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### ► To cite this version:

Benedetta Ferrara, Cataldo Pignatelli, Melissande Cossutta, Antonio Citro, José Courty, et al.. The extracellular matrix in pancreatic cancer: description of a complex network and promising therapeutic options. Contemporary Southeast Asia, In press. hal-03331434

**HAL Id: hal-03331434**

**<https://hal.science/hal-03331434>**

Submitted on 1 Sep 2021

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## REVIEW

### **The extracellular matrix in pancreatic cancer: description of a complex network and promising therapeutic options**

Benedetta Ferrara, Cataldo Pignatelli, Melissande Cossutta, Antonio Citro, José Courty, Lorenzo Piemonti

#### **Abstract**

Pancreatic ductal adenocarcinoma (PDAC) presents an abundant fibrotic stroma characterized by a stiff extracellular matrix (ECM) which is constantly remodeled to create a microenvironment promoting tumor growth and metastasis. The importance of stroma in driving and supporting PDAC progression is becoming clear as well as its relevance in reducing the efficacy of current therapies. In fact, the dense ECM can act as a natural physical barrier impairing drug penetration. The homeostatic restoration of the fibrotic stroma in combination with an anticancer therapy might be an appealing option for improving drug penetration and thereby PDAC treatment. Several approaches have been adopted in order to target the PDAC stroma, such as the depletion of ECM components and the targeting of cancer associated fibroblasts (CAFs), which are responsible for the increased matrix deposition in cancer. Even though many stroma-targeting strategies have reported disappointing results in clinics, the ECM offers a wide range of potential therapeutic targets that are now being investigated. The leaky and collapsing blood vessels characterizing the tumor might be normalized thus restoring blood perfusion and allowing the penetration of drugs within the tumor mass. The dense ECM might be by-passed by implementing nanoparticle-based systems or by using mesenchymal stem cells as drug carriers. The present review aims at giving an overview of the principal mechanisms involved in the ECM remodeling and of new promising therapeutic strategies for PDAC.

#### **1. Introduction**

Pancreatic ductal adenocarcinoma (PDAC) is a malignancy with a really dramatic clinical course and is the third cause of cancer-related death in the US, with a 5-year survival rate of lower than 10% [1]. The clinical stage of PDAC includes four classes: 1 (resectable tumor comprised between 2 and 4 cm), 2 (tumor > 4 cm, localized to the pancreas), 3 (unresectable tumor expanded to the nearby blood vessel or lymph

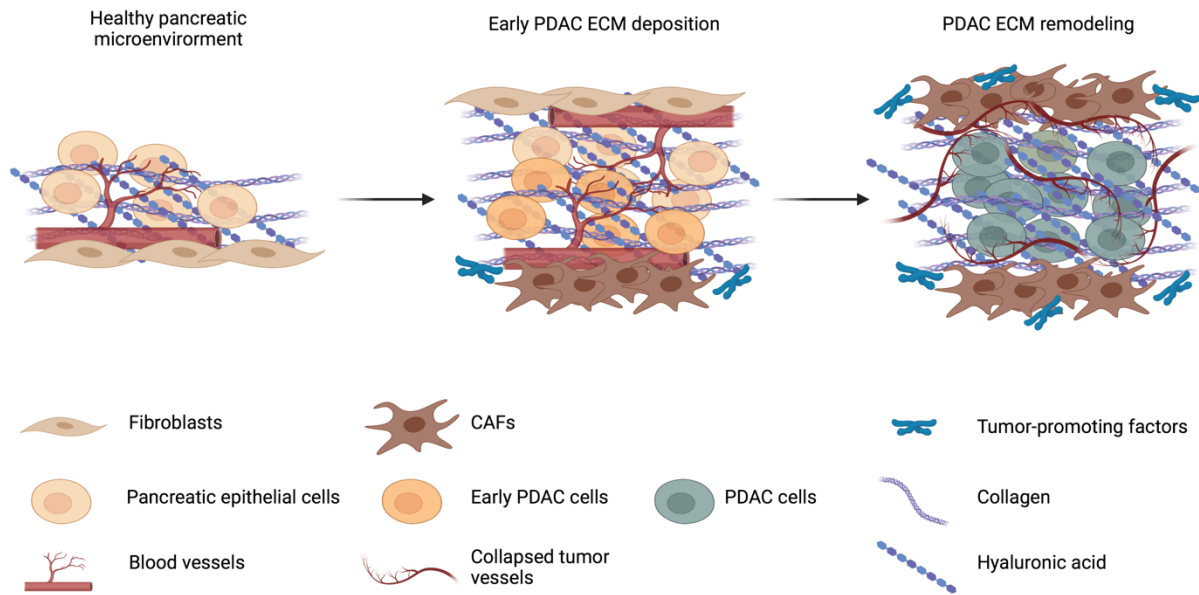
nodes), 4 (metastatic disease) [2]. As for now, the PDAC is a “silent” disease due to the absence of biomarkers and non-specific symptoms especially in the early stages [3]. Concordantly, 80-85% of patients display a locally advanced or metastatic disease at the time of diagnosis, thereby making chemotherapy or radiotherapy the primary treatment options [4]. Even for the small subset of cases eligible for surgical resection, the prognosis remains poor with a high risk of recurrence especially within the first two years post-surgery [5]. In recent years, some advancements in the chemotherapeutic regimens have modestly improved the overall survival of patients. Conventional chemotherapeutic monotherapy based on gemcitabine (GCB) has been widely used in the past as PDAC standard treatment [6]. However, the therapeutic responses using GCB were disappointing. Among the tested strategies, the combination of GCB and nab-paclitaxel reported to significantly improve the overall patients survival, the progression-free survival and the response rate [7]. Moreover, a combination of chemotherapeutic drugs (FOLFIRINOX: oxaliplatin, irinotecan, leucovorin and 5-fluorouracil) was developed and demonstrated to prolong patients survival when compared to GCB alone [8]. Modified (m)FOLFIRINOX was further obtained removing the 5-fluorouracil bolus from the regimen and became the preferred adjuvant therapy for patients with PDAC who have undergone surgical resection and have not received neoadjuvant chemotherapy [9]. Despite these treatments, the drug resistance of PDAC still leads to extremely poor outcomes. The dense fibrous stroma surrounding the tumor mass, together with the abnormal vasculature network and the immune-suppressive microenvironment typical of this cancer type, are among the causes of this drug resistance [10]. The tumor microenvironment (TME) in PDAC is composed of a stiff extracellular matrix (ECM) based on collagen I, elastin and fibronectin, as well as hyaluronan and other sulfated glycosaminoglycans, which create a dense network together with surrounding fibroblasts, endothelial cells and infiltrating immune cells [11]. The remarkable ECM stiffness and desmoplasia surrounding PDAC tumor cells do not only constitute an anatomically supporting tissue, but dynamically contribute to generate a specific microenvironment facilitating tumor growth, metastasis and survival (Fig. 1) [12–15] and can constitute a barrier for chemotherapeutic drugs [16,17]. PDAC stroma is hypovascularized, presenting tortuous, compressed and poorly functional blood vessels and this phenotype is determined by different factors that can be extrinsic to blood vessels (related to the physical and chemical properties of the ECM) or intrinsic

to blood vessels (related to endothelial cell activation and tumor angiogenesis) [18]. In the last years, an increasing number of scientific evidence has highlighted the influence of the physical and mechanical properties of the tumor comprising stiffness, hypoxia and chaotic vascularization, on the drug-resistance or metastasizing ability which are typical of this cancer [19–23]. Several preclinical and clinical studies have been investigating numerous systems to target the ECM in PDAC. In this review, besides describing the principal mechanisms and key players involved in the ECM remodeling, we will focus our discussion on the existing or future therapeutic strategies to overcome the dense ECM of PDAC.

