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INFLAMMATORY LANDSCAPE AFTER NEOADJUVANT CHEMOTHERAPY IN PANCREATIC DUCTAL ADENOCARCINOMA

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1 ABSTRACT

Background. Pancreatic Ductal Adenocarcinomas (PDACs) are characterized by a high mortality rate and a lack of effectiveness of therapies, especially in locally advanced and metastatic tumors. The tumor microenvironment (TME) is believed to be responsible, at least in part, for the resistance to conventional therapies, like chemotherapy. Thus, an accurate assessment of TME could be useful for the development of effective therapeutic strategies.

Aim of the study. The aim of the study is to investigate the immune landscape of PDACs and the composition and distribution of the immune and inflammatory infiltrate in PDACs, comparing patients who received neoadjuvant chemotherapy followed by resection and patients who underwent upfront surgery at first.

Materials and methods. A total of 81 cases were analyzed: 65 cases of patients who underwent upfront surgery and 16 cases who received chemotherapy as a firstline treatment, instead. The specimens underwent a histopathologic, immunohistochemical (IHC) and molecular characterization to evaluate: grading, Tumor Infiltrating Lymphocytes (TILs), Tumor Associated Macrophages (TAMs), extracellular matrix and PD-L1 expression.

Results. The majority of the infiltrate was concentrated in the intra and peritumoral area, rather than in the periacinar compartment of the normal tissue; therefore the intra and peritumoral area was the most inflamed compartment. The analysis found out that the majority (45.5%) were PD-L1+/TILs+ in both groups and no statistically relevant differences were pointed out concerning the inflammatory infiltrate, the extracellular matrix and PD-L1 expression.

Conclusions. Chemotherapy doesn't seem to impact on PD-L1 status and inflammatory infiltrate, which could play a predictive role in the response to immunotherapy. Considering that the majority of our cases were "hot" in terms of infiltrate (PD-L1+/TILs+), a possible therapeutic strategy could be immunotherapy in doublets or in addition to chemotherapy as a first line treatment in patients with locally advanced or metastatic PDACs.

RIASSUNTO

Introduzione. Gli adenocarcinomi duttali del pancreas sono tumori ad altra mortalità, in cui c'è una scarsa risposta alle terapie specialmente nei tumori localmente avanzati e metastatici. Il microambiente tumorale viene considerato, almeno in parte, come responsabile della mancata risposta alle terapie tradizionali come la chemioterapia. Dunque, un'accurata valutazione del microambiente tumorale potrebbe rivelarsi utile nella ricerca di efficaci protocolli terapeutici.

Scopo dello studio. L'obiettivo è quello di valutare il microambiente immunitario e la composizione e la distribuzione dell'infiltrato infiammatorio nei PDACs, confrontando pazienti che hanno fatto chemioterapia neoadiuvante e chirurgia con pazienti sottoposti a chirurgia in prima battuta.

Materiali e metodi. Un totale di 81 casi sono stati analizzati: 65 casi di pazienti che hanno fatto la chirurgia come prima linea terapeutica e 16 casi di pazienti che sono stati sottoposti a chemioterapia neoadiuvante in prima battuta. I campioni sono stati sottoposti ad analisi istopatologica, immunoistochimica e molecolare con lo scopo di valutare: il grading, l'infiltrato linfocitario (TILs), l'infiltrato macrofagico (TAMs), la matrice extracellulare e l'espressione di PD-L1.

Risultati. La maggior parte dell'infiltrato era concentrato nell'area intra e peritumorale rispetto al compartimento periacinare del tessuto non neoplastico adiacente; dunque il compartimento intra e peritumorale è l'area maggiormente interessata dal processo infiammatorio. L'analisi ha rilevato che la maggioranza (45.5%) erano PD-L1+/TILs+ in entrambi i gruppi e che non c'era alcuna differenza statisticamente rilevante tra i due

gruppo per quanto riguarda l'infiltrato infiammatorio, la matrice extracellulare e lo stato di espressione del PD-L1.

Conclusioni. La chemioterapia non sembra avere un impatto sull'infiltrato infiammatorio e sullo stato del PD-L1, che sono due fattori predittivi di risposta all'immunoterapia. Considerando che la maggior parte dei tumori erano PD-L1+/TILs+, una possibile strategia terapeutica potrebbe prevedere l'immunoterapia come prima linea terapeutica in doppiette o insieme ad un trattamento chemioterapico in pazienti con tumori localmente avanzati o metastatici.

2 INTRODUCTION

2.1 EPIDEMIOLOGY

Incidence and mortality

In Europe, pancreatic cancer is estimated to be the fourth deadliest cancer in men after lung, colorectal, and prostate cancers [1]. Similarly, pancreatic cancer was found to be the fourth deadliest cancer in women after breast, colorectal and lung cancers [1]. With a life expectancy of ∼5% at 5 years, the prognosis of this cancer type has not improved over the past 20 years, and incidence and mortality rates are very similar.

The epidemiology in the US is comparable, with 60 430 new diagnoses in 2021 [2]. The incidence is rising at a rate of 0.5% to 1.0% per year, and pancreatic cancer is projected to become the second-leading cause of cancer death by 2030 in the US [2,3]. The survival improvements have been modest and attributed primarily to multiagent cytotoxic therapies [4,5].

Pancreatic ductal adenocarcinoma (PDAC) encompasses >90% of all pancreatic tumors and has by far the poorest prognosis of all solid tumors [6].

Currently, surgery in combination with adjuvant therapy remains the most effective therapeutic option. However, most patients are not amenable to surgery at the time of diagnosis.
Although there have been multiple recent improvements in the treatment of PDAC, including combination chemotherapies that have improved survival, such as gemcitabine plus capecitabine in the adjuvant setting as well as in advanced disease and the use of fluorouracil, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) in advanced disease, the overall 5 years survival rate remains 8% [2,4].

Median age, gender prevalence and presentation at diagnosis

The median age at diagnosis in the US is 71 years, and PDAC is slightly more common in men than in women (5.5 *versus* 4.0 per 100 000 individuals)[7]. At presentation, 50% of patients have metastatic disease, 10%-15% have localized disease amenable to surgery, and 30%-35% have locally advanced (usually unresectable disease due to the extent of tumor-vascular involvement) [2].

Modifiable and inherited risk factors

Among lifestyle risk factors, current cigarette smoking has the strongest association with PDAC. A meta-analysis of 12 case-control studies that included 6507 patients with pancreatic cancer and 12 890 control patients reported an odds ratio (OR) of 1.74 (95% CI, 1.61-1.87) for the association of current smoking with pancreatic cancer [8,9]. According to a meta-analysis of 19 prospective studies reporting outcomes from 4 211 129 individuals (relative risk, 1.22 [95% CI, 1.03-1.45]; absolute rates not reported), there is a modest association between alcohol use and PDAC, when intake exceeded 30 g per day (approximately 3 drinks per day) [10] . Another modifiable risk factor of pancreatic cancer is obesity. Tumorigenesis is enhanced by excess adipose tissue, probably through the mechanism of abnormal glucose metabolism. Obesity [body mass index (BMI) > 30 kb/m2] is associated with a 20%–40% higher mortality rate from pancreatic cancer. Meta-analyses have demonstrated associations between both type 1 and type 2 diabetes mellitus and pancreatic cancer, with ORs of ∼2.0 and 1.8, respectively [11]. Chronic pancreatitis was associated with a 13-fold increased risk for PDAC in a pooled analysis of 14 prospective cohort studies of 862 664 individuals (relative risk, 13.3 [95% CI, 6.1-28.9]) [12]. Diets of processed meat, high-fructose beverages, and saturated fat were associated with obesity, diabetes, and pancreatic cancer [13].

Only a small proportion (<10%) of PDAC are due to inherited germline mutations. Germline mutations in *BRCA2, ATM, STK11, PRSS1/PRSS2, SPINK1, PALB2*, and *DNA mismatch repair (MMR) genes* are associated with varying degrees of increased risk for pancreatic carcinoma [11]. Familial pancreatic cancers, defined as at least two first-degree relatives with pancreatic cancer, account for only 5%–10% of all pancreatic cancer cases. Mutation in BRCA2 is probably the most common inherited disorder in familial pancreatic cancer. Other familial syndromes linked to pancreatic cancer are: hereditary pancreatitis, hereditary non-polyposis colorectal cancer, hereditary breast and ovarian cancers, Peutz–Jeghers syndrome, ataxia telangiectasia, familial atypical multiple mole melanoma syndrome and Li–Fraumeni syndrome [14].

2.2 THE ROLE OF IMMUNE SYSTEM IN TUMOR PROGRESSION

The mechanism by which the immune system can initially protect a host from tumor growth, but in some cases subsequently promotes cancer progression, is termed cancer immunoediting[15]. Cancer immunoediting is a dynamic process consisting of three phases, (1) elimination, when the immune system overcomes and eliminates the tumor before it can progress to a clinically relevant disease; (2) equilibrium, when the immune system does not eliminate the tumor, but controls tumor growth; and (3) escape, which occurs when the tumor has evaded the immune system and progresses to a clinically apparent disease. This third stage is generally seen as a failure of the adaptive immune system to provide long-term protection from tumor development due to selection of less immunogenic tumor cell variants during the equilibrium stage. Additionally, tumor escape can be facilitated by active immunosuppression induced by the tumor itself or some form of immune compromise or immune deficiency [16]: in fact, the escaped tumor cells will create a tumor microenvironment (TME) suitable for the growth of the early lesions. In the last phase, tumor cells recruit immunosuppressor cells like marrow-derived suppressor cell (MDSC),

tumor associated macrophages (TAM), and regulatory T cells (Treg cells), to help establishing an immunosuppressive TME, therefore escaping from host immune surveillance [22].

2.3 PANCREATIC TUMOR MICROENVIRONMENT (TME)

The development of PDAC has been shown to progress because of an activating mutation in the *KRAS* oncogene, resulting in acinar to ductal metaplasia, followed by subsequent progression through increasing grades of pancreatic intraepithelial neoplasia (PanIN) and ultimately PDAC, after acquiring additional somatic mutations in multiple tumor suppressor genes, including *p16/CDKN2A*, *TP53*, and *SMAD4*, and the overexpression of HER2-2/neu [15,16,17,18]. The progression from acinar cell to PDAC is accompanied by a profuse fibrotic stromal desmoplasia, which is the basis of a complex tumor microenvironment (TME) [19].

