Article

A comprehensive transcriptional signature in pancreatic ductal adenocarcinoma reveals new insights into the immune and desmoplastic microenvironment

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Simple Summary:

Pancreatic ductal adenocarcinoma (PDAC) is a highly lethal disease with few curative options. Desmoplastic stroma and immune system evasion in PDAC represent challenges to the success of therapeutic strategies that function well in other tumor types. Characterizing the PDAC microenvironment (including the immune environment) remains critical to developing safe and efficient therapies. Here, we present a comprehensive meta-analysis identifying 1153 significantly dysregulated genes, which mainly impact extracellular matrix remodeling and the immune system. We identify two signatures of twenty-eight immune-related genes and eleven stroma-related genes influencing PDAC patient survival. Additionally, five immune genes are associated with PDAC prognosis for the first time.

Abstract:

Pancreatic ductal adenocarcinoma (PDAC) prognosis and treatment response remains devastatingly poor due partly to the highly heterogeneous, aggressive, and immunosuppressive nature of this tumor type. The intricate relationship between stroma, inflammation, and immunity remains vaguely understood in the PDAC microenvironment. Here, we performed a meta-analysis of stroma-, and immune-related gene expression in the PDAC microenvironment to improve disease prognosis and therapeutic development. We selected twenty-one PDAC studies from the Gene Expression Omnibus and ArrayