D has been connected to PDAC exactly by its functions as a cell proliferation modulator. It was established that VDR expression was positively connected to better prognosis. In one study, VDR was detected in all healthy tissues but in only 62.5% of highly differentiated cancer tissues. In 75.7% of tissues with moderate or low differentiation VDR levels were very low or indetectable. It was also present in higher amounts in smaller tumors. [106]. The studies in the prognostic role of 25D levels are, however, inconsistent. [105]

In an important study, Sherman et al. [107] verified the presence of VDR in PDAC stroma, especially in PSCs, that express high levels of this receptor. They used calcipotriol (cal), a nonhypercalcemic vitamin D analog in models of PDAC and demonstrated that it reduces the number of activated PSCs, inhibits negative regulators and supports positive regulators of angiogenesis and reduces SMAD3 binding in the promoter regions of fibrogenetic genes, acting also as an inhibitor of the TGF-β pathway. Cal-treated mice had attenuated inflammation and fibrosis when compared to the control ones. It was also verified the decrease of activation of tumor-connected genes, such as those expressing ECM components, growth factors and cytokines. They also established that stromal VDR activation suppresses the tumor supporting PSC secretoma, muting the crosstalk between PSCs and cancer cells. Use of cal also enhanced intratumoral angiogenesis, with an accompanying

increase in gemcitabine delivery. Tumor size and stromal reaction extension were successfully reduced. Compared to gemcitabine alone, KPC mice that received gemcitabine+cal had a rise of 57% in median survival. These results opened doors for clinical trials.

There was a phase II study that explored the combination of calcitriol with docetaxel in patients with advanced PDAC. The median PFS of the patients treated with this combination was 15 weeks and median OS of 25 weeks; it represented a modest increase of these parameters when compared to docetaxel alone but it came up short when compared to gemcitabine therapy. [108]

There are currently several clinical trials that are testing the use of paricalcitol, a calcitriol analog, in PDAC. Particularly, a phase I study is evaluating the combination of paricalcitol and pembrolizumab with or without chemotherapy in resectable cancers (NCT02930902); and a pilot pharmacodynamic trial is studying paricalcitol as a neoadjuvant to target the stroma in resectable PDAC (NCT02030860).

Pirfenidone

Pirfenidone ([5-methyl-1-2-[1H]-pyridone]) is a pyridine compound with established therapeutic value in certain fibrotic malignancies. Its use showed a suppression of the PSC-related stromal growth, stalling proliferation of the stellate cells and reducing the synthesis and secretion of factors involved in the tumor-stroma crosstalk, as HGF, PDGF, collagen I, fibronectin and periostin. It also inhibits tumor formation and growth in vivo and it contributed to a lower number of peritoneal disseminated nodules and incidence of liver metastasis, effects that were enhanced by combination with gemcitabine. [109]

Ji et al. [110] developed a liposome, responsive to a MMP2, which integrated antifibrotic and chemotherapeutic drugs for modulation of PSCs and enhanced targeted

delivery of cytotoxic agents. Upon MMP2 cleavage the liposome sliced into two functional parts, one containing pirfenidone, that inhibited TGF-β expression and collagen I deposition, and the other gemcitabine, targeting tumor cells. It showed decreased stroma reactions and increased drug perfusion in the tumor cells, which successfully provided restriction of tumor growth. It is a promising step in the development of nanomaterials to improve PDAC treatment.

Until this date there are no Pirfenidone clinical trials in PDAC yet.

CONCLUSIONS AND DISCUSSION

PDAC is a highly aggressive and lethal neoplasm, that usually presents with locally advanced or with metastatic disease. Its 5 year survival is, at its best, of about 7%. [8] Current management options for unresectable PDAC, such has regimens of gemcitabine with nab-paclitaxel and FOLFIRINOX, offer a very small increase in survival, which highlights the pressing need for new therapeutic strategies. [10,11]

In the last few years the knowledge about PDAC stroma has increased immensely. It is understood, now, that the desmoplastic reaction that nurses the cancer cells is not only a passive bystander in PDAC development, but plays instead a central role in carcinogenesis, metastatic spread and chemotherapy resistance.