As a non-immunogenic tumor, the immune profile of PDAC and immunologic milieu of its TME is unique relative to other malignant tumors that are responsive to immunotherapy. PDAC bears a low-moderate mutational burden and has lower immunogenic potential [23]. The TME of PDAC has increased the infiltration of immunosuppressive cells, like MDSCs and Treg cells, and is characterized by increased infiltration of carcinoma associated fibroblasts (CAFs) resulting in collagen deposition with an elevated fibrotic response [24]. This desmoplastic microenvironment may compromise tumor blood perfusion and oxygen delivery, thus generating an obstacle for drug delivery. This barrier has been hypothesized to lead to intrinsic resistance to chemotherapy regimens, including gemcitabine [25].

The dense stroma of PDAC is composed of extracellular matrix (ECM), pancreatic stellate cells, fibroblasts, myofibroblasts, a variety of immune cells, cytokines, and growth factors, all of which contribute to tumor

proliferation and the promotion of metastasis through an intricate interaction [26].

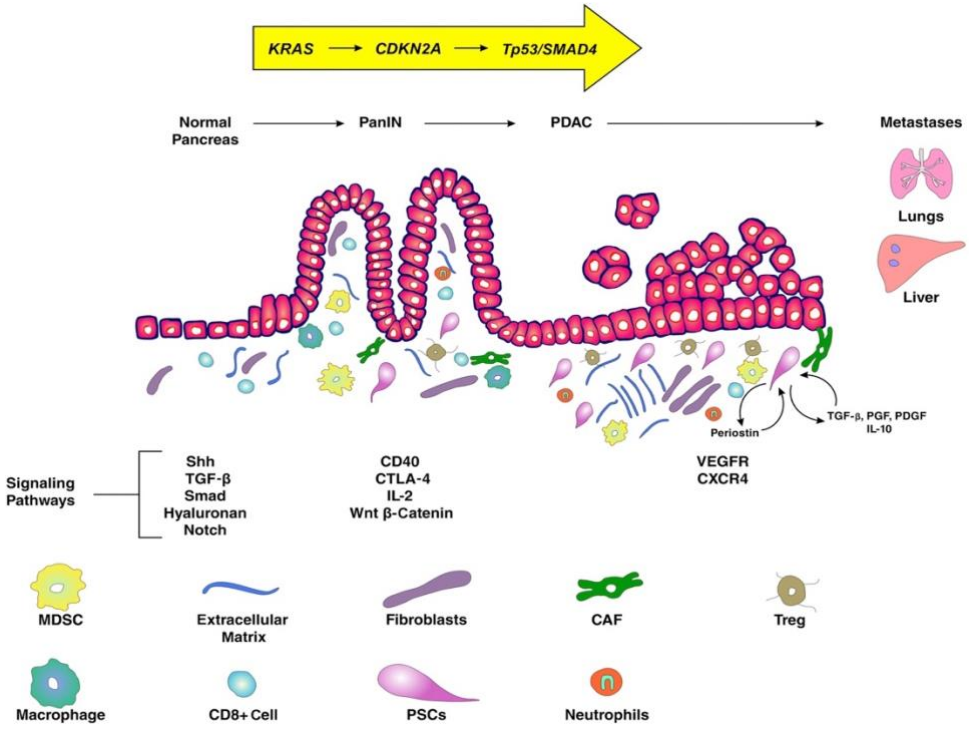

Figure 1. An overview of the progression in terms of mutations and tumor microenvironment from normal pancreas to pancreatic ductal adenocarcinoma

Cytotoxic T cells and T helper cells

Cytotoxic T cells (CTLs) are CD8+ and they are preferred immune cells for targeting cancer cells.

For durable and efficient immune responses, naïve T cells are primed in the lymph nodes with tumor antigens through interactions with APCs (Antigen-presenting cells). Upon activation, they rapidly proliferate, differentiate into antigen-specific CTLs and migrate to tumor sites to perform their cytotoxic functions [27]. Elimination of tumor cells by CTLs occurs *via* the release of cytotoxic granzymes, IFN-γ and tumor necrosis factor α (TNF-α), or by induction of FasL-mediated apoptosis [28]. Following a cytotoxic immune response, the majority of CTLs will undergo apoptosis while a small fraction of them will further differentiate into diverse subsets of multipotent, long-lived memory $CD8^+$ T cells endowed with self-renewal ability [27]. The integration of three coordinated signals regulates T cells

activation, expansion, survival, and memory formation: T cell receptor (TCR) stimulation by antigens, engagement of co-stimulatory molecules (CD28, CD27, 4-1BB, and OX40) expressed by $CD8^+$ T cells, and the release of inflammatory cytokines. In the absence of co-stimulatory signals, antigenic stimulation induces tolerance or clonal deletion in peripheral lymphoid organs [29]. The pro-inflammatory cytokines IL-12, IL-2 and IFN-γ, are crucial for satisfactory naïve $CD8^+$ T cell activation, expansion and differentiation whereas IL-7 and IL-15 are predominantly required for formation maintenance of memory $CD8^+$ T cells. In pancreatic cancer patients, both number and functions are altered within the $CD8^+$ T cell population. These patients show a decrease in circulating $CD8^+$ T cells and a decrease in perforin expression within these cells compared to healthy subjects. Moreover, intra-tumoral $CD8^+$ T infiltrates often display abnormal exhausted phenotype [30].

$CD4^+$ helper T cells (Th) can differentiate into Th1, Th2, Th17 and Tregs. $CD4^+$ Th1 cells secrete the pro-inflammatory cytokine interferon-γ (IFN-γ) which activates and supports CTLs cytotoxicity, while $CD4^+$ Th2 cells exhibit tumor-promoting functions by producing a plethora of cytokines (IL-4,IL-5,IL-6,IL-9,IL-10, IL-13) [30], sustaining fibrosis through ECM (Extracellular matrix) and collagen deposition, and contributing to the differentiation of macrophages into a M2-immunosuppressive phenotype. Polarization towards Th2 cell subset is a common trait in pancreatic cancer, and this shift from Th1 to Th2 cells is correlated with decreased patient survival [31].

Regulatory T cells (T_{regs})

They are defined as CD4+, CD25+,FOXP3 + T cells.

T_{reg} cells can be classified into two subtypes according to the sites at which they develop. Thymus-derived T_{reg} (tT_{reg}) cells are generated in the thymus as a functionally mature T cell subpopulation with a specialized role in immunosuppression. Under certain conditions, some $FOXP3^+$ T_{reg} cells differentiate from T_{conv} cells in the periphery and are, therefore, termed induced T_{reg} (iT_{reg}) cells [32].

There is an alternative classification of T_{reg} cells according to the expression levels of a marker of naïve T cells, CD45RA, as well as CD25 and FOXP3 , with compelling associations with immunological phenotypes and functions. Using this classification, $FOXP3^+CD25^+CD4^+$ T cells can be categorized into three fractions: naive T_{reg} cells ($CD45RA^+FOXP3^{lo}CD25^{lo}CD4^+$); effector T_{reg} (eT_{reg}) cells ($CD45RA^-FOXP3^{hi}CD25^{hi}CD4^+$); and non-$T_{reg}$ cells ($CD45RA^-FOXP3^{lo}CD25^{lo}CD4^+$). Naive T_{reg} cells are cells that have egressed from the thymus but have not yet been activated in the periphery and possess weak immunosuppressive activity. Upon TCR stimulation, naive T_{reg} cells proliferate rapidly and differentiate into highly immunosuppressive eT_{reg} cells. By contrast, $FOXP3^+$ non-T_{reg} cells are not immunosuppressive but rather are immunostimulatory, producing inflammatory cytokines, such as IFNγ and IL-17[33].

T_{reg} cells exert their immunosuppressive activity through various cellular and humoral mechanisms: competition for and consumption of IL-2, thereby limiting the amount of this cytokine that is available to T_{conv} cells [34,35]; CTLA-4-mediated suppression of APC function, which inhibits the priming and/or activation of T_{conv} cells [36,37]; production of immunosuppressive cytokines (such as IL-10, IL-35 and TGFβ) [38,39,40,41]; conversion of ATP into adenosine [42,43], an immunomodulatory metabolite that can prevent optimal T cell activation; and secretion of granzyme and/or perforin to destroy effector cells [44].

The majority of the T-lymphocytes in PDAC are $CD4^+$ Tregs, supporting an immunosuppressive phenotype. Tregs are significantly increased in the blood of PDAC patients as well as in the pancreatic tissue [45]. They are found typically in the stromal areas of the tumor, and only occasionally in association with tumor epithelial cells [46]. Hiraoka et al. [46] examined clinical samples of pre-malignant lesions and found that Treg accumulation correlates with the progression of both of the major preneoplastic lesions in pancreatic cancer, PanINs and intraductal papillary mucinous neoplasms (IPMN). The association of Tregs with IPMN progression has been independently confirmed [47]. Additionally, Tregs correlate with metastasis [48] and tumor grade, and negatively correlate with patient survival [46].

Tumor associated macrophages (TAM)

Macrophages play critical roles in both innate and adaptive immunity and are known for their remarkable phenotypic heterogeneity and functional diversity.

Tumor-associated macrophages (TAMs) are macrophages that participate in the formation of the TME. TAMs are widely present in various tumors [50], they can promote tumor growth, invasion, metastasis, and drug resistance [49]. It has been proposed that functional difference of macrophages is closely related to the plasticity of macrophages, and its functional phenotype is regulated by molecules in tumor microenvironments [51].

Many classifications have been proposed. However, the most used one focuses on macrophage functional polarization: macrophages can be divided into two different polarization states, M1 type macrophages (M1) and M2 type macrophages (M2).

M1 can respond to dangerous signals transmitted by bacterial products or IFN-γ, which result in attracting and activating cells of the adaptive

immune system; an important feature of M1 is that it can express nitric oxide synthase (iNOS) and reactive oxygen species (ROS) [52,53] and cytokine IL-12 [54]. M1 also has the function of engulfing and killing target cells.

M1-type macrophages have anti-tumor effects, which can distinguish tumor cells from normal cells. By identifying tumor cells and ultimately killing tumor cells, studies have found that M1 type macrophages have two different effects on killing tumor cells mechanism. M1 type macrophages directly mediate cytotoxicity to kill tumor cells: macrophage-mediated cytotoxicity is a slow process (generally requires 1 to 3 days) and involves multiple mechanisms. For example, macrophages release tumor killing molecules such as ROS and NO, which have cytotoxic effects on tumor cells [57]. The other is antibody-dependent cell-mediated cytotoxicity (ADCC) killing tumor cells: ADCC requires less time to kill tumor cells (generally within a few hours) and requires the participation of anti-tumor antibodies [58].