## **2. Cellular component of PDAC microenvironment: heterogeneity and plasticity of cancer-associated fibroblasts (CAFs)**

Fibroblasts are present in all solid organs. One of the most important characteristics of these cells is that they produce and release several components of the matrix, cytokines and growth factors and play a role in the regulation of the homeostasis. In cancer, these proliferative activated cells have an important function in the regulation of tumor growth, dissemination and metastasis [24]. As compared to the other cancers, PDAC is characterized by a prominent desmoplasia in which the stroma components occupy more than 70% of the total tumor volume [25]. This dense desmoplastic stroma is functionally structured into several parts including acellular components derived from ECM and cellular ones that include endothelial and perivascular cells, immune cells, neurons and fibroblasts [26]. It is now well established that all these components interact and participate to promote the growth of PDAC [27]. Among the cellular actors involved in the regulation of the tumor growth and invasive behavior, cancer-associated fibroblasts (CAFs) are activated fibroblasts found into the TME or around it [28]. This cell type originates from activated resident fibroblasts [29], transdifferentiation of epithelial cells or pericytes [30] or differentiation from mesenchymal progenitor cells located into the tumor [31], adipocytes [32] or stem cells [33,34]. Several studies have shown that fibroblasts differentiation into CAFs is triggered by various growth factors, chemokines or inflammatory cytokines, such as FGF-2, TGF- $\beta$ , IL-6, IL-10 or PDGF expressed by cell components of the TME [35–37]. CAFs can display various phenotypes and functions according to the tumor tissue in which they are located [34,38–40]. Depending on the biological function, several subpopulations of CAFs can be defined

accounting for a certain heterogeneity. Using several experimental biological models including co-cultured cells and organoids, followed by validation of the data in human pancreatic tumors, two types of CAFs have been described by the Tuveson's research group [41]. The first one is represented by the inflammatory CAFs (iCAF) which display a low expression of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and a high expression of inflammatory cytokines such as interleukin-6 (IL-6), IL-11 and LIF. The second one is the CAFs identified as myofibroblasts (myCAF), with a high expression of  $\alpha$ -SMA and low production of inflammatory cytokines. Interestingly, immunohistological studies have indicated that there is a different localization of these two CAFs types in the TME. Indeed, while myCAFs are located close to tumor cells, the majority of iCAFs are found distantly to the tumor foci [41]. Using single-cell RNA sequencing, the existence of myCAF and iCAF was further confirmed and a gene signature has been defined [42]. Using this approach, a third class of CAFs expressing MHC class II-related genes was also identified [42]. This subclass of CAFs named « antigen-presenting CAFs » (apCAFs) induces T-cell receptor ligation in CD4<sup>+</sup> T cells in an antigen-dependent manner. The existence of this heterogeneity in CAFs and their plasticity to acquire different phenotypes has been confirmed by other studies [43][44]. In contrast to the fact that normal fibroblasts have the capacity to inhibit cancer growth [45,46], several reports demonstrated that CAFs promote tumor growth through different pathways including an abnormal production of ECM components as well as matrix-remodeling molecules such as heparanase and matrix metalloproteinases (MMP) [47–49]. CAFs also express regulatory molecules such as growth factors that affect tumor angiogenesis and participate to the activation of quiescent fibroblasts [50–52], chemokines generating an immunosuppressive TME [53] and cytokines inducing inflammation [41,54]. All of these characteristics make CAFs a real "cellular conductor" that truly control tumor growth in the primary tumor and metastases [55–57], playing a role also in the acquisition of drug resistance [58–61]. The protumorigenic potential of CAFs has prompted studies to target this cell type as a therapy for the PDAC.



**Figure 1.** The extracellular matrix (ECM) modifications in pancreatic ductal adenocarcinoma (PDAC). In physiological conditions, pancreatic epithelial cells are surrounded by: the ECM with its molecular components; cells providing structural and nutritive support, such as fibroblasts; and the vasculature network. During tumor transformation, the enhanced ECM deposition by cancer associated fibroblasts (CAFs), also thanks to the help of molecular messengers such as TGF- $\beta$  or SHH, forms a dense and stiff matrix around early PDAC cells. This complex meshwork, together with the formation of new collapsed and leaky blood vessels, creates a tumor microenvironment, which favors PDAC growth and invasiveness, activating intracellular pathways that induce pro-tumorigenic programs.

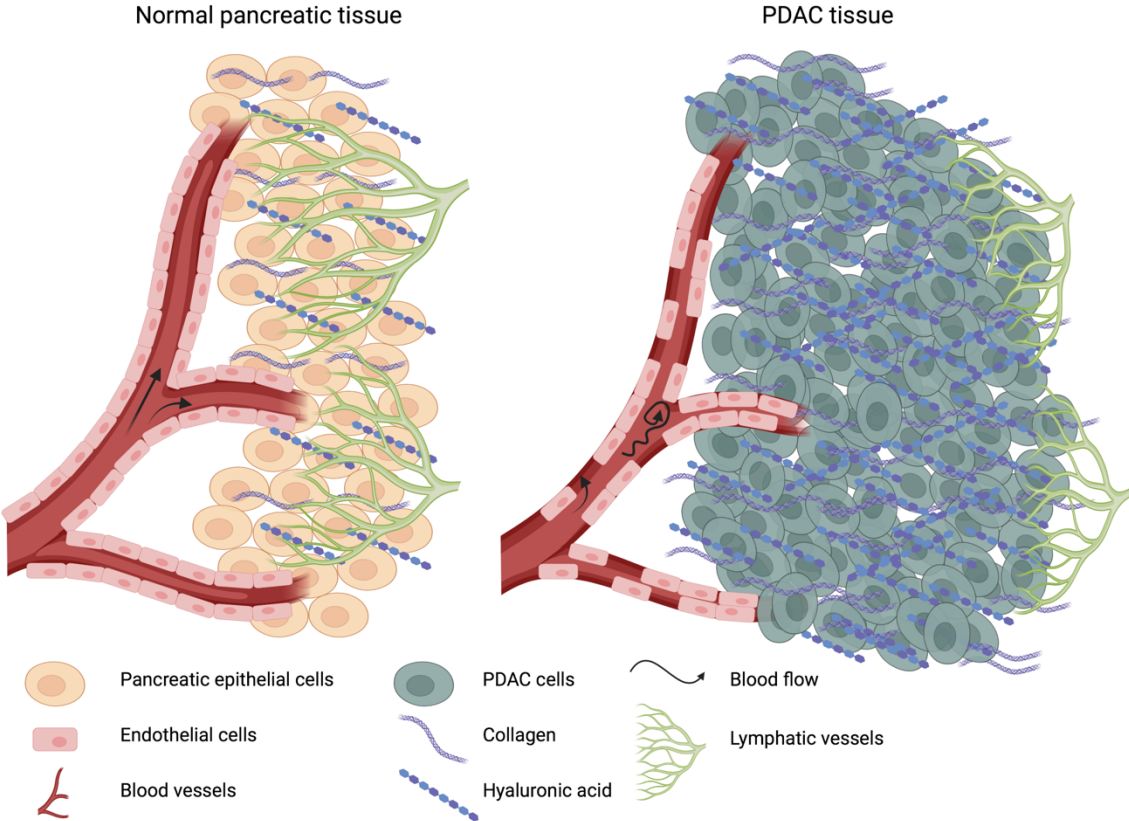
### 3 Physical and mechanical modifications of the matrix in PDAC

#### 3.1 Fibrosis and ECM remodeling affect pancreatic microenvironment

Fibrosis is a pathological process that induces changes in ECM composition and organization, leading to scar formation within tissues during dysregulated wound repair. It brings to substitution of normal structures with fibrotic ECM, invasion and proliferation of mesenchymal cells, completely affecting tissue functions [62]. The fibrotic process is caused by an aberrant activity of the ECM remodeling machinery. Consequently, the ECM modification might induce an altered cellular response that, in chronic wound healing processes, can culminate in malignant proliferation and migration of cells, which is the prelude to tumorigenesis [16,63]. Fibrosis characterizes the desmoplasia of PDAC (Fig 1) [64]. Here, pancreatic tumor cells can exploit fibrotic mechanisms in order to sustain and maintain an environment suitable for their proliferation and an invasiveness ability. In fact, native interstitial and

basement membrane ECM are replaced by a huge amount of fibrotic ECM, which consists of collagen, especially type I, III and IV, hyaluronic acid (HA) and fibronectin [65]. They are mainly synthesized by PDAC cells and CAFs, which are firstly recruited by tumor cells upon secretion of sonic hedgehog (SHH), TGF $\beta$ 1, FGF2 and PDGF and subsequently stimulated by immune cells, such as macrophages, attracted by the inflammatory environment [66,67]. CAFs acquire myofibroblast phenotype expressing  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) upon activation and show an enhanced collagen synthesis and deposition. Moreover, they are further activated by TGF $\beta$ 1 autocrine signaling, which elicits a harmful self-sustaining mechanism [68]. In healthy condition, type I and type III collagen fibrils are present and confer structural thickness and stiffness to the ECM [69]. Collagen fibrils assembly is favored by cross-links between lysine residues through a process catalyzed by extracellular enzyme lysyl-oxidases (LOX) [70]. LOX enzymes are overexpressed in PDAC increasing collagen fibers cross-linking thus stiffening the ECM [68,71]. Due to their intense crosslinking, LOX can alter cell migration, invasion and increase resistance to treatments [72]. In fact, the use of neutralizing antibodies versus these enzymes has showed that collagen crosslinking as well as proliferation of metastases were reduced suggesting that LOXs exert important roles in tumor progression and invasiveness. Moreover, after their inhibition, vessels density was increased [63,68]. In PDAC, abnormal new collagen deposition increases the density, affects the composition and organization of the fibrils and interstitial ECM physical properties are inevitably affected. Fibrillar collagen type I is one of the molecules mostly involved in desmoplasia [73]. Additionally, type IV collagen is part of the basement membrane and is similarly over synthesized, causing modifications in the architecture of surrounding microenvironment [69]. Hyaluronic acid (HA) has been largely investigated in PDAC, since it is overexpressed in neoplastic and stromal cells [74]. HA is a polysaccharide and non-sulfated GAG component of ECM characterized by important viscoelastic properties and it is involved in water uptake of tissues [17,68,69,75,76]. Its receptor CD44 leads to the activation of different intracellular signaling pathways, including the PI3K-AKT ERK, RhoA and RAS ones, thereby promoting cell survival, invasion and epithelial to mesenchymal transition [77,78]. Deposition of high content of collagen I and HA within the neoplastic tissue (both primary or metastatic tumor) was negatively associated to survival. Other proteins are involved in ECM remodeling and are similarly important in PDAC progression. MMPs