As this review has brought to light, preclinical trials targeting cancer stroma have held great potential, with very good results in depleting tumor progression and prolonging survival. However, when brought to the clinical phases, most of them have failed to demonstrate efficacy. Those which have held good results still need to undergo more robust clinical trials.

Recent works have offered some measure of explanation to the failures in clinical investigation. Tumors with depleted stroma have been shown to exhibit more aggressive characteristics, with undifferentiated phenotypes, an increment in vascularity and accelerated cancer cell proliferation. [12] The decrease in the number of α-SMA expressing fibroblasts was also correlated with shortened survival. [13] This points to a more complex interaction between stromal components and cancer cells than the one that was used as premise for the investigation of stromal-targeting therapies: stroma doesn't have a purely promoting role when it comes to carcinogenesis, some parts of it also act as a protecting factor.

The role of stroma in chemotherapy resistance has also been established, with improved drug delivery when suppressing stroma [59] but then reiterated by the work of Rhim et al., that suggested that the discrepancy between the good results of SHh inhibition in preclinical trials and the failure of the clinical studies may be due to the fact that short-term, beneficial effects of increased drug delivery are eventually surpassed by the negative effects of long-term stroma inhibition. [12]

These findings point towards a different direction for stroma targeting therapies' investigation: the modulation of the stroma microenvironment, rather than its blunt suppression. The coupling of several targeting therapies may be a way to move forward. There is need to find a point of balance between a chemotherapy regimen and targeted therapies that suppress stroma interactions but heighten immune surveillance and drug delivery. There are currently a vast number of studies exploring different combination therapies that may be groundbreaking. Also, a better understanding of the tumor-restraining role of stroma is needed, as a way to surely know which stromal components should be suppressed and which could be enhanced.

Another step that should be explored in PDAC management is the tailoring of therapies according to the cancer "subtype". Moffitt et al. [111], through advanced bioinformatic methods, have been able to discriminate gene signatures from tumor cells and stromal cells that independently predict patient outcome. This opens a door for personalized, more effective, targeted therapies.

ABBREVIATIONS

α-SMA - α-smooth muscle actin

ACEi - Angiotensin-converting-enzyme inhibitor

AngII – Angiotensin II

ARB – Angiotensin II receptor blocker

AT(1/2)R – Angiotensin II type1/2 receptor

Cal - Calcipotriol

CTGF - Connective tissue growth factor

CTLA-4 - Cytotoxic T lymphocyte antigen 4

DCs – Dendritic cells

ECM – Extracellular matrix

EGF - Epidermal growth factor

ET – Endothelin

FAK - Focal adhesion kinase

FAP - Fibroblast activation protein-α

GM-CSF - Granulocyte-macrophage colony stimulating factor

HA – Hyaluronan

HGF – Hepatocyte growth factor

HT - Hypertension

IFN - Interferon

IGF – Insulin-like growth factor

IL – Interleukin

IL-1ra – Interleukin-1 receptor antagonist

MAPk – Mitogen-activated protein kinase

MDSCs- Myeloid derived suppressor cells

MMPs- Matrix metalloproteinases

MT-MMP – Membrane-type matrix metalloproteinase

OS – Overall survival

PanIN – Pancreatic intraepithelial neoplasm

PD-1 - Programmed cell death 1 receptor

PDAC – Pancreatic ductal adenocarcinoma

PDGF - Platelet derived growth factor

PFS – Progression-free survival

PI3K – Phospoinositide 3-kinase

PSCs – Pancreatic stellate cells

RHAMM - Receptor for hyaluronan-mediated motility

SHh – Sonic Hedgehog

Smo - Smoothened

TAMs - Tumor associated macrophages

TANs – Tumor associated neutrophils

TFF1 - Trefoil factor 1

TGFβ – Transforming growth factor β

Th – T helper

TIMPs - Tissue inhibitors of metalloproteinases

TME – Tumor microenvironment

TNF – Tumor necrosis factor

Treg – Regulatory T cells

VEGF – Vascular endothelial growth factor

VDR – Vitamin D receptor

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