M2 expresses a large number of scavenger receptors, which is related to the high-intensity expression of IL-10, IL-1β, VEGF and matrix metalloprotein (MMP) [55,56]. M2 has the function of removing debris, promoting angiogenesis, tissue reconstruction and injury repairments, as well as promoting tumorigenesis and development [44]. More specifically M2-like TAMs, which are generated under the influence of several cytokines such as IL-10 and transforming growth factor (TGF)-β, activate Th2-type immune responses and promote tumorigenesis and development [59]. They mainly promote upregulation of the expression of anti-inflammatory cytokines and chemokines, including IL-10, TGF-β, CC chemokine ligand (CCL) 17, CCL18, CCL22, and CCL24 [60]. Such secretion is involved in tumor invasion and metastasis. Surface proteins, such as CD206 (mannose receptor-1), CD204 and CD163 (macrophage scavenger receptors), are also overexpressed[61]. M2-like TAMs have critical roles in facilitating epithelial-mesenchymal transition (EMT), angiogenesis and

immunosuppression [62,63]. Moreover, M2-like TAMs may hamper the efficacy of chemotherapy and radiotherapy through suppression of $CD8^+$ T cell function, leading to tumor progression and poor outcomes [61, 64,65]. Tumor associated macrophages can regulate T cell function directly or indirectly. TAMs can directly inhibit cytotoxic T lymphocyte (CTL) responses through three distinct mechanisms. First, immune checkpoint engagement *via* their expression of molecules (such as programmed cell death 1 ligand 1 (PD-L1)): PD-L1 expression by CD68+ macrophages is observed in multiple cancer tissues; it should be noted that PD-L1 expression by macrophages has not been established as an independent predictor of response to PD-L1 blockade. Second, production of inhibitory cytokines (such as IL-10 and transforming growth factor-β (TGFβ)) and their metabolic activities, including the depletion of metabolites (such as L-arginine). IL-10 is known to suppress CD8+ T cell stimulation by reducing colocalization of CD8 protein with the T cell receptor. Third, production of reactive oxygen species (ROS) via iNOS. In addition to potential direct effects of nitric oxide on T cells, production of peroxynitrites can prevent the interaction of the T cell receptor with MHC through nitration of proteins.

TAMs also inhibit T cell responses indirectly by controlling the immune microenvironment. This includes control through TAM-mediated recruitment of immunosuppressive populations (such as regulatory T cells) or inhibition of stimulatory populations (such as dendritic cells) by cytokines production (IL-10). TAMs also blunt T cell recruitment through distinct mechanisms: *via* regulation of vascular structure and increase of adhesion molecules (through expression of VEGFα) and *via* the exclusion T cells from intratumoral regions through the regulation of the extracellular matrix (ECM): increasing the degree of fibrosis can be another mechanism by which macrophages could shield tumors from T cell infiltration [66].

Pancreatic stellate cells (PSCs)

It has been conclusively proven that 80% of the PDAC volume is composed of desmoplastic stroma, and cumulating evidence substantially corroborate the two-way interactions between tumor cells and stromal components [71,72]. Desmoplastic stroma in the PDAC is predominantly composed of fibrous components laid down by PSCs along with cellular components (lymphocytes, endothelial cells, and mast cells), non-cellular ECM proteins (collagen, elastin, fibronectin, and laminin) and non-ECM components (stellate or cancer cell-derived growth factors) [70,73].

PSCs are mainly distributed around the pancreatic glands they are able to synthesize matrix proteins, matrix metalloproteinases (MMP), and MMP inhibitors that regulate ECM turnover [67]. PSCs can be activated by factors including pro-inflammatory cytokines, oxidant stress, and by factors of particular interest in PDAC such as hypoxia, hyperglycemia, and increased interstitial pressure [68]. The activated PSCs can secrete various growth factors to promote the growth and proliferation of pancreatic cancer cells, inhibit their apoptosis, and enhance their invasion ability [69,70]. PSCs have been confirmed to be the predominant source of collagen in the tumor stroma, and able to secrete ECM proteins like α-smooth muscle actin and collagen.

Since fibrosis is an early event to PDAC development, initially it was believed that PSC-derived stroma is protective against the tumor progression. However, the opinion is eventually shifted towards the concept that stellate cell-stromal-cancer cell interactions are dynamic, stage and context dependent which may be protective at the earliest stage, however obviously harmful at the later stage. Evidence showed that two-way interactions between PSCs and cancer cells that significantly influence each other are essential for tumor growth. For instance, PDAC cells produce factors such as PDGF, TGF-β, cytokines, and chemokines. In

return, PSCs produced growth factors that enhance tumor growth and MMPs degrade the basement membrane which facilitates tumor cell migration and invasion [71].

Due to the central and decisive role of PSCs in the PDAC desmoplasia, these are considered as an attractive target for treatment. Several experimental studies that targeted pro-fibrogenic PSCs have shown favorable results in regulating PDAC progression and metastasis.
Based on the immunosuppressive role of activated PSCs that regulate T-cell migration, alteration in PSC function was found as an effective mode to restore anti-tumor response [75]. Since PDAC stroma has been found to be associated with hypoxia and drug resistance, drugs that degrade stroma are expected with good clinical outcome [76, 77, 78].

Cancer associated fibroblasts (CAFs)

Fibroblasts are supportive cells of mesenchymal origin that are present in substantial quantities in nearly every solid organ. In physiological condition they are critical to homeostatic mechanisms by providing structural support and secreting soluble factors and ECM proteins. In the setting of cancer, there is an growing body of evidence that demonstrates that CAFs are not mere cellular bystanders but active players during the process of cancer initiation, progression and metastasis. The contribution of CAFs to the biology of PDAC and other carcinomas has generally been held to be tumor-promoting, making targeting of CAFs an attractive therapeutic strategy [79].

2.4 THE ROLE OF ICB IN PDAC

The targeting of PD-1 and PD-L1 has revolutionized cancer therapy. To date, two PD-1 inhibitors (nivolumab and pembrolizumab) and three PD-L1 inhibitors (atezolizumab, avelumab, and durvalumab) have been

approved by the US FDA for various indications. These drugs work by blocking the PD-1 or PD-L1 immune checkpoint pathway to reactivate T cell mediated antitumor immunity. Several reports have now shown that PD1 and PDL1 blockade drive *de novo* peripheral immune responses culminating in new effector T cell infiltration in the TME, rather than reinvigorating terminally exhausted TILs (tumor infiltrating lymphocytes) that are incapable of key effector functions.

Although immune checkpoint inhibitors have successfully achieved durable responses in many different types of malignant diseases, they are only effective in a fraction of patients in each type of cancer. Therefore, the use of immune checkpoint inhibitors is not limited to specific cancer types but is more likely limited to malignant diseases with specific immunobiologic characteristics. PD-L1 expression has been suggested to predict the response to anti-PD-1/PD-L1 antibody therapies. However, no consensus on a reliable PD-L1 staining assay has been made. A more prominent immunobiologic characteristic of immune checkpoint inhibitor sensitive malignant diseases is abundant effector T cell infiltration. This is better characterized in melanoma treated with immune checkpoint inhibitors [80], but is also seen in other cancer types [81]. Essentially the checkpoint inhibitor sensitive tumors are usually abundantly infiltrated with CD8+ T cells and can be classified as “immune active” tumors. “Immune quiescent” tumors, which lack infiltration of effector T cells are almost always resistant to single agent checkpoint inhibitor treatment. All pancreatic cancers, except those with mismatch repair deficiencies (which occurs approximately in 1-2% of PDACs [82]), are considered to be immune quiescent tumors and are insensitive to therapeutic single agent checkpoint inhibitors[83].

There are various explanations for ICB (immune checkpoint blockade) failure in PDAC tumors, including low mutational burden and expression of neoantigens, minimal intra-tumoral infiltration of $CD8^+$ T cells, expression of multiple inhibitory receptors in $CD8^+$ T cells that infiltrate tumors, as well as decreased tumor and myeloid expression cell expression of PD-L1

[84,85]. To improve PADC response to ICB, combined approaches have been investigated. Multi-agent immunotherapeutic protocols targeting multiple inhibitory receptors is a promising approach, and has proved more effective than single inhibitory receptor blockade in reversing dysfunctional $CD8^+$ T cells PDAC[86,87]. In the same way, strategies with the goal to prime effector $CD8^+$ T cells to increase their immunogenicity and responsiveness before the use of checkpoint inhibitor treatment represents an exciting opportunity in cancer immunotherapy [86,88 ,89].

The anti-PD-1 immune checkpoint inhibitor pembrolizumab is the only immunotherapy that is FDA-approved for the treatment of patients with advanced PDAC which is mismatch repair deficient (dMMR) or with microsatellite instability (MSI). In May 2017, pembrolizumab was approved for patients with unresectable or metastatic, MSI/dMMR solid tumors with progression on prior treatment with no satisfactory alternative treatment options, including PDAC [90].

Mismatch repair deficiency and hypermutation in pancreatic cancer

The mismatch repair (MMR) system plays a pivotal role in the repair of DNA sequence mismatches during replication. Defects in the MMR system (dMMR) or loss of function of one of the MMR proteins (MLH1, MSH2, MSH6 and PMS2) causes errors in DNA replication, leading to the high burden of mutations that accumulate in microsatellites (defined as short tandem repeats that are prone to DNA replication errors), resulting in MSI (microsatellite instability)[91]. A defective MMR system leads to an accumulation of somatic mutations, resulting in a higher neoantigen load, which promotes proinflammatory cytokines and activation of T cells. Increased neoantigens and cytotoxic T cell recruitment contributes to the

immunogenicity of dMMR tumors and hence, sensitivity to immunotherapy [92].

Tumors with dMMR/MSI can develop either as a result of a germline mutation in the MMR gene (Lynch syndrome) or more commonly, as epigenetic inactivation of the *MLH1* gene [93]. Tumor mutational load (TML) is defined as the total number of mutations per coding area of a tumor gene, and tumors that are MSI typically have high TML [94]. Evidence suggests that tumors with high TML status have increased sensitivity to immunotherapy [95].

Due to the recently approved site agnostic indication for pembrolizumab, a recent update in the National Comprehensive Cancer Network guidelines encourage MSI testing for locally advanced and metastatic PDAC [96].

2.5 DEFINITION OF RESECTABILITY STATUS IN PDAC

Locally advanced and borderline resectable pancreatic cancers are being increasingly recognized as a result of significant improvements in imaging modalities.

Borderline resectable pancreatic cancer has been difficult to categorically define. However, it is known as a tumor that is localized to the pancreatic bed, which has limited involvement of the surrounding vascular structures and where a reconstruction of this vasculature is possible. Even with this definition, there is a lack of a universally accepted classification regarding the degree of vascular involvement that would be considered possible to reconstruct.

For our purposes we adopted the resecability criterias from 2022 NCNN guidelines.

Resectability status	Arterial	Venous
Resectable	No arterial tumor contact (celiac axis [CA], superior mesenteric artery [SMA], or common hepatic artery.	No tumor contact with the superior mesenteric vein (SMV) or portal vein (PV) or ≤180° contact without vein contour irregularity.
Borderline resectable	Pancreatic head/ uncinate process: • Solid tumor contact with CHA without extension to CA or hepatic artery bifurcation allowing for safe and complete resection and reconstruction. • Solid tumor contact with the SMA≤180°. • Solid tumor contact with variant arterial anatomy (ex: accessory right hepatic artery, replaced right hepatic artery, replaced CHA, and the origin of replaced or accessory artery) and the presence and the degree of tumor contact should be noted if present, as it may affect surgical planning. Pancreatic body/tail: • Solid tumor contact with the CA of ≤ 180°	Solid tumor contact with the SMV and PV of > 180°, contact of ≤180°with contour irregularity of the vein or thrombosis of the vein but with suitable vessel proximal and distal to the site of involvement allowing for safe and complete resection and vein reconstruction. Solid tumor contact with the inferior vena cava (IVC).
Locally advanced	Pancreatic head/ uncinate process: • Solid tumor contact >180° with the SMA or CA. Pancreatic body/ tail: • Solid tumor contact of >180° with the SMA or CA. • Solid tumor contact with the CA and aortic involvement	Unreconstructible SMV/ PV due to tumor involvement or occlusion (can be due to tumor or bland thrombus)

Table 1. Definition of resectability status from NCNN guidelines 2022.

2.6 GRADING OF POSTOPERATIVE PANCREATIC FISTULA

Postoperative pancreatic fistula (POPF) is a common complication in pancreatic surgery. A pancreatic fistula is typically diagnosed by measuring the amylase content of fluid from the peripancreatic drain; a drain amylase content greater than three times the serum amylase on or after POD 3 is pathognomonic for a fistula. Rates of pancreatic fistula remain similar for

both PD and distal pancreatectomy, ranging from 3% to 28% for both operations [97].

According to their clinical impact, they are classically classified as biochemical leak (BL), grade B and grade C POPF.

BL applies to the original “grade A” POPF, and no longer is considered a true pancreatic fistula or an actual complication. As called a “biochemical fistula” in the literature, the BL has by definition no clinical impact. In particular, a BL implies no deviation in the normal postoperative pathway and therefore, does not affect the normal postoperative duration of stay. In some cases, a drain may remain in place even after discharge for observation purposes for up to 3 weeks after operation, before it might be considered to have a clinical impact on the patient. The patient, however, remains clinically well, fed orally, and can adhere to an enhanced recovery pathway.

Grade B POPF refers to a properly defined fistula involving increased amylase activity in the fluid from any drain in association with a clinically relevant condition. A grade B POPF requires a change in the management of the expected postoperative pathway. Unlike the BL, the pancreatic drains might be left in place for an extended period (defined as 3 weeks/21 days after operation), or there may be a need to reposition the operatively placed drains through interventional, image-guided means to “decompress” an undrained intra-abdominal fluid collection. Alternatively, percutaneous or endoscopic ID is warranted for the same purpose. If a POPF-related hemorrhage or pseudo-aneurism occurs, transfusions and/or angiography usually are necessary. Whenever reoperation is needed or organ failure occurs, the fistula shifts to a grade C POPF. In most cases, the POPF is associated with signs of mild infection (leucocytosis and mild fever) requiring only antibiotic administration; however, once single or multiple organ dysfunctions occurs, the fistula would shift to a C grade POPF. Finally, if sudden death occurs (for example, secondary to myocardial infarction, fatal pulmonary embolus, or renal failure), the grade B POPF

might shift into a C in case the fistula represents the initiating/triggering factor.

Because of these grade B POPF-related complications, patients may be kept on nothing per mouth and supported with either enteral or parenteral nutrition and sometimes therapeutic somatostatin analogues.

Whenever a grade B POPF leads to organ failure or to clinical instability such that a reoperation is needed, the POPF becomes a grade C. Often, stay in an ICU is necessary, and the hospital stay becomes excessively prolonged secondary to the POPF-related problems. For the purpose of POPF classification, postoperative organ failure is defined as the need for reintubation, hemodialysis, and/or use of inotropic agents for >24 hours because of respiratory, renal, or cardiac insufficiency, respectively. Reoperation usually is performed after attempts at percutaneous and/or endoscopic ID have failed to improve the clinical outcome, and is specifically addressed to treat the fistula. Obviously, reoperation potentially is associated with relevant morbidity and mortality. In addition to the above, if a subsequent POPF-specific mortality takes place even without a reoperation, the POPF becomes a grade C POPF [98].

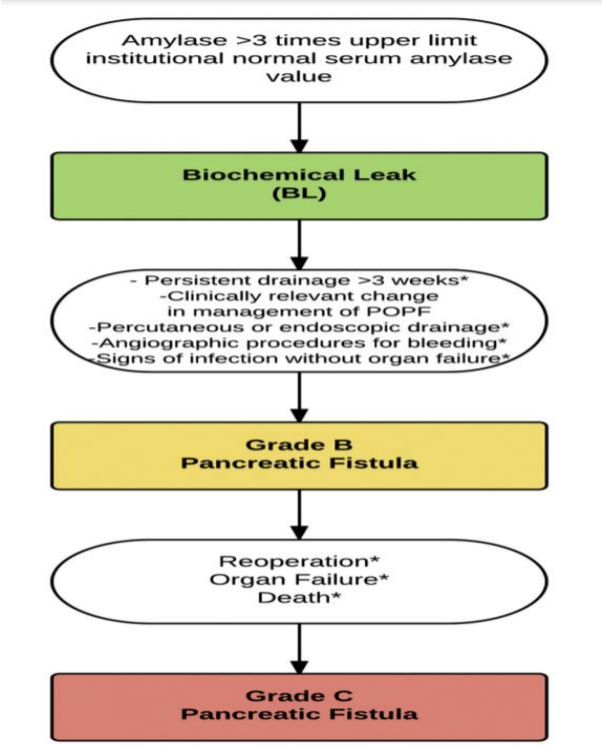

Figure 2. Definitions of pancreatic fistulas

2.7 OPTIMAL REGIMEN FOR NEOAJUVANT CHEMOTHERAPY IN PDAC

Two major regimens for PDAC have been provided as standard based on the results of RCTs. Conroy et al. first demonstrated the superiority of combination chemotherapy FOLFIRINOX compared to single-agent gemcitabine. Von Hoff et al. also reported the superiority of another combination regimen, gemcitabine plus nab-paclitaxel, compared to single-agent gemcitabine [99].

Neoadjuvant FOLFIRINOX consisted of a 2-hour intravenous infusion of oxaliplatin 85 mg/m^2 followed by a 90-min intravenous infusion of irinotecan 180 mg/m^2 and a 2-hour infusion of leucovorin 400 mg/m^2, followed by an intravenous bolus of 5-FU 400 mg/m^2 and a 46-hour continuous infusion of 5-FU 2,400 mg/m^2, administered every 2 weeks, as described in the PRODIGE 4 trial.

Neoadjuvant Gemcitabine plus nab-paclitaxel consisted of intravenous infusion of 1,000 mg/m^2 gemcitabine and 125 mg/m^2 nab-paclitaxel on days 1, 8, and 15 of each 28-day cycle, as described by Miyasaka et al., 2021.

2.8 AJCC 8th EDITION OF TNM STAGING FOR PANCREATIC CANCER

The seventh edition of the AJCC TNM staging system (2009) has been criticized for its poorly applicable and nonspecific T stages, in which nearly all cases of PDAC are classified as extrapancreatic. The preponderance of T3 tumors, because of the absence of a true capsule around the pancreas, reduced distribution in the T stage and subsequently the discriminative ability of the seventh edition. The N stage of the seventh edition was found to be outdated because of its dichotomous nature, since numerous studies now support the prognostic value of both the number of positive lymph nodes and the lymph node ratio (the number of disease-positive lymph nodes divided

by the total number of lymph nodes) in patients with pancreatic cancer [100,101]. These previously mentioned disadvantages limited the clinical applicability and usefulness in the daily practice of the seventh edition of the TNM staging system.

As of January 2018, the eighth edition of *The AJCC Cancer Staging Manual*, including the TNM staging system for tumors arising from the exocrine pancreas, is in use. In the eighth edition, extension beyond the pancreas is no longer considered stage T3, because staging in the T stage has been replaced by a size-based system (except for pT4 tumors). Furthermore, the eighth edition subdivided the N1 stage from the seventh edition into N1 and N2 according to the number of positive regional lymph nodes [102].

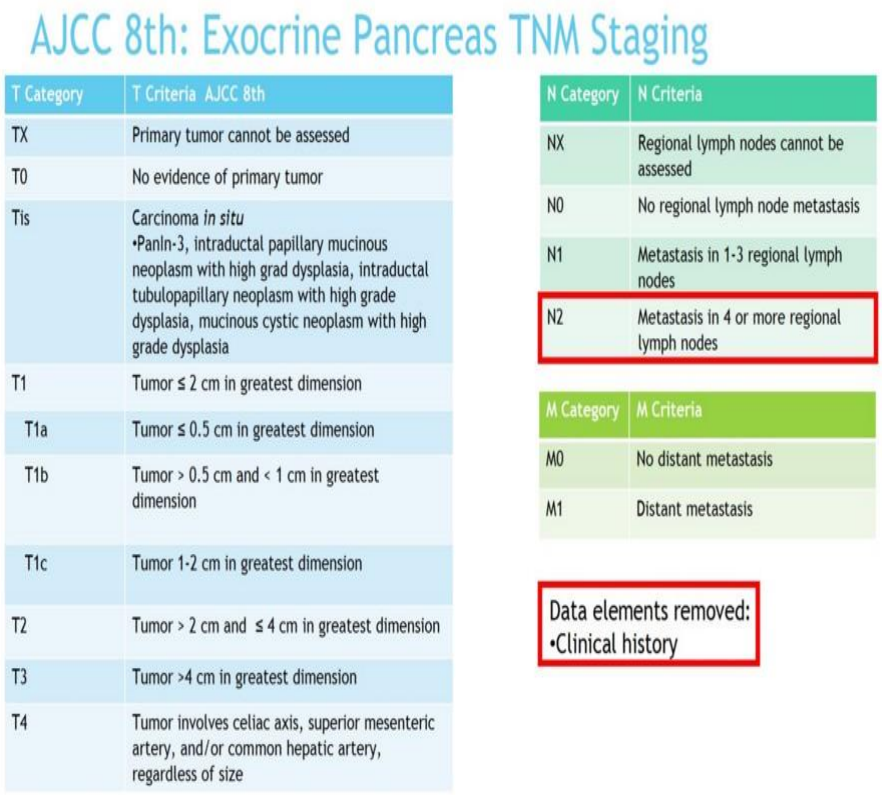

Figure 3. TNM staging of exocrine pancreas

3 AIM OF THE STUDY

The aim of the study is to investigate the immune landscape pf PDACs and the composition and distribution of the immune and inflammatory infiltrate in PDACs, with particular focus on the comparison between from patients who received neoadjuvant chemotherapy followed by resection and patients who underwent upfront surgery at first.

4 METHODS AND MATERIALS

4.1 CASE SELECTION

A series of mono-Institutional formalin-fixed and paraffin-embedded (FFPE) 81 pancreatic ductal adenocarcinomas (PDACs) samples were collected from the archives of the Surgical Pathology Unit at Padua University between January 2001 and September 2021.

The 81 samples of PDACs were divided in two groups as follows :

- 65 PDAC samples from upfront surgery;
- 16 PDAC samples which underwent neoadjuvant chemotherapy at first.

Regarding PDACs, cancers originating from the pancreatic head, body and tail were considered for comparisons, while pancreatic ductal carcinomas that secondarily involved the ampulla were excluded.

Baseline clinical and pathological data were retrospectively collected from the electronic archive.

Clinical data included patient sex, age at surgery, type of surgical procedure, tumor location, tumor size and type of neoadjuvant chemotherapy.

Pathological data consisted of tumor size and grade, presence of lymphovascular infiltration and perineural invasion, lymph node ratio, status of surgical margins and tumor stage. (AJCC 8^{th} edition of TNM staging for pancreatic cancer).

4.2 HISTOLOGIC EVALUATION

Routine H&E staining from formalin-fixed paraffin-embedded (FFPE) tissue was performed to evaluate cell morphology and architecture, in order to determine histologic variant, grading and areas of stromal and intra-

epithelial tumor infiltrating lymphocytes (TILs) and tumor associated macrophages (TAMs) for each sample.

4.3 TISSUE MICROARRAY CONSTRUCTION

To construct the tissue microarray, the formalin-fixed, paraffin-embedded archival tissue blocks and their matching H&E-stained slides were reviewed and screened for representative tumor regions and normal pancreatic parenchyma by a gastrointestinal pathologist. Representative tumor regions included tumor center, invasion front and areas of TILs. For each patient, four cores of tumor and two cores of paired normal pancreatic parenchyma were sampled from representative areas using a 1.0-mm punch. If little tumor material was available, only three cores of tumor and one core of normal paired tissue were obtained. All surgical samples were processed using the Galileo CK3500 Arrayer (www.isenet.it), a semiautomatic and computer-assisted Tissue microarray (TMA) platform. The constructed TMA blocks were sealed with paraffin, and 3-4-mm-thick slides were cut from the TMA blocks for immunohistochemical staining.

4.4 IMMUNOHISTOCHEMICAL INTERPRETATION

PD-L1 expression

The immunohistochemical stains for PD-L1 were evaluated for the presence of partial or complete membrane staining in tumor cells or the presence of membrane and/or cytoplasmic staining of mononuclear inflammatory cells (lymphocytes and macrophages) within the tumor and/or adjacent supporting stroma.

PD-L1 expression was measured using the CPS scoring system. The *combined positive score* (CPS) is calculated as the number of PD-L1–positive cells (tumor cells, lymphocytes, and macrophages) divided by the total number of viable tumor cells multiplied by 100. The CPS is expressed

by the following formula: CPS = (number of PD-L1–stained cells: tumor cells, lymphocytes, macrophages/ total number of viable tumor cells) × 100. All samples were confirmed to include at least 100 viable tumor cells, which is regarded as adequate for PD-L1 assessment.(Xue et al., 2020)
According to CPS, PDACs were dichotomized into two groups: tumors with low PD-L1 expression, if CPS was < 1 (designed as “PD-L1 negative” or “PD-L1 neg”); tumors with high PD-L1 expression, if CPS was ≥1 (designed as “PD-L1 positive” or “PD-L1 pos”).

Tumor infiltrating lymphocytes (TILs)

Stromal Intra-tumoral and peri-tumoral (e.g., detected at the invasive neoplastic front) (sTILs) as well as intra-epithelial lymphocytes in the tumor spots were considered; Tertiary Lymphoid Structures (TLSs), however, were excluded from the evaluation.
CD3 stained lymphocytes were counted manually in five high-power fields (HPFs) among all tumor spots and in three HPFs among all spots obtained from paired normal pancreatic parenchyma. The average lymphocytes number was calculated across HPFs. The result was that each patient had a final score for the tumor area regarding both intra-tumoral/ peri-tumoral (e.g., stroma infiltrating lymphocytes) and intra-epithelial lymphocytes. Moreover, a final score for the non-neoplastic normal pancreatic tissue regarding intramucosal and intraepithelial infiltrating lymphocytes for PDACs was obtained. The same method was applied for CD4, CD8, FOXP3 and CD20 stained lymphocytes evaluation for PDACs.
According to recent studies on TILs in colorectal cancers (CRCs) (Loupakis et al., 2019) and SBAs (Jun et al., 2020) PDACs were dichotomized into two groups based on CD3+ iTILs: tumors with low CD3+ iTILs (designed as “TILs negative” or “TILs neg”), if the mean CD3+ intra-epithelial T cell count among 6 HPF was < 2; tumors with high CD3+ iTILs (designed as “TILs positive” or “TILs pos”), if the mean CD3 intra-epithelial T cell count among

6 HPF was ≥ 2. On the basis on PD-L1 expression and iTILs levels, tumors were subsequently classified in four groups according to *Teng et al*. [103] (PD-L1 pos/ TILs pos, PD-L1 pos/ TILs neg, PD-L1 neg/ TILs pos, PD-L1 neg/ TILs neg).

Tumor associated macrophages (TAMs)

CD163 and iNOS (Inducible nitric oxide synthase) stained intra-tumoral and peri-tumoral TAMs (TAMs) were counted manually in five HPFs among all tumor spots for each patient. Intra-epithelial as well as intra-glandular macrophages were omitted. Finally, the *average* of CD163 and iNOS stained *macrophages number* was calculated for each sample.
Moreover, CD163 and iNOS stained intra-mucosal, acinar and periacinar macrophages were counted manually in three HPFs among all spots obtained from normal pancreatic parenchyma, obtaining the average CD163+ macrophage and iNOS+ macrophage number for each case.

Extracellular matrix

CD44 standard isoform (CD44s) expression was semi quantitatively analyzed on the basis on recent studies on gastric and breast cancer (Wu et al., 2016). The protein expression was scored according to the intensity of cellular staining and the proportion of stained tumor cells. The staining intensity was scored as 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellow brown) and 3 (strong staining, brown). The proportions of stained tumor cells were classified as 0 (≤5% positive cells), 1 (6-25% positive cells),2 (26-50% positive cells) and 3 (≥51% positive cells). The multiplication for intensity and proportion scores was utilized to represent the level of CD44 protein abundance. According to the final staining score, 1-3 was grouped to low expression, while ≥ 4 was classified

into high expression. Staining in non-neoplastic tissue provided an internal control in sections with negatively staining tumors.

Scoring of CD168 immunohistochemistry was adapted from Wartenberg and colleagues (Wartenberg et al., 2018). In more details, score 0 was assigned when no positivity was observed; score 1 was assigned when high-power magnification (×20-×40) was needed to detect expression; score 2 was recorded when staining was observed under medium power (×10) and score 3 was assigned when positive staining was observed at low magnification (×5).

4.5 STATISTICAL ANALYSIS

Chi-square, Fisher and Wilcoxon-Mann-Whitney tests were used, where appropriate. P values <0.05 were considered statistically significant.

5 RESULTS

5.1 CLINICAL CHARACTERISTICS

We identified 81 cases of pathologically confirmed pancreatic ductal adenocarcinomas (PDACs). These included 41 (50.6%) men and 40 women (49.4%). They were mostly sporadic, as only one case of PDAC was familial (mBRCA2). The overall mean age was 69 years (range, 34 to 84 y); BMI ranged from 14 to 40.4 (mean of 24.9), while the majority of the patients were classified as ECOG 1 (63.0%).

The main approach was upfront surgery (80.3%), while a minority of patients received a neoadjuvant chemotherapy as a first line treatment (19.7%), mainly FOLFIRINOX. Operative procedures included 68 Whipple pancreaticoduodenectomy (84.0%), 7 distal splenopancreasectomy (8.6%) and 6 total pancreatectomy (7.4%).

For what concern postoperative complications fistulas and hemorrhages were taken into account. Fistulas occurred in 9 patients (11.1%), mostly grade B (66.7%), while hemorrhages occurred in 10 patients (12.3%).

The detailed distribution of clinical characteristics of our case series is reported in Table 1.