are zinc-containing endopeptidase, which are responsible of ECM degradation during migration and invasion of cells and therefore allow metastasis. More specifically, MMP-2, 7, 9 and 14 were identified to be overexpressed in PDAC patients. Particularly MMP2 secreted by activated fibroblast turn on membrane-associated MMP14 at filopodia level of tumor cells, degrading the basement membrane and inducing cells extravasation. Moreover, MMP7 deficient mice with Kras-driven PDAC showed smaller tumor mass and less liver and lymphatic metastasization, suggesting a role in PDAC progression as well [79–81]. Besides inducing new ECM deposition, stromal cells can also secrete diffusible factors acting on blood vessels. For instance, tumor-associated macrophages (TAMs) have been shown to produce several factors, such as vascular endothelial growth factor-A (VEGF-A), basic fibroblast growth factor (bFGF), urokinase-type plasminogen activator (uPA) and matrix metalloproteinase 9 (MMP9), promoting tumor angiogenesis and vascular permeability [82–84]. It is important to note that bFGF promotes endothelial cell migration *in vitro*, increases VEGF synthesis and induces the synthesis of collagen, fibronectin and proteoglycans by endothelial cells, reinforcing both tumor angiogenesis and the desmoplastic reaction in PDAC tumors [85,86].





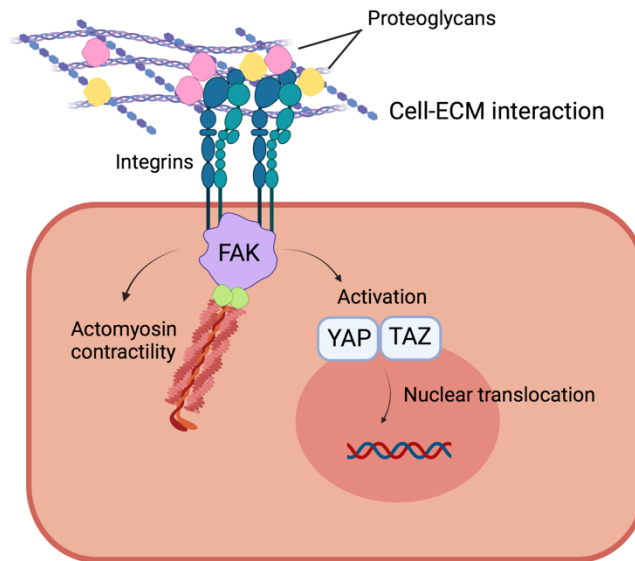
**Figure 2.** Consequences of ECM stiffness and solid stress. In normal conditions, HA and collagen fibers are well organized in the pancreatic tissue. There is no stress throughout the tissue and fluids can easily flow from the blood vessels to the interstitium. Functional lymphatic vessels can drain out the interstitium, keeping its fluid pressure lower than intravascular pressure. In PDAC, the high deposition of new ECM increases the stiffness as well as the solid stress within the tumor mass. Meanwhile, HA increases the osmolarity of the interstitial space, inducing an augmented water uptake from the blood vessel. This, along with the fluid leakage and collapse of both blood and lymphatic vessels, increases the IFP, causing an altered fluid flow.

### **3.2 ECM stiffness, solid stress and interstitial fluid pressure**

As mentioned above, the deposition of new ECM not only affects microarchitecture of pancreatic tissue, but also increases the TME stiffness [63,68]. HA and collagen deposition and intense crosslinking fibrils make ECM more dense and poorly porous (Fig 2). Several studies have investigated PDAC stiffness compared to normal pancreatic tissue. They used direct rheological analysis or elastography techniques. Through analysis of the steady-state modulus, it was possible to demonstrate that PDAC biopsies are stiffer than normal pancreatic tissues, while through ultrasound-based elastography it was possible to generate a map based on tissue stiffness [87–91]. *In vitro* analysis of collagen I and HA hydrogels likewise showed the role of both polymers within the ECM. Collagen is predominant in stiffening matrices, as its concentration increases [92]. Contrarily, HA concentration augmentation have shown a decreasing in stiffness and shifting towards fluid-like properties, but elevated resistance to compressive stress increasing failure stress [93–96]. The elevated resistance to compression is due to hydraulic resistance granted by HA [92].

Furthermore, as reported by Nia *et. al.*, other two physical aberrancies can be identified in the desmoplastic TME: elevated solid stress and elevated interstitial fluid pressure [97–99]. If the stiffness is basically related to the ECM composition and organization, solid stress is due to the cellular components of stroma. Physical forces involved in solid stress are created by: the cytoskeleton filaments involved in cell movement, migration and proliferation; the interaction of cells (tumor cells or CAFs) with ECM; the interplaying forces between the host tissues and tumor. Specifically, it is the combination of all the physical forces derived from the tumor growth. So, if the tumor applies a certain force towards the host tissue, the host tissue tries to respond with similar counter forces. Moreover, although ECM stiffness could remain similar

during the tumor progression, the solid stress could increase, becoming less dependent on the ECM stiffness. Thus tumor mass can expand [98]. While in normal tissues solid stress is null, PDAC displays an elevated solid stress [19]. In particular, primary tumors display a higher solid stress than metastatic ones, while ECM stiffness results similar [99]. Such high solid stress at primary lesions leads to lymphatic and blood vessels collapse when subjected to this pressure [97–100]. Previously, PDAC in *Kras*<sup>LSL-G12D/+</sup>; *Trp53*<sup>LSL-R172H/+</sup>; *Cre* (KPC) mice have mostly shown constricted and collapsed vessels (up to 75%), and HA and especially collagen seems to contribute to this phenomenon [17,101,102]. Therefore, both high stiffness and solid stress cause abnormalities in fluid flow within the tumor mass. Normally, fluids exchange between blood vessels and interstitial space is mainly ruled by intra-vasculature pressure (IVP), which is higher than the interstitial fluid pressure (IFP). Therefore, fluids tend to flow out of blood vessels mainly through convection process, reaching the interstitial space. Here, lymphatic vessels drain the interstitium collecting the fluids. This prevents an increase of pressure within the interstitium. As previously shown, in desmoplastic tumors, HA participates in increasing IFP. Its high deposition induces a higher water uptake within the tissues, swelling the fibrotic ECM and creating a gel fluid phase that contributes to vessels collapsing [17,103]. Oppositely, the stiff collagen matrix acts against ECM swelling, limiting the over-absorption of fluids within tumor [100]. Additionally, since there are no functional lymphatic vessels, the interstitium cannot be drained and IFP further increases. On the other hand, IVP is decreased in PDAC as blood vessels lose their integrity, leading to leakage of fluids (Fig 2) [20,99]. In this condition IFP exceeds IVP, hindering normally fluid flow [97]. So, convection transports become negligible, while diffusion is the dominant mechanisms through which exchanges occur [19]. Therefore, likewise small molecules, also macromolecules, which in normal condition are transported through convection mechanisms, are subjected to diffusive processes, reducing their penetration within tumor mass. Furthermore, altogether, these phenomena lead to reverse the pressure gradient, inducing fluids to be oozed from tumor mass towards the surrounding microenvironment. This brings tumor fluids – loaded with tumor growth factors, cytokines and cancer cells – to be spilled out to adjacent tissues [19,97,100]. Finally, the collapsing of blood vessels provokes a lack of nutrient and oxygen supply to tumor mass, resulting in an acidic and hypoxic environment promoting tumor progression [104].



**Fig 3.** PDAC cell response to stiffness and solid stress. The interaction between ECM and PDAC cells occurs through the binding to adhesion proteins, such as integrins, expressed on the cell surface, and it is further stabilized by proteoglycans. This interaction triggers the actomyosin contractility and the activation of YAP/TAZ which translocate to the nucleus to induce gene transcription.

### ***3.3 Cellular response to stiffness and solid stress***

Some evidences demonstrated that epithelial-mesenchymal transition (EMT), which is the prerequisite to invasion and metastasis, is also elicited by ECM stiffening. In this processes, cellular mechanosensors are involved, including proteins at focal adhesion [65]. Among adhesion proteins, integrin  $\beta 1$  was shown to be overexpressed in cells cultured in stiff matrix [105]. Cells with high number of focal adhesion with matrix have an elevated cytoskeleton tension, which is reflected by higher phosphorylation of the actomyosin systems, responsible of cells contractility and movements [79,106]. Some heparane sulfate proteoglycans (HSPGs) intervene in stabilizing integrin  $\beta 1$  interaction with ECM, allowing tumor cells and CAFs to sense mechanical modifications: agrin and perlecan. Both were observed to be overexpressed within PDAC, through mass spectroscopy analysis. They enhance the response to mechanical cues inducing polymerization and reorganization of actin during cell contraction, elevating actomyosin contractility and increasing the activation of Yes-associate protein (YAP) and its nuclear translocation (Fig 3) [81,107–109].

EMT is triggered by accumulation of YAP and transcriptional coactivator with PDZ-binding motif, also known as WWTR1 (TAZ) within the nuclei [110–114]. At this

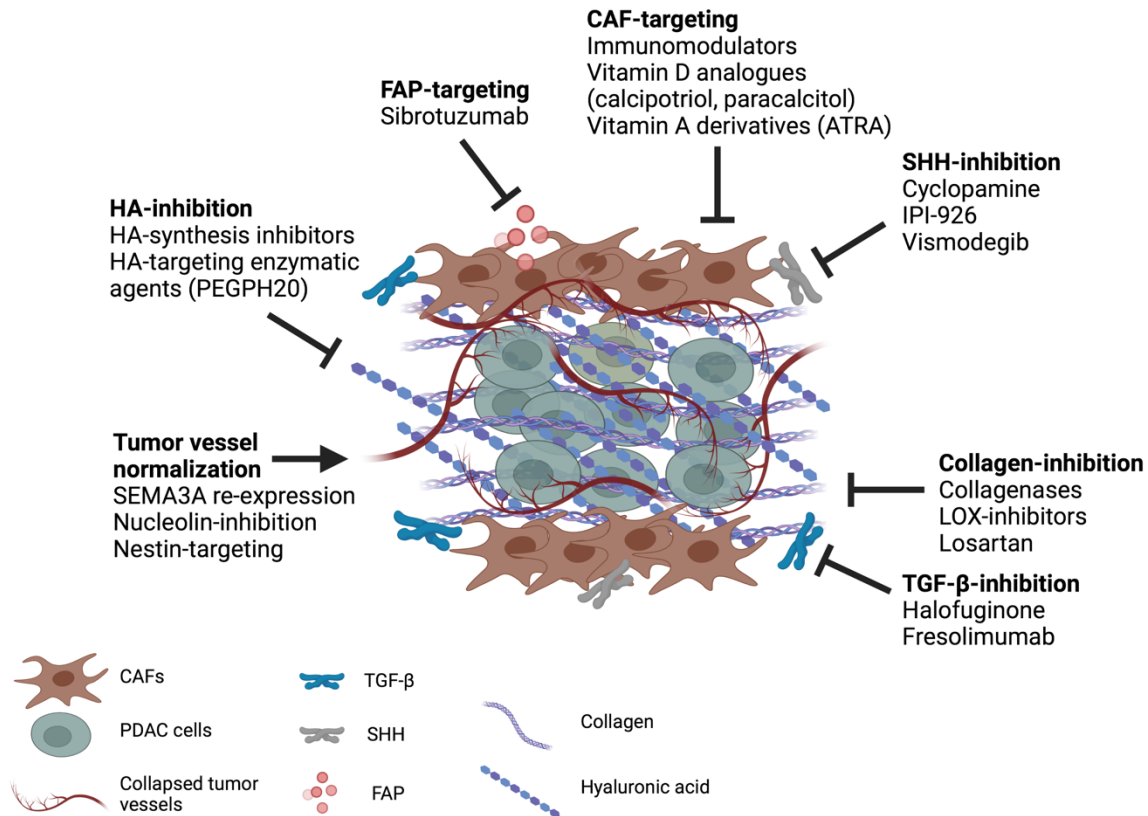
step, cells show less protein designated to maintain cell-cell interactions such as E-cadherin causing a loss of tissue polarity, while they increase expression of vimentin, which is a marker of mesenchymal cells [68,112]. Additionally, integrin  $\beta 1$  has been demonstrated to be involved in inducing the activation of ECM-bound TGF- $\beta$  through mechanical processes. Normally, latent TGF- $\beta$  binding protein (LTBP) retains inactive TGF- $\beta 1$  bound to ECM. During the ECM remodeling and stiffening, integrin  $\beta 1$  of pancreatic stellate cells (PSCs) induces the release of TGF- $\beta$ , activating it. This process is induced by the tension caused upon the activation of the actomyosin system. [79,115]. Through mechanosensors machinery, PDAC cells have the ability to migrate and invade stiffer substrates: that is called durotaxis. Regarding this, activated PSCs have shown to migrate according to substrate rigidity by means of integrin  $\beta 1$  [116]. Additionally, *in vitro* analysis on different pancreatic tumor cell lines in order to evaluate cellular stiffness have shown growing invasiveness ability as stiffness increases [117].

Tumor solid stress can similarly induce several cell responses. It has shown to reduce cell proliferation and induce apoptosis, suggesting a role in regulating tumor morphology and growth. However, recently it reported a role in the induction of pancreatic cell migration *in vitro* by affecting the cytoskeleton organization. During PDAC development, this induces in fibroblasts an increased expression and secretion of growth differentiation factor 15 (GDF15) which is implicated in the mechanisms of tumor cell migration and invasion [98,118].

**Table 1.** List of completed, active or recruiting clinical trials for targeting the ECM in PDAC.

Drug	Design	Status	Phase	Stage
<b>PEGPH20</b>				
NCT01959139	PEGPH20+FOLFIRINOX	Active	I/II	Metastatic PDAC
NCT03193190	Atezolizumab+PEGPH20	Active	I/II	Metastatic PDAC
NCT01839487	PEGPH20+GCB/nab	Completed	II	Metastatic PDAC
NCT04058964	PEGPH20+pembrolizumab	Withdrawn	II	Metastatic PDAC
NCT02921022	PEGPH20+GCB/nab+rivaroxaban	Active	NA	Advanced PDAC
<b>Paricalcitol</b>				
NCT03520790	Paricalcitol+GCB/nab	Active	I/II	Metastatic PDAC
NCT03300921	Paricalcitol+Pembrolizumab	Active	I	Resectable PDAC
NCT03883919	Liposomal Paricalcitol+5-FU/leucovorin	Active	I	Advanced PDAC progressed on GCB-based therapy
NCT03519308	Nivolumab+GCB/nab Paricalcitol	Recruiting	I	Resectable PDAC
NCT04617067	Paricalcitol+GCB/nab	Recruiting	II	Advanced PDAC
NCT04524702	Paricalcitol+Hydroxychloroquine+nab	Recruiting	II	Advanced metastatic PDAC
NCT02930902	Pembrolizumab + Paricalcitol+ GCB/nab	Active	I	Resectable PDAC
NCT03138720	Paricalcitol+nab+GCB+Cisplastin	Active	II	Untreated, resectable, borderline and locally advanced PDAC
NCT03415854	Paricalcitol+Cisplatin+GCB/nab (single arm)	Active	II	Metastatic PDAC
<b>Hydroxychloroquine</b>				
NCT01494155	Hydroxychloroquine Capecitabine+Radiation (single arm)	Active	II	Resectable PDAC
NCT04132505	hydroxychloroquine+binimetinib	Recruiting	I	KRAS mutated metastatic PDAC
NCT03825289	Hydroxychloroquine+trametinib	Recruiting	I	Stage II,III,IV unresectable and metastatic PDAC
NCT04386057	Hydroxychloroquine+LY3214996	Recruiting	II	PDAC
NCT01506973	Hydroxychloroquine+GCB/nab	Active	I/II	Advanced and metastatic PDAC
<b>ATRA</b>				
NCT04241276	ATRA+GCB/nab	NA	II	PDAC
<b>Losartan</b>				
NCT03563248	Losartan+nivolumab+SBRT	recruiting	II	Localized PDAC
NCT01821729	Losartan+radiation	active	II	PDAC
NCT04106856	Losartan+Rx after chemotherapy	recruiting	I	Borderline resectable or locally advanced PDAC
<b>Volociximab</b>				
NCT00401570	Volociximab+GCB	Completed	II	Metastatic PDAC
<b>IPI-926</b>				
NCT01130142	IPI-926+GCB	Completed	I/II	Metastatic PDAC
NCT01383538	IPI-926+FOLFIRINOX	Completed	I	Advanced PDAC
<b>Dexamethasone</b>				
NCT00002689	dexamethasone+FU+radiation	Completed	II	PDAC

GCB/nab, Gemcitabine + nab-Paclitaxel; FU, Fluorouracil; SBRT, stereotactic body radiotherapy; Rx, X-radiation.