Clinical characteristics

All patients n= 81	
Age (Mean±SD)	69 +/- 10
Sex	
- M	41 (50.6%)
- F	40 (49.4%)
ECOG	
- 0	30 (37.0%)
- 1	51 (63.0%)
BMI	24.9 +/- 6
Comorbidities	
- Arterial hypertension	12 (14.8%)
- Hypertensive cardiomyopathy	5 (6.1%)
- Ischemic cardiomyopathy	4 (4.9%)

- Diabetes	6 (7.4%)
- Obesity	4 (4.9%)
- Atrial fibrillation	2 (2.4%)
- Chronic renal insufficiency	2 (2.4%)
- Patients with previous neoplasm	13 (16.0%)
Resectability	
- Resectable	63 (77.7%)
- Borderline resectable / locally advanced	18 (22.3%)
cT stage	
- T1	13 (16.0%)
- T2	29 (35.8%)
- T3	14 (17.2%)
- T4	4 (4.9%)
cN stage	
- Nx	37 (45.6%)
- N0	14 (17.2%)
- N1	6 (7.4%)
- N2	3 (3.6%)
Fistula	
- No	72 (88.8%)
- Yes	9 (11.1%)
Grade of the fistula	
- BL	3 (33.3%)
- B	6 (66.7%)
- C	0 (0%)
Hemorrhage	
- Yes	10 (12.3%)
- No	71 (87.7%)
Neoadjuvant	
- Yes	16 (19.7%)
- No	65 (80.3%)
Type of neoadjuvant	
- FOLFIRINOX	7 (43.8%)
- GEMCITABINE-NABPACLITAXEL	3 (18.8%)
- GEMCITABINE-ABRAXANE	4 (25.0%)
- GEMOX	1 (6.2%)
- PAXG	1 (6.2%)
Number of cycles	6 (range, 2-12)
Inheritance	
- Sporadic	80 (98.8%)
- Heredofamilial (BRCA2)	1 (1.2%)

Table 2. Clinical characteristics of the case series

5.2 PATHOLOGIC CHARACTERISTICS

Four patients who underwent neoadjuvant chemotherapy at first, had no residual tumor; instead the other samples revealed a tumor size ranging from 1.5 to 7.0 cm (mean, 3.40 cm). All patients were classified as M0, whereas the pathologic T and N stages of the 81 cases were as follows—T0: 4(4,9%); T1: 9(11.1%); T2: 34(41.9%); T3: 33(40.7%); T4: 1(1.4%); N0:

30 (30.0%); N1: 33(47.8%); N2: 18(22.2%). Ultimately the final stages were—0: 4(4.9%); I: 20(24.7%); II: 20(46.9%); III: 38(23.5%); IV(0%).

As regards grading, PDAC samples were classified as low grade (G1-G2)(40.8%) and high grade (G3)(59.2%).

Lymphovascular and perineural invasion were identified in 65 (80.3%) and 71 (87.7%) tumors, respectively. The examination of the margins of resection pointed out that the majority showed a negative state (R0-72.8%).

The detailed distribution of pathologic characteristics of our case series is reported in Table 3.

Pathologic characteristics

All patients n= 81	PDACs (*n*= 81)
Tumor size (Mean +/- SD cm)	3.4 +/- 1.4
pT stage	
- 0	4 (4.9%)
- 1	9 (11.1%)
• a	0 (0%)
• b	0 (0%)
• c	4 (100%)
- 2	34 (42.0%)
- 3	33 (40.8%)
- 4	1 (1.2%)
pN stage	
- 0	30 (37%)
- 1	33 (40.8%)
- 2	18 (22.2%)
Stage	
- 0	4 (4.9%)
- I	20 (24.7%)
• A	9 (45.0%)
• B	11 (55.0%)
- II	38 (46.9%)
• A	5 (13.1%)
• B	33 (86.9%)
- III	19 (23.5%)
- IV	0 (%)
Lymphovascular invasion (LVI)	
- Absent	16 (19.7%)
- Present	65 (80.3%)
Perineural invasion	
- Absent	10 (12.3%)
- Present	71 (87.7%)

Histological grade - Low grade (G1-G2) - High grade (G3)	 31 (40.8%) 45 (59.2%)
Surgery - R0 - R1	 59 (72.8%) 22 (17.2%)

Table 3 Pathologic characteristics of the case series

5.3 AN OVERVIEW OF THE INFLAMMATORY MICROENVIRONMENT WITHIN THE TUMOR AND NORMAL TISSUE

Correlation between CD3, CD4 and CD8 T cells

In the intra and peritumoral compartment CD3+ T cells revealed to be higher than in the periacinar compartment of the normal tissue ($p<0.00001$). In the same way, the concentration of CD3+ T cells was higher in the periacinar compartment, rather than in the intraepithelial one ($p<0.00001$).

In the intra and peritumoral compartment CD4+ T cells revealed to be significantly higher than in the periacinar compartment of the normal tissue ($p<0.00001$). Similarly, the density of CD4+ T cells in the latter compartment revealed to be higher compared to the intraepithelial compartment of PDACs ($p<0.00001$).

Immunohistochemical analysis found out greater concentration of CD8+ T cells in the intra and peritumoral compartment, rather than in the periacinar area of the healthy tissue ($p<0.00001$) that, in turn, was higher compared to the intraepithelial compartment of tumors ($p<0.00001$).

Ultimately, CD3+, CD4+ and CD8+ T cells were mainly concentrated within intra and peritumoral compartment rather than in the normal pancreatic parenchyma.

Correlation between FOXP3 T cells and CD20 B cells

The spatial localization of these cells followed the trend seen for CD4+ and CD8+ T cells. Both FOXP3+ T cells and CD20+ B cells, were found mostly in the intra and peritumoral compartment rather than in the periacinar compartment of the normal tissue ($p<0.00001$, $p<0.00001$ respectively). As expected, the area with less inflammatory infiltrate was the intraepithelial compartment of tumor ($p<0.00001$).

Correlation between TAMs

For TAMs only intra-peritumoral and periacinar compartment were evaluated. Both iNOS+ TAMs (M1) and CD163+ TAMs (M2) were more concentrated in the intra and peritumoral compartment, rather than in the periacinar area ($p<0.00001$).

	CD3+	CD4+	CD8+	CD20+	FOXP3+	iNOS+	CD163+
A	98.1+/-51.8	45.9+/-26.8	52.4+/- 36.6	30.1+/-54.0	18.0+/-12.6	6.0+/-5.7	104.8+/-42.5
B	3.5+/-7.0	1.1+/-2.2	2.9+/- 6.7	0.1+/-0.3	0.7+/-1.4	2.4+/-3.4	71.7+/-34.1
C	33.3+/-20.5	12.6+/-13.2	28.2+/- 20.9	4.5+/-6.9	3.8+/-5.0		
D	0.3+/-1.3	0.11+/-0.9	1.0+/- 2.8	0.0+/-0.0	0.1+/-0.3		

Table 4. Distribution of inflammatory cells in each compartment; A= Intra and peritumoral; B= Intraepithelial; C= Periacinar; D=Intra-acinar

5.4 DISTRIBUTION OF TUMOR IMMUNE MICROENVIRONMENT (TIME) PHENOTYPES

In order to define the microenvironment phenotypes distribution between the PDACs, CD3+ iTILs count and PD-L1 expression were taken into account. 4 of 81 patients had no tumor left after neoadjuvant

chemotherapy, consequently no further analyses could be done on these specimens. In the remaining 77 cases intraepithelial CD3+ TILs were high (≥2/HPF; designated as “positive”) in 47 (61.0%) PDACs and low in 30 (39.0%), whereas PD-L1 (CPS≥ 1; designated as “positive”) was expressed in 61 (79.2%) PDACs and not expressed in 16 (20.7%).

The distribution of the four TIME phenotypes (PD-L1 pos/TILs pos; PD-L1 pos/TILs neg; PD-L1 neg/TILs pos; PD-L1 neg/TILs neg) was as follows—PD-L1 pos/TILs pos: 35(45.5%); PD-L1 pos/TILs neg: 26(33.8%); PD-L1 neg/TILs pos: 12(15.6%); PD-L1 neg/TILs neg: 4(5.1%).

Subsequently we determined, for each one of the four TIME phenotype, the quantity, in terms of mean, of every cell we made correlations with before.

The detailed distribution of TIME phenotypes and the cells within each TIME phenotypes are reported in figure 4 and table 5 respectively.

All patients n= 77	PD-L1 pos/ TILs pos (n=35)	PD-L1 pos/ TILs neg (n=26)	PD-L1 neg/ TILs pos (n=12)	PD-L1 neg/ TILs neg (n=4)
CD3+ TILs				
- A	104.8+/-51.2	102.3+/-52.2	86.7+/-51.8	45.2+/-39.7
- B	5.6+/-7.1	1.0+/-7.0	4.1+/-2.3	0.7+/-2.1
- C	34.5+/-20.7	27.5+/-20.5	37.5+/-20.3	39.4+/-23.7
- D	0.3+/-1.3	0.6+/-1.3	0.0+/-0.0	0.0+/-0.0
CD4+ TILs				
- A	50.2+/-27.1	47.0+/-27.0	40.0+/-26.2	19.4+/-25.0
- B	1.5+/-1.9	22.5+/-2.2	0.8+/-1.2	0.5+/-1.7
- C	12.7+/-9.7	13.5+/-9.6	10.9+/-1.2	8.6+/-11.8
- D	0.0+/-0.0	0.0+/-0.0	0.7+/-1.2	0.0+/-0.0
CD8+ TILs				
- A	60.0+/-36.8	47.3+/-36.7	50.2+/-37.5	25.7+/-14.6
- B	4.1+/-6.8	0.8+/-6.8	4.0+/-2.7	2.9+/-2.6
- C	30.5+/-21.1	25.4+/-21.0	31.2+/-21.9	11.0+/-18.3
- D	0.8+/-2.8	0.8+/-2.8	0.8+/-3.1	6.2+/-4.6
FOXP3+ TILs				
- A	20.6+/-12.8	18.6+/-12.6	12.0+/-12.4	9.0+/-7.2
- B	1.0+/-1.4	0.5+/-1.4	0.7+/-1.2	0.2+/-1.0
- C	3.8+/-5.0	4.1+/-5.0	4.0+/-5.6	3.0+/-4.2
- D	0.1+/-0.3	0.1+/-0.3	0.0+/-0.0	0.2+/-0.3
CD20+ TILs				
- A	22.1+/-53.3	53.1+/-54.4	12.6+/-59.0	3.9+/-12.9
- B	0.2+/-0.4	0.1+/-0.4	0.2+/-0.3	0.0+/-0.0
- C	5.7+/-7.0	4.0+/-7.0	3.0+/-6.0	4.9+/-9.5
- D	0.0+/-0.0	0.0+/-0.0	0.0+/-0.0	0.0+/-0.0

iNOS+ TAMs - A - C	5.1+/-33.8 2.7+/-3.4	7.0+/-34.2 2.0+/-3.5	7.3+/-36.1 3.4+/-2.9	5.9+/-36.0 1.4+/-1.6
CD163+ TAMs - A - C	106.4+/-42.5 76.0+/-33.8	106.4+/-42.8 68.4+/-34.2	89.9+/-34.3 67.3+/-34.8	104.6+/-39.5 82.3+/-35.9

Table 5. Distribution of inflammatory cells within each TIME phenotypes; A= Intra and peritumoral(T)l; B= Intraepithelial(T); C= Periacinar(N); D=Intra-acinar(N)

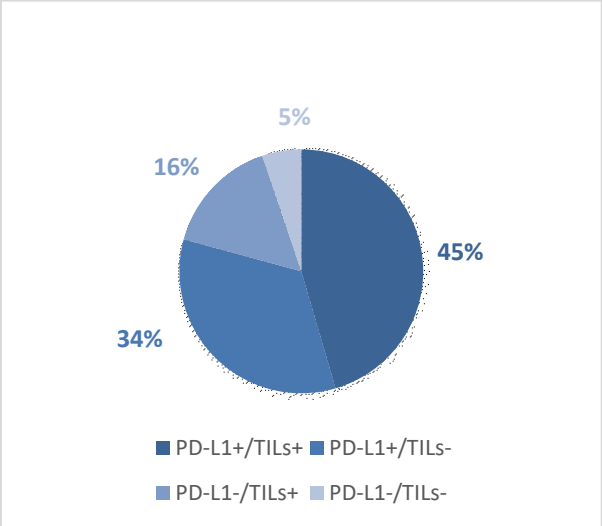

Figure 4. Distribution of TIME phenotypes

At last, we focused on the stromal immunophenotype and analyzed the expression of CD44 and CD168. CD44 expression had been dichotomized in CD44+ (IRS≥1) and CD44- (IRS=0), whereas CD168 expression was dichotomized in CD168+ (intensity score≥1) and CD168-(intensity score=0). CD44 was found positive in 49 PDACs (63.7%) and negative in 28(36.3%), whereas CD168 was found positive in 62 PDACs (80.5%) and negative in 15(19.5%).