**Fig 4.** Schema of the principal drugs and pharmaceutical tools used and proposed for targeting the different components of PDAC stroma.

## 4 Pharmacological tools targeting the stromal barrier: from CAFs to ECM components and vessels normalization

### 4.1 CAF targeting as therapeutic strategy: a double-edged sword

While many studies have focused on epithelial cells in search of anti-PDAC therapy for several years, more recently a large number of therapeutic strategies targeting CAFs has been developed and tested (Table 1; Fig 4). The rationale of these studies comes from their tumor-promoting functions, their ability to produce tumor stromal constituents and their association with poor prognosis in cancer patients [28,119,120]. Several approaches have been undertaken including the inhibition or the reprogramming of CAFs toward a normal phenotype. CAFs activation might be prevented by targeting the SHH signaling pathway. With this aim, cyclopamine was proposed. It is a natural steroidal alkaloid which resulted able to reduce fibronectin content and to improve tumor vascularization in a PDAC xenograft mouse model. Moreover, in combination with PTX-NPs, it increased the inhibition of tumor growth by

63,3% [121,122]. In a preclinical murine model of pancreatic cancer, the administration of IPI-926, which is an inhibitor of SHH receptors, was combined with GCB. The treatment significantly enhanced the bioavailability of GCB in tumor tissue, inducing tumor regression. However, data obtained from clinical studies were disappointing such to interrupt the study due to the reduced patient survival (NCT01130142) [123]. Similar results were obtained in several clinical trials involving combination of GCB or FOLFIRINOX with vismodegib, another SHH inhibitor [124–127]. Another approach targeting CAFs expressing fibroblast activation protein (FAP) has been investigated using diphtheria toxin. The study demonstrated that the toxin is able to enhance the anti-tumorigenic cytotoxicity of CD8<sup>+</sup> T cells and to reduce tumor growth. Moreover, histological analysis of the tumor showed a reduction of CAFs migration toward the metastatic niche [14,128,129]. Analogous observations have been reported in breast and lung cancer preclinical models [14,128,130]. FAP-targeting strategies through immunotherapy have been also proposed. Sibrotuzumab, an anti-FAP antibody was used in a phase II clinical study. The results obtained were very contrasting with no significant effect on tumor development [131].

The role of the stroma is controversial, since it can act not only as a barrier for drug delivery but also as a protective defense mechanism that could prevent and restrain the growth of PDAC tumor. A complete stroma depletion might lead to a more aggressive cancer with a poor survival rate [26,132]. In line with these findings, other studies reported that the depletion of CAFs yielded unexpected results. In a very elegant study using transgenic approaches, mice with the ability to deplete  $\alpha$ SMA<sup>+</sup> myofibroblasts in pancreatic cancer were generated. In this model, the depletion of myofibroblasts resulted in invasive tumors with increased hypoxia and metastasis, as well as an increased infiltration of immunosuppressive cells, such as regulatory T cells, and thus decreased animal survival [133]. Similarly, targeting SHH might lead to augmented tumor progression. In a study, exploiting a well-defined mouse model of PDAC, SHH was deleted and the resultant tumors were reported to be more aggressive, presenting undifferentiated histology with an increase of tumor angiogenesis despite the reduction of the stromal volume [134].

However, among the current therapeutic developments targeting CAFs, immunotherapy could still bring new therapeutic hopes. Indeed, strategies aimed to a vaccination against the FAP antigen in *in vivo* models of colon, breast and lung

cancer have been proposed [135,136]. Tumor reduction was observed in the lung and pancreatic cancer by immunogenic administration of a chimeric T antigen receptor specific for FAP [137–139]. The targeting of CAFs either by a specific antibody or by immunotherapy remains a challenge to be accomplished by clinical validation. Many upstream studies are still required, particularly concerning the type of CAFs to be targeted. This regards the heterogeneity of CAFs, which is caused by their plasticity that could bring to a specific phenotype for one given patient. Therefore, there will be the necessity to address the studies to personalized medicine.

Faced with these difficulties caused by the existence of a great heterogeneity of CAFs, several studies have been undertaken on the deactivation or reprogramming of CAFs into so-called "normal" fibroblasts [31,140]. Among developed approaches, targeting vitamin D receptor (VDR) through the use of analogues of vitamin D resulted in a successful reduction of fibrosis. For instance, calcipotriol revealed to reprogram CAFs into quiescent fibroblasts by stimulating lipid droplets accumulation of vitamin D, which normally occurs in normal fibroblasts, and decreasing the expression of  $\alpha$ SMA [68,141]. In addition, the administration of calcipotriol in combination with GCB demonstrated to enhance the survival of KPC mice [141]. Interestingly, a positive correlation was found between patient survival and the expression level of the VDR [142]. Currently, several trials up to phase III targeting the VDR are being investigated. For example, a randomized phase II study is being evaluated in metastatic PDAC using the combination of GCB, nab-paclitaxel and paricalcitol (NCT 03520790). Another phase Ib or II study testing paricalcitol in patients with resectable pancreatic cancer is ongoing (NCT 03300921, NCT03331562) also using hydroxychloroquine (NCT04524702).

Other studies have also tested natural and synthetic derivatives of vitamin A, such as all-trans retinoic acid (ATRA). These molecules are strongly involved in the control of cell differentiation, growth and apoptosis. In an experimental KPC mouse model, it has been shown that ATRA administration leads to quiescence of CAFs bringing to a reduction of activated stroma, a reduction in the number of cells in the activated stroma as well as a reduction of cancer cell proliferation [143]. Investigation of the mechanism of action indicates that this effect is mediated by inhibition of Wnt [143]. Interestingly, it has also been shown that ATRA administration increases infiltration of CD8-positive T cells in a KPC mouse model [144]. A randomized phase II trial testing



the combination of ATRA with GCB/nab paclitaxel is currently underway (NCT04241276).

#### **4.2 Targeting ECM components**

As for now, several approaches have been investigated to target the dense and stiff matrix of PDAC. Collagen-targeting strategies were proposed to alleviate its excessive deposition in this tumor (Fig 4). The use of collagenases was proposed to degrade collagen, reducing the ECM stiffness and allowing a better delivery of drugs into the tumor site [145]. However, depending on the tissues of delivery, these enzymes can have different *in vivo* half-life, bringing to their inactivation. Therefore, some solutions were proposed for stabilizing these molecules and deliver them to the lesion site. For instance, Zinger A. et al. proposed collagozome which is a 100-nm liposome encapsulating collagenase. The authors demonstrated that the treatment of xenografts PDAC bearing mice with collagozome reported a strong reduction of tumor size (reduction by 87%) when compared to mice treated with the empty liposomes and PTX [146]. However, collagen degradation may induce the release of growth factors and cytokines which could initiate an inflammatory cascade and also tumor progression [147]. Therefore, the right time point for initiating this treatment should be cautiously defined and validated. Another approach for reducing collagen deposition might be to inhibit its synthesis, by blocking TGF- $\beta$  signaling which is crucial during this process. Halofuginone is an anticoccidial which revealed the ability to reduce collagen synthesis by inhibiting TGF- $\beta$  signaling in preclinical models of several solid cancers, including PDAC [148]. Fresolimumab is a monoclonal antibody targeting TGF- $\beta$  and it is currently being investigated in several clinical trials for cancer therapy (NCT01401062 and NCT02581787) [149]. However, also a treatment targeting TGF- $\beta$  should be cautiously defined since it is involved in both inflammatory and tumor processes [150]. Another solution for inhibiting collagen synthesis might be the use of the anti-hypertensive drug losartan, which demonstrated to contribute to the inhibition of collagen synthesis in both preclinical [151] and clinical trials (clinicaltrials.gov identifier: NCT01821729). Furthermore, the inhibition of collagen cross-linking by means of LOX inhibitors may represent a promising strategy to target ECM stiffness. Even if LOX inhibition demonstrated to improve the delivery of chemotherapeutic agents in mouse models of PDAC [152], it might not work for cancers with an existing mature collagen mesh.

Depletion of fibrotic ECM can occur also through the silencing of protein stabilizing mRNA of ECM components. Poly(rC)-binding protein 2 ( $\alpha$ CP2, encoded by the PCBP2 gene) stabilizes type I collagen mRNA. Li et al. proposed a siRNA for silencing the expression of the PCBP2 gene. They evaluated the silencing effect analyzing the expression of collagen I in human PSCs and NIH 3T3 mouse fibroblasts. The treatment with human or mouse PCBP2 siRNA significantly silenced the gene with a 97% knockdown of PCBP2 mRNA expression [153].