Afterwards we focused on the distribution of the tumor immune microenvironment phenotypes (TIME) between patients who underwent upfront surgery at first, and patients who received a neoadjuvant chemotherapy as a first line treatment. There was no statistically significant association between the distribution of the TIME phenotypes and therapeutic choice (p=0.639). The exact partition of each phenotype is displayed in table 6 and in figure 5 and 6.

All PDACs n = 77	Upfront surgery (n=65)	Neoadjuvant (n=12)
PD-L1+/TILs+ (n=35)	29 (45.0%)	6 (50.0%)
PD-L1+/TILs- (n=26)	23 (35.0%)	3 (25.0%)
PD-L1-/TILs+ (n=12)	9 (14.0%)	3 (25.0%)
PD-L1-/TILs- (n=4)	4 (6%)	0 (0.0%)

Table 6. Distribution of TIME phenotypes in patients who underwent upfront surgery or neoadjuvant chemotherapy

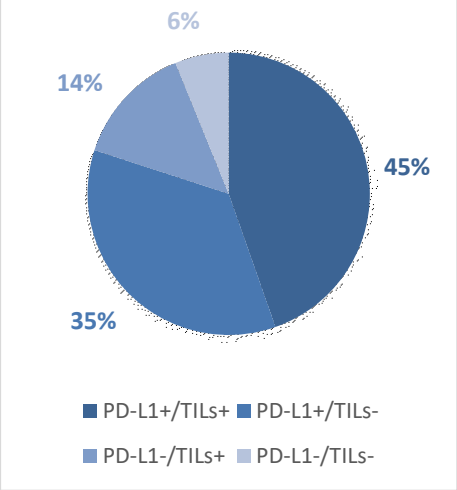

Figure 5. TIME phenotypes in patients who underwent upfront surgery

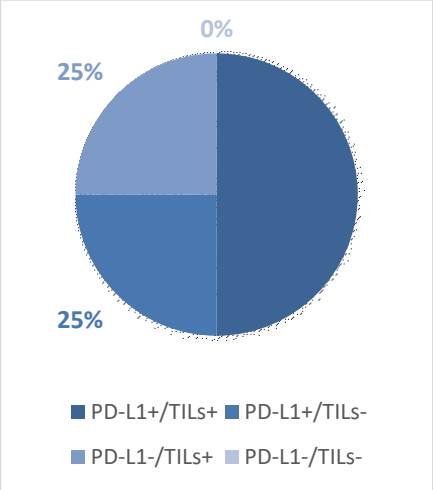

Figure 6. TIME phenotypes in patients who underwent neoadjuvant chemotherapy

5.5 OVERALL SURVIVALS (OS) ANALYSIS

At this point we had carried out OS analysis in our cohort and in each TIME phenotypes. 12 patients out of 81 were excluded because we could not

find any data on the electronic archive. In the remaining 69 cases we observed so far 42 events and 27 censored data. The median OS was 27.7 months, the lower and the upper extremities were respectively 18.9 and 37.7 months. The survival curve is displayed in figure 7.

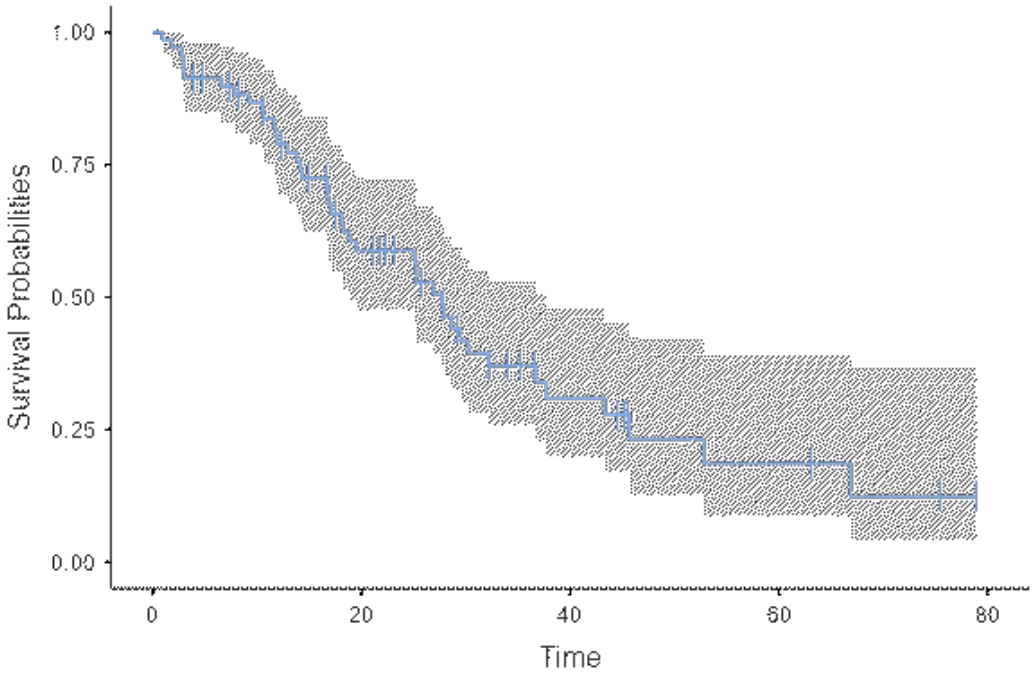

Figure 7. OS curve of the case series

Afterwards, we analyzed the OS rates in each TIME phenotypes, which were relabeled as follows: PDL1+/ TILs+ = 0; PD-L1+/ TILs- = 1; PD-L1-/TILs+ = 2; PD-L1-/TILs- = 3.

PDL1+/ TILs+ patients were 33, with a mean and median OS of 24.8 and 25.2 months respectively, whereas PD-L1+/ TILs- were 22, with a mean and median OS of 35.8 and 29.5 months respectively. PD-L1-/TILs+ were 11, with a mean and median OS of 45.2 and 32.2 months respectively, while PD-L1-/TILs- were 3, with a mean and median OS of 25.5 and 30.2 months respectively. The exact partitions and OS rates are shown in table 7.

Median Survival Table: Levels for C

						95% Confidence Interval	
Levels	**Records**	**Events**	**rmean**	**se_rmean**	**Median**	**Lower**	**Upper**
C=0	33.00	21	24.8	2.72	25.2	16.80	NaN
C=1	22.00	12	35.8	6.00	29.5	16.70	NaN
C=2	11.00	6	45.2	9.10	32.2	25.20	NaN
C=3	3.00	3	25.5	9.72	30.2	2.90	NaN

Table 7. OS rates in each TIME phenotypes

The comparison between OS rates of each TIME phenotypes did not find any statistical relevant difference (p=0.36). The Kaplan-Meyer curves are shown in Figure 8.

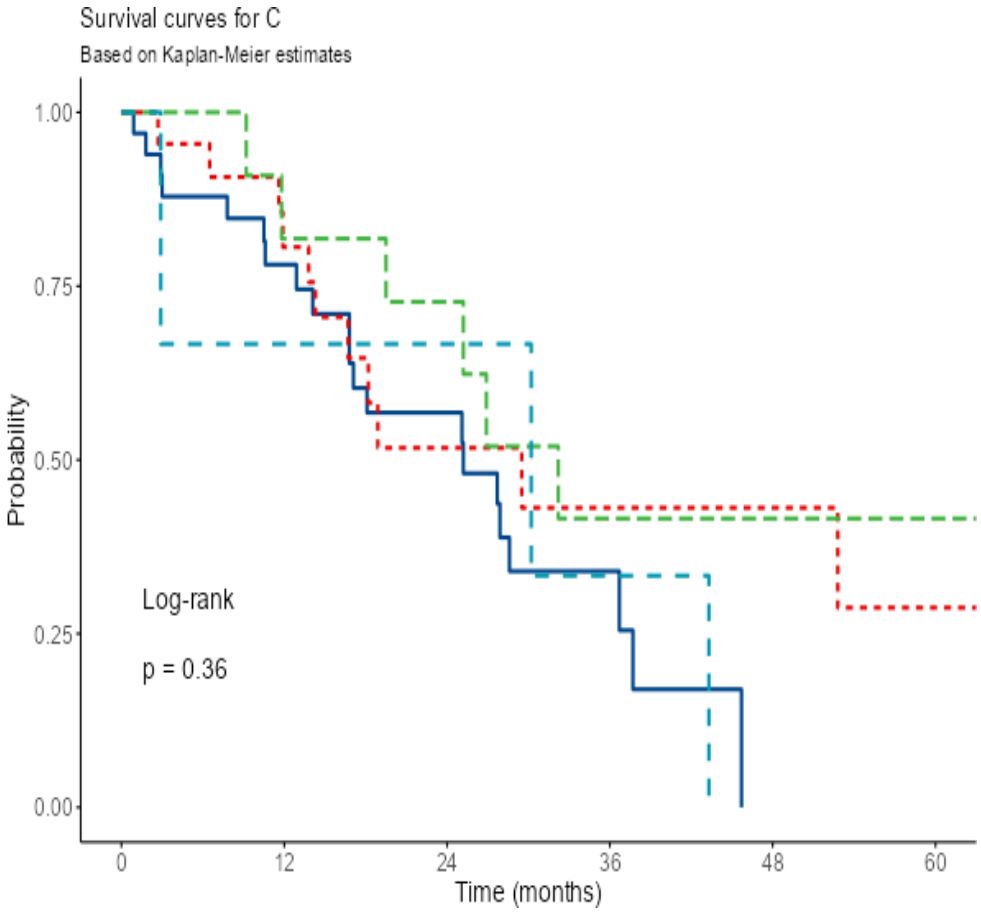

Figure 8. Kaplan- Meyer curves of each TIME phenotypes; PDL1+/ TILs+ = blue; PD-L1+/ TILs- = red; PD-L1-/TILs+ = green PD-L1-/TILs - = light blue

5.6 THE INFLAMMATORY MICROENVIRONMENT WITHOUT NEOADJUVANT CHEMOTHERAPY AND AFTER NEOADJUVANT CHEMOTHERAPY

Once we had evaluated the localization of the inflammatory cells in PDACs, we carried on our study on the inflammatory microenvironment searching for differences, in term of density of inflammatory infiltrate, between patients who underwent upfront surgery and patients who received neoadjuvant chemotherapy instead. In order to do that, we focused on the intra and peritumoral compartment. Only four patients out of 81 showed no tumor left in the specimen after neoadjuvant chemotherapy; thus they were excluded from the analysis.