Also, the employment of some anti-angiotensin vasodilators has shown to deplete stromal collagen and HA in tumors, simultaneously enhancing the penetration of nanomaterials throughout the tumor stroma [102,154–156]. Within this context, Chen *et al.* proposed a sequential delivery strategy by combining GCB to nitric oxide (NO), which has a role in ruling vascular tone and remodeling [157]. Indeed, NO demonstrated to attenuate fibrosis through the activation of cyclic guanosine monophosphate (cGMP) and soluble guanylyl cyclase (sGC) signaling [158–160]. These pathways seem to interfere with the activation of TGF- $\beta$  signaling, inhibiting fibroblasts activation [158]. To exploit NO property, a system based on liposomes (Lip) loaded with S-nitroso-N-acetylpenicillamine (SNAP), which is a stable NO donor, or GCB has been developed. As expected, the expression of the intratumoral ECM marker  $\alpha$ -SMA and collagen I was significantly reduced after treatment with Lip-SNAP and this system reported a therapeutic efficacy *in vivo*.

To reduce ECM stiffness, integrins have been similarly investigated as pharmaceutical target. At focal adhesion, they are widely expressed by cancer and stromal cells and several preclinical studies assessed that their inhibition could strongly reduce tumor progression [161]. Volociximab is a monoclonal antibody targeting integrin  $\alpha$ 5 $\beta$ 1 and revealed a therapeutic efficacy for the treatment of pancreatic, ovarian, peritoneal and renal cancer patients during clinical trials [162,163].

In addition to the above-mentioned drugs, other pharmaceutical tools developed to directly target PDAC stroma include MMP inhibitors, anti-fibrotic agents, drugs targeting fibronectin and TGF- $\beta$ R. A few studies have reported that tranilast, pirfenidone, fasudil, metformin, and dexamethasone could reduce matrix stiffness in tumors [164–168]. In addition, traditional drugs, such as hydroxychloroquine, retinoic acid receptor agonists, and FAK inhibitors, have the potential to attenuate matrix stiffness in tumors [68,150].

### ***4.3 Reducing the interstitial fluid pressure in the TME***

As previously mentioned, both small and large molecules reach the tumor site mainly through diffusion because the desmoplastic stroma may influence their distribution in the tumor. Similarly, drug transport can be considerably reduced [169,170]. Among the pharmaceutical tools developed with the aim to target the ECM components, different strategies were proposed to reduce the IFP and to by-pass the ECM physical barrier (Table 1; Fig 4). A formulation encapsulating pegylated recombinant human hyaluronidase 20 (PEGPH20) was proposed to enable the degradation of HA [17,171]. Its administration to tumor-bearing KC and KPC mice could deplete HA in the stroma resulting in a decreased IFP and increased diameter of intratumor vessels. Mice receiving PEGPH20 in combination to gemcitabine reported an important reduction of tumor volume, demonstrating that the tumor perfusion of chemotherapeutic agents was enhanced by the treatment. Also a clinical trial (phase Ib/II) was performed using PEGPH20 combined with mFOLFIRINOX in 138 metastatic PDAC patients [172]. Unfortunately, the median survival rate was lower in this group of patients, compared to the only mFOLFIRINOX-receiving group. However, 4 patients receiving the combined treatment were the only patients, among the total 138, who reported a complete response, which is a really rare event in PDAC.

The randomized, blinded phase III study HALO-301 compared PEGPH20 plus nab-paclitaxel/gemcitabine (AG) versus placebo plus AG [173]. The objective response rate was higher with PEGPH20 plus AG, but there was no improvement in the duration of the response. The safety outcomes were consistent with the established profiles of PEGPH20 and AG [7,174,175]. However, the addition of PEGPH20 did not improve the overall survival in the group of treated patients. 494 patients were enrolled and surely a larger study would have had a different outcome. These findings strengthen the concept that more preclinical or retrospective studies need to be performed for reducing the IFP thus targeting the stroma remodeling. However, the concept of reducing the IFP can be promising in order to overcome the ECM physical barrier and new pharmaceutical tools still need to be explored.

### ***4.4 Tumor vessels normalization***

To date, it seems that none of the different combinations using anti-angiogenic agents significantly improve the overall survival of PDAC patients, underlying the urgent need to design new strategies to overcome vascular-induced resistance to pancreatic cancer therapy. Within this context, vascular normalization has the aim to restore tumor vessel structure and functions to decrease the hypoxia-induced mechanisms of resistance to treatment (Fig 4). Structurally, it has been shown in several tumor models that normalization (i) improves the shape and junctions between endothelial cells (ECs), (ii) promotes the covering of vessels by perivascular cells and (iii) restores the composition and rigidity of the basement membrane [176]. Functionally, vascular normalization has reported: (iv) to improve tumor oxygenation and therefore to decrease hypoxia and angiogenesis, (v) to improve tumor perfusion promoting chemotherapy and immunotherapy access to cancer cells, (vi) to limit intravasation of cancer cells and metastasis and (vii) to increase the anti-tumor immune response by promoting tumor infiltration by anti-tumor T lymphocytes and polarization of macrophages from the pro-tumor type M2 phenotype towards the anti-tumor type M1 phenotype [176].

It has been shown that semaphorin 3A (Sema3A) is expressed in ECs during angiogenesis, where it serves as an endogenous inhibitor of angiogenesis that is present in premalignant lesions and lost during tumor progression in human uterine cervical cancer [177]. Genomic and transcriptional analyses of a wide cohort of PDAC patients identified SEMA3A gene as a transcriptional target that is down-regulated by N-terminally truncated p63 (TP63 $\Delta$ N) in squamous PDAC subtype [178]. Maione et al showed that long-term re-expression of Sema3A induces vascular normalization in RIP-Tag2 pancreatic tumor mouse model [177]. On the contrary, some molecules are upregulated during tumor progression. Nucleolin is a glycoprotein located in the nucleus of resting cells but translocated to the cell surface and the cytoplasm of proliferative cells, such as cancer cells and activated ECs [179–181]. Nucleolin is a cell marker of angiogenic vessels [181] and its expression is significantly increased in PDAC patients [182]. Nucleolin targeting by a synthetic antagonist, N6L, inhibits Ang-2 secretion and participates to a program of EC loss-of-activation that increases the recruitment of perivascular cells and normalizes tumor vessels [182]. Nestin is a class VI intermediate filament protein reported to be a progenitor cell marker in various tissues. Nestin expression level is increased in various tumor cells and its expression has been shown in proliferating vascular ECs

[183–185]. The expression of nestin is specific to small, highly proliferative blood vessels in PDAC tissues, whereas CD34 is expressed in all-sized vessels [186]. Micro-vessel density (MVD) is often reported to correlate with prognosis in various gastrointestinal cancers [187,188]. Future studies could allow to clarify if nestin can be a predictive and prognostic marker of MVD [189]. Furthermore, nestin targeting by small interfering RNA (siRNA) has shown a tumor inhibitory effect *in vivo* via the inhibition of tumor angiogenesis in a mouse model of pancreatic cancer [186], suggesting that nestin could be a potential therapeutic target of tumor angiogenesis. It is interesting to note that nucleolin and nestin are expressed both by tumor cells and angiogenic ECs in pancreatic tumors, suggesting that potential targeted therapies could act *via* different cell types of the TME.

## 5. Identifying new ECM targets

Besides improving the existing systems for targeting the matrix or enhancing the efficacy of currently used drugs, novel potential therapeutic targets may be found in the ECM. Different strategies have been developed to identify and characterize the principal players involved in the ECM remodeling. The proteomic approach has been explored for comprehensively profiling the dynamic changes in the composition of the ECM. New proteomic protocols and pipelines were developed to selectively enrich for ECM proteins based on their insolubility compared to the intracellular proteins [190–192]. Also the strategy to deplete highly abundant ECM molecules, such as collagen, may increase and facilitate the proteomic coverage of the proteins less abundant in decellularized tissues [193]. The proteomic approach may be combined with bioinformatic pipelines to reconstitute the concept of the matrisome, which refers to both ECM and ECM-associated proteins. The matrisome project aims to generate different reference matrisomes for the organisms (MatrisomeDB; <http://matrisomeproject.mit.edu/>) by giving information about proteins genomically predicted to encode ECM proteins, as defined by InterPro domain-based structures which represent a classical hallmark of matrix proteins [194]. 55 domains were found in matrix proteins and the goal is now to map the expression patterns related to organ development and disease. Based on these findings, the ECM Atlas was constituted through the compilation of proteomic data sets of ECM molecules obtained from different tissues and diseases. This platform would furnish an interesting reference to access and use information from a huge pool of data [195].