There were no statistically relevant differences between the mean of the two groups for what regards the distribution of CD3+ cells(p=0.40), CD4+ T cells(p=0.46), CD8+ T cells(p=0.13), FOXP3+ T cells(p=0.30), CD20+ B cells(p=0.42), iNOS TAMs(p=0.65), CD163+ TAMs(p=0.13).

The means and the standard deviation for each type of cells are reported in table 8.

	CD3+	CD4+	CD8+	FOXP3+	CD20+	iNOS+	CD163+
Upfront surgery (n=65)	99.5+/-50.7	45.1+/-27.5	51.8+/-36.3	18.9+/-13.2	31.0+/-56.6	5.7+/-35.0	106.4+/-43.2
Neoadjuvant chemotherapy (n=12)	90.5+/-59.4	50.2+/-23.4	55.9+/-39.4	12.8+/-7.2	25.4+/-39.1	7.9+/-23.0	96.0+/-38.6

Table 8. Distribution of inflammatory cells in patients who underwent upfront surgery and neoadjuvant chemotherapy

The same goal was pursued for the analysis of the stroma, considering this time CD44 and CD168 expression of the tumoral stroma in the two groups mentioned above. As we said in the previous paragraph, CD44 expression had been dichotomized in CD44+ (IRS≥1) and CD44- (IRS=0), whereas

CD168 expression was dichotomized in CD168+ (intensity score≥1) and CD168-(intensity score=0).

Both CD44 and CD168 expression were not related to the type of treatment (p=0.12, p=0.34, respectively).

The detailed distribution of CD44 and CD168 are reported in table 9 and table 10.

	CD44+	CD44-	
Neoadjuvant chemotherapy	10	2	12
No neoadjuvant chemotherapy	39	26	55
	49	28	77

Table 9. Distribution of CD44 in the cohort

	CD168+	CD168-	
Neoadjuvant chemotherapy	7	5	12
No neoadjuvant chemotherapy	55	10	65
	62	15	77

Table 10. Distribution of CD168 in the cohort

6 DISCUSSION

PDACs are tumors with high mortality, commonly diagnosed at an advanced stage with poor prognosis and limited therapeutic options. Surgery and systemic chemotherapy are the mainstay of therapy for locoregional and metastatic disease, respectively.

The introduction of checkpoint immunotherapies has recently revolutionized the oncological targeted therapy paradigm; however, clinical trials in PDACs have been largely unsuccessful. Pembrolizumab, an anti-PD1 monoclonal antibody effective in many solid tumors with microsatellite instability, has already been approved by the U.S. Food and Drug Administration (FDA) in 2017 for the treatment of a variety of advanced solid tumors either with MSI or MMRd, including PDACs [90].

The predictive biomarkers in use in this therapeutic setting in gastrointestinal tract cancers, are the expression of the immune checkpoint programmed cell death protein 1 (PD-1) and its ligand PD-L1, the presence of high tumor mutational burden (TMB), and microsatellite instability (MSI) [104]. However considering that $CD8^+$ cytotoxic T lymphocytes (CTLs) encounter dysfunction and exhaustion due to immunerelated tolerance and immunosuppression within the tumor microenvironment (TME), and that programmed death-1 receptor (PD-1)–ligand (PD-L1) and CTL-associated antigen 4 (CTLA-4) are checkpoint receptors that can be targeted for relieving exhaustion of $CD8^+$ T cells and renewing their priming, it is clear that the presence of infiltrating $CD8^+$ T cells in combination with increased PD-L1 expression/amplification is positively associated with the therapeutic efficacy of PD-1 blockade [105]. In light of the above it's reasonable that the study of the TME, in terms of immune infiltrate, could play an important role as a predictive biomarker of immunotherapy response.

With this biologic and therapeutic rationale, the TMEs of 81 PDACs were evaluated.

Firstly, clinical and pathologic features of PDACs were studied. Pancreatic tumor present at an older age, generally ≥2 cm(≥T2), at stage II and with high grade, mostly G3, confirming their well-known aggressive behaviour. Consistently, lymphovascular and perineural invasion was reported to be really common in PDACs, highlighting once again the reason behind the poor prognosis in this type of tumors.

Eventually, patients presenting with pancreatic cancer, should be evaluated for a possible occult underlying predisposing condition, such as familial pancreatic cancer, hereditary pancreatitis; if reasonable other more rare conditions, like hereditary non-polyposis colorectal cancer, hereditary breast and ovarian cancers, Peutz–Jeghers syndrome, ataxia telangiectasia, familial atypical multiple mole melanoma syndrome and Li–Fraumeni syndrome, should be investigated, especially in presence of previous neoplasms in the anamnestic history.

Secondly, the inflammatory microenvironment was studied. TAMs were among the most abundant cells in TME, playing an important role in tumor progression as highlighted in many previous publications [30, 106, 107, 108, 109]. As expected, all the inflammatory cells, regardless of the subgroup they belonged to, tended to be more concentrated within the intra and peritumoral area, instead of the intraepithelial, intra-acinar and peri-acinar compartment of the normal pancreatic tissue.

Afterwards the distribution of tumor immune microenvironment phenotypes was evaluated. According to *Teng et al*. [100], PDACs were classified on the basis on PD-L1 expression and iTILs levels in four tumor immune microenvironment (TIME) phenotypes as follows: PD-L1 pos/ TILs pos; PD-L1 pos/ TILs neg; PD-L1 neg/ TILs pos; PD-L1 neg/ TILs neg.

Among PDACs, PD-L1 pos/ TILs pos phenotype was reported to be the most frequent, both in patients who underwent upfront surgery and in patients

who received neoadjuvant chemotherapy at first. Thus, showing high density of TILs and PD-L1s, this subgroup is thought to be the most responsive to immune checkpoint blockade. However, considering the high prevalence of M2 macrophages, whose immunosuppressive role is well established, more evidence is needed on whether being PD-L1 pos/ TILs pos is enough to respond to ICBs and to counteract the protumor effect of M2 TAMs.

PD-L1 pos/ TILs neg phenotype in this series of PDCAs was the second most frequent phenotypes on both groups. Moreover, they were once again characterized by low iNOS and high CD163+ TAMs levels, suggesting once more the importance of immune ignorance mechanisms.

PD-L1 neg/ TILs positive, which was third in terms of prevalence in both groups, was characterized by a lower inflamed status: in fact the TME was poorer in infiltrate; however, once again, the most representative cells were CD163+ TAMs and CD3+ TILs.

The last phenotype, PD-L1 neg/ TILs neg, showed the lowest rate of TILs, along with high levels of CD163+ TAMs. Giving its immunologic ignorance, it should not be suitable for immune checkpoint blockade; surgery and chemotherapy remains the mainstay of the therapy.

For what regards the overall survival rates in each TIME phenotypes, PD-L1-/TILs showed a longer survival in absolute rates, however the difference in relation with the other phenotypes was not relevant.

At last we evaluated the differences in immune landscape between the two groups of patients, those who underwent upfront surgery and those who had neoadjuvant chemotherapy. M2 macrophages were the leading cells in term of numbers and there were no statistically relevant differences between the two groups; thus, it's reasonable to say that chemotherapy didn't have a major impact on the immune landscape, even though in literature is reported the use of chemotherapy to make a tumor "hotter" in terms of immune infiltrate [110, 111].

This study has several limitations. Notably, giving the rarity of PDACs and especially of patients suitable for neoadjuvant chemotherapy followed by resection, further studies are needed to confirm these data. Another limitation is the use of TMAs rather than the whole section.

7 CONCLUSIONS

The PDAC tumors microenvironment is characterized by complex fibrotic stroma with substantial infiltration of tumors-promoting immunosuppressive cells and pronounced T cell exhaustion, favoring immune evasion that results in immunotherapeutic failures and poor clinical outcome. Therefore, understanding the complexity of PDAC immune landscape and the mechanisms involved in T cell dysfunction may contribute to identifying new immunotherapeutic strategies.

The prevalence of PD-L1 expression, intraepithelial CD3+ tumor infiltrating lymphocytes (iTILs) and CTLs density could be used as markers in order to detect patients who are more suitable for immune checkpoint blockade, such as PD-L1 pos/ TILs pos PDACs. However, considering that PDACs in our series were mostly PD-L1 pos/ TILs pos, biopsy is performed only in third level structure and that neoadjuvant chemotherapy did not seem to change neither the TME nor the PD-L1 status, it is reasonable to think of immunotherapy as a first line treatment in doublets or in addition to the chemotherapy in locally advanced or metastatic PDACs, regardless the PD-L1/ TILs status resulting from the biopsy. A similar approach was considered in the TOPAZ-1 study, where patients with inoperable advanced biliary tract cancer were randomly assigned to receive durvalumab plus gemcitabine–cisplatin or a placebo plus gemcitabine–cisplatin; the results showed that patients who received durvalumab had a longer OS (12.8 months vs 11.5 months), without any serious side effect. These results could potentially change the therapy paradigm of advanced PDACs, however, more evidence on larger cohorts is needed in terms of clinical data.

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In science novelty emerges only with difficulty, manifested by resistance, against a background provided by expectation.

Thomas Kuhn