The following step regards the determination of novel ECM proteins and modulators in tissue samples from tumor progression models in order to understand how the ECM regulates disease progression. Pearce et al. defined a matrix index useful for predicting poor prognosis in high-grade serous ovarian cancer [196]. The technique Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) has gained interest as it combines the specificity and sensitivity of mass spectrometry with spatial information, in order to map the distribution of molecules in the tissue [197,198]. In a single experiment, it is possible to identify and quantify several analytes with a high spatial resolution. On 2D tissue sections, a digital MALDI can be created for each ECM component. Since cancer samples present heterogeneous histological ECM structures, the MALDI-MSI may be used as a successful approach in this context because it preserves the spatial distribution and the histology of the samples. It has been recently employed in combination with decellularization approaches for improving the identification and mapping of ECM components in tissues [199]. Indeed, a better characterization of native ECM composition, distribution and organization is important for providing a deeper understanding of its function in diseases such as cancer [200]. The decellularized ECM is assumed to be identical to the composition of native ECM and to possess native mechanical properties. The *In situ* decellularization of tissues (ISDoT) was developed by et Mayorca-Guiliani et al. for allowing tissue decellularization without collapse not to destroy the delicate ECM architecture [201]. They demonstrated that ISDoT enriched ECM molecules making it possible to perform a proteomic analysis to register ECM changes during cancer progression. This enrichment reported a high increase in proteomic coverage when compared to non-decellularized tissues. Using ISDoT samples, it is possible to catalogue the ECM and to map the spatial distribution of its components in 3D in high resolution in the normal context vs. the tumor one. The authors provided for the first time a detailed 3D characterization of the metastatic niche in breast cancer progression and identified several ECM components not previously described. The ability to map the ECM from a spatial point of view is crucial for understanding how it influences the cancer cell invasivity, migratory and proliferative capacity.

A further approach for investigating the principal players involved in the PDAC matrix might be to explore the signature of extracellular vesicles (EVs). As far as known, the cellular interactions and communication occur not only through direct contact

between cells such as cellular gap junctions, but also by EVs which include nano- or micro-vesicles secreted by almost every cell type both in physiological and pathological conditions. [202]. EVs can carry different molecules including mRNAs, miRNAs, long non-coding RNAs, proteins, lipids and carbohydrates [203–205]. Since EVs can be easily obtained from the different biological fluids, the detection of the molecules that they carry makes them a potential source of biomarkers for several diseases including cancer [202,203]. Moreover, they might deliver aberrantly expressed genes or oncogenic proteins [206–208]. Interestingly, EVs showed to contain matrix-degrading enzymes such as matrix MMPs, heparanases, hyaluronidases, ECM metalloproteinase inducer (EMMPRIN) and tissue inhibitors of MMPs (TIMPs). These MMPs presented on EV surface seem to govern different proteolytic activities for the turnover of the ECM thus contributing to matrix remodeling [209–212]. For example, the localization of MMP-9 or  $\alpha$ 5 $\beta$ 1 integrin and their shedding into EVs deriving from cancer cells participate in the localized degradation and proteolysis of ECM during cell migration and thus metastasis [213]. Tumor-derived EVs demonstrated also to induce the expression of MMPs in target cells. Indeed, EV-associated heat shock protein-90 released by cancer cells could induce the expression of MMP-2 which activates plasmin, which is a protease inducing cancer cell invasion [214]. The presence of hyaluronidase Hya11 in EVs derived from prostate cancer induce the prostate stromal cell motility by activating FAK-mediated integrin signaling, reporting that the high Hya11 promotes the progression of this cancer [215].

As previously discussed, the potency of cancer cells to migrate and invade other tissues is largely due to the acquisition of a mesenchymal cell state. The EMT is often characterized by the secretion of MMPs, can weak the intercellular adhesion and reduce cell polarity with implications in metastasis [216–220]. Several studies evidenced that EVs are involved in the EMT [221–223]. Indeed, once secreted from one cell type, they can induce the EMT in the recipient cells [224]. Multiple carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) and ECM proteins were identified in the EVs isolated from pancreatic duct fluid of PDAC patients, indicating a potential implication in the carcinogenesis and diagnosis of PDAC [224].

As such, the presence of proteolytic molecules or proteins implicated in the EMT among EVs may constitute one of the novel sources for identifying new possible

targets which modulate the structural architecture and dynamics of ECM occurring during cancer progression.

## **6. Nanomedicine as therapeutic strategy: improvement of nanoparticle-based systems for by-passing the ECM**

It is well known that nanoparticles (NPs) constitute a successful platform for drug delivery since they can improve the bioavailability and solubility of carried drugs. They can specifically reach the tumor site thanks to the enhanced permeability retention (EPR) effect, which is due to the leakiness of vessels occurring during tumor angiogenesis and to the impairment of lymphatic drainage [225]. Even though the use of NPs has been largely investigated in preclinical and clinical trials, the tools based on NPs vehiculating anticancer molecules provided only modest benefits in term of survival [226]. Indeed, the abnormal TME and the heterogeneity of each tumor can negatively affect the EPR effect. The difficult tumor vasculature and the dense basement membrane may limit the vascular and interstitial transport of nanocarriers. In pancreatic cancer, where the interstitial space is thick and collagen fibers are crosslinked generating a stiff matrix, the extravasation of NPs into the tumor interstitium is limited [225]. The physicochemical properties of NPs can be exploited to overcome these limitations. Indeed, PEGylated NPs which are steric small particles with a size <50 nm are able to penetrate through stroma-rich tumors, as demonstrated on BxPC3 pancreatic cancer cell line, better than larger NPs >50 nm [227]. Regarding particle charge, PEGylated NPs and neutrally charged liposomes display the ability to easily diffuse in ECM hydrogel and deep penetrate into tumors, while cationic NPs remain entrapped in the hydrogel [228]. However, cationic NPs exhibit a better transvascular transport by targeting endothelial cells [229]. Other studies revealed that NPs with linear and semi-flexible shape can diffuse and penetrate more efficiently through the interstitial matrix compared with solid spherical NPs of similar size [225,230]. The *in vivo* biodistribution of NPs can be influenced also by the interaction of NPs with biological fluids, which make them acquire a surface corona of biomolecules, such as proteins or lipids [231]. For example, the FDA approved albumin-bound form of paclitaxel, Abraxane<sup>TM</sup>, was generated using this approach, that allow it to acquire a prolonged circulation time [232].



As an acidic pH characterizes PDAC, smart NPs with ultrahigh pH sensitivity switching size in the acidic TME of PDAC were developed by Lucero-Acuna and Guzman, and with this system the penetration of encapsulated anticancer drugs was improved [233]. pH-sensitive NPs were developed also by Fan et al (2021), who proposed a system composed of membrane-disruptive macromolecules to facilitate the penetration of drugs through the stromal barrier. This nano-formulation displayed an acid-activated cytotoxicity towards both cancer cells and fibroblasts, by disrupting the cell membrane integrity in an acid-dependent manner. Therefore, the permeabilization of the stromal barrier allowed to target and to inhibit cancer cells. This effect was demonstrated *in vitro*, using 3D spheroids containing both BxPC-3 cells and fibroblasts, and also *in vivo* on xenograft BxPC-3 tumor bearing mice, where tumor growth was strongly inhibited without severe side effects [234].

Colby et al also proposed a novel formulation by using an expansile unit comprising a pH-responsive group, a polymerizing methyl methacrylate group and a hydrophilic triol-linker. The diameter of the formulation could expand until 10 times in water and it was triggered by an acid environment, so that the NPs could release the drugs directly in the tumor site. Indeed, the efficacy with paclitaxel-loaded expansile NPs was superior than the one of free paclitaxel in an *in vivo* model of pancreatic cancer [235].

A class of novel theranostic NPs conjugated to insulin-like growth factor 1 (IGF1) for both imaging and targeted delivery of doxorubicin (Dox) was developed for PDAC treatment. Iron oxide NPs (IONPs) were employed to target IGF1 receptor (IGF1R) which is highly expressed in many tumor cells (including PDAC), stromal fibroblasts and macrophages [236]. Moreover, IGF1R expression is increased in drug-resistant cells [237,238]. Therefore, the effect of these NPs was assessed *in vivo* on human pancreatic cancer patient tissue derived xenografts (PDXs). A near infrared (NIR) dye was conjugated to the NPs, to monitor the targeting by both non-invasive optical imaging and MRI. The nano-formulation demonstrated to accumulate in the tumor site, as further confirmed by histological analysis. In line with this finding, tumor growth was significantly reduced in the animals treated with IGF1-IONP-Dox compared to control groups. These results demonstrated that IGF1-IONPs for theranostics is an effective system, by overcoming the tumor stromal barrier and delivering Dox directly to pancreatic cancer cells [239].

As described, several efforts have been made for improving drug delivery reducing the ECM barrier. Another strategy for employing nanocarriers regards the inhibition of ECM material production [27]. For example, metalloproteinase (MMP)-2 peptides were encapsulated in hybrid liposomes for delivering an agent downregulating ECM production, which is pirfenidone. In a PDAC model, this system demonstrated to reduce the production of ECM material thus increasing the penetration of the small molecules [240]. Also, collagenase was delivered by liposomes to break down the ECM in PDAC, so that the enzyme was protected and went to localize at the tumor. The pre-treatment allowed paclitaxel micelles to directly reach the tumor site and the tumors of mice which received both treatments were 87% smaller than tumors of mice which received only empty liposomes before paclitaxel micelles [146].

## **7. Cell therapy-based approach: mesenchymal stem cells as carriers for delivering anticancer drugs**

Another innovative system to deliver drugs into the tumor site is the one based on mesenchymal stem cells (MSCs). MSCs have been recently investigated as cellular vehicles of anticancer drugs, since they present several advantages, such as feasible isolation, availability, ex-vivo expansion capacity, multipotent differentiation, immunomodulatory and non-immunogenicity properties [241]. MSCs can be isolated from many sources, including bone marrow, adipose tissue, umbilical cord tissue, placenta and amniotic fluid [242–246]. The observation that MSCs migrate toward inflammatory microenvironment and engraft into tumor stroma after systemic administration suggested new therapeutic approaches for delivering anti-cancer molecules directly within the tumor. Indeed, MSCs demonstrated to migrate specifically to the tumor site, because they respond to tissue damage, hypoxia and inflammation. MSCs can do homing to the tumor stroma being attracted to several cytokines, growth factors and proteases of the tumor [247]. Various studies confirmed the ability of MSCs to localize at the tumor site and to be distributed among many cancer cell lines, including pancreatic cancer [248–250]. The high rate of migration and distribution was reported in *in vitro* co-cultures and *in vivo* xenografts. Several cytokines and chemokines were found to interact with MSCs receptors [251]. A number of cytokine-receptor pairs were found to be associated with the MSCs migratory ability, including SDF-1, SCF/c-Kit, HGF/c-Met, VEGF/VEGFR and adhesion molecules such as  $\beta$ 1 and  $\beta$ 2 integrins [252–254]. Furthermore, even

though the role of MSCs in the TME needs still to be investigated and depends on the tumor type, MSCs reported to exert also intrinsic antitumor properties. For example, in a SCID mice model of pancreatic cancer, the tumor growth rate was significantly reduced after the injection of MSCs [255].

Taken together, all these findings make MSCs a suitable candidate for a therapy targeted to the tumor site. Several therapeutic approaches based on the cell-based delivery of anti-cancer agents by MSCs have been developed. After demonstrating that Dox could be uptaken by mouse bone marrow derived MSCs (BM-MSCs) in a significant amount without showing evident signs of toxicity, Pessina et al (2011) investigated whether human and mouse MSCs could be loaded with the anticancer drug paclitaxel (PTX) and exert a toxic effect towards tumor cells. Therefore, they primed the cells with a concentration of drug not toxic for the MSCs, which rapidly incorporated PTX and slowly released it in a time-dependent manner. BM-MSCs were able to acquire and exert a potent anti-tumor and anti-angiogenic dose-dependent effect *in vitro*. Furthermore, when injected in immunodeficient mouse models of melanoma, they significantly reduced tumor growth. By confocal microscopy, PTX was seen to accumulate in hMSCs primed cells and to co-localize with Golgi apparatus and derived vesicles. Despite the mechanism in which the cells release PTX, once they reached cancer cells, they released the drug in a quantity sufficient to inhibit proliferation *in vitro* and *in vivo*. It was estimated that about 25-30% of PTX was retained by PTX-primed BM-MSCs and never released [256].

Also adipose tissue derived MSCs (AT-MSCs) reported the ability to be loaded with PTX and released the drug inhibiting tumor cell proliferation *in vitro* [257]. AT-MSCs revealed to be resistant to the cytotoxic effect of PTX and to release the drug to the higher extent within the first 24 hours. The cell conditioned medium (CM) collected after treatment with PTX was tested on different models of human tumors, such as osteosarcoma and prostate cancer, where cell proliferation was inhibited *in vitro*. In co-culture assay, AT-MSCs loaded with PTX were effective against the proliferation of a leukemia cell line. The adipose tissue represents an attractive source of stem cells since it is ubiquitous and easily obtainable without using invasive methods [258]. Also clinical trials using AT-MSCs in regenerative medicine have confirmed their safety so far [259,260].

Bonomi et al (2015) demonstrated also that MSCs loaded with GCB were able to inhibit the *in vitro* growth of a human PDAC cell line. For the first time they showed

that BM-MSCs can be loaded *in vitro* with GCB. A concentration of 2000 ng/mL allowed GCB to block cell division but maintaining cell viability and drug accumulation. They could also inhibit the proliferation of the PDAC cell line CFPAC-1. Moreover, they showed that very high concentrations of GCB did not affect the secretome of BM-MSCs, which is interesting in terms of potential application in regenerative medicine, since MSCs produce many factors having autocrine/paracrine functions. BM-MSCs seem to regulate epithelial mesenchymal transition of a tumor initiating cell population and to maintain it, therefore a therapy based on MSCs could be integrated into the tumor mass and deliver the drug in situ at very high concentrations difficult to obtain by intravenous injection [261,262].

Human MSCs were primed *in vitro* also with sorafenib (SFN) in the context of glioblastoma (GB) by Clavreul et al (2017). After demonstrating the cytotoxicity of the released SFN in vitro, SFN-primed MSCs were administered by intranasal delivery on an orthotopic model of GB. MSCs could penetrate the brain from the nasal cavity and infiltrate the tumor with a higher accumulation after seven days. They observed that MSCs could migrate towards large or small tumors and it is clinically relevant since GB is highly invasive. Even if they did not obtain an anti-proliferative effect in vivo, the treatment with SFN-MSCs consistently reduced tumor angiogenesis [263].

MSCs can be introduced into the body through local delivery into the tissue or systemically. The vascular route is often preferable since it is more feasible and less invasive, but with this methodology there are some hurdles to overcome in order to allow these cells to reach the target tissue. Firstly, they have to pass through the lungs before to distribute throughout the body. Since MSCs have a diameter of 20-50  $\mu\text{m}$ , while the lung microvessels are around 10, they are often entrapped in the lungs [264–266]. Preclinical but also clinical evidence demonstrated that the lung entrapment occurs after iv injection of MSCs. A low engraftment level was found after iv administration of MSCs to treat graft versus host disease (GvHD), or when co-infused with hematopoietic stem cells (HSCs) to promote HSC engraftment [267,268]. The adding of a vasodilator may solve the problem of MSC entrapment in microvessels. Moreover, since MSCs secrete mediators that exert a paracrine effect on nearby cells and tissues, they may need to be administered in very close proximity to the injury site [269]. The intrahepatic arterial injection of MSCs demonstrated to bypass the lung vasculature [270]. Also the intracoronary injection of MSCs resulted in a significant cell retention in the cardiac tissue [271].

Moreover, current developed techniques allow us to easily detect and track MSCs once injected *in vivo*. Previously, the *in vivo* cell tracking required a post-mortem analysis of sectioned tissues, but the developments in recent years in whole body and vital *in vivo* imaging have allowed higher resolution and more accurate long-term analyses. MSCs can be fluorescently labelled using fluorophores linked to a specific molecule on target cells or transduced with a bioluminescent protein reporter gene. These methods require an *ex vivo* cell preparation before administration but are well characterized and largely employed [272–274].

For many diseases, a clinical utility of MSCs has been published. Several clinical trials reported interesting results in terms of safety and efficacy of MSCs in patients with GvHD, autism, Crohn's disease, multiple sclerosis, systemic lupus erythematosus and type 1 diabetes. A successful use of these cells has been observed in multiple organs regarding the repair of cardiovascular, spinal and lung injuries, bone and cartilage diseases [275–278]. Therefore, thanks to the versatility of these cells, they may constitute a promising strategy for an anticancer therapy specifically directed to the tumor site by-passing the dense ECM barrier.

## **Conclusion**

The high ECM stiffness typical of PDAC allows the tumor to displace the host tissue and grow in an uncontrolled manner. Indeed, PDAC cells take advantage of the fibrotic mechanisms occurring in the ECM for sustaining and maintaining an optimal environment for their proliferation. Moreover, the desmoplastic stroma acts as a physical barrier impairing the delivery of anticancer molecules to the tumor site. The compression of blood vessels exerted by the stroma, together with the poor tumor perfusion, limit the access of chemotherapeutic compounds reducing their effectiveness. In recent years, several drugs targeting the ECM components and the CAFs have been developed and most of them are currently under preclinical or clinical investigation. In fact, by targeting the stroma, the penetration of anticancer agents would be enhanced. Also, the improvement of NP-based systems by exploiting their physicochemical properties, such as size, charge or pH-responsiveness demonstrated to increase the drug delivery. Furthermore, the use of MSCs for drug delivery has been achieving interest since it presents several advantages in terms of feasibility and intrinsic properties. Altogether the initial results

of these strategies seem to be promising, even though deeper research is required to characterize new therapeutic targets in the ECM and to improve the existing systems.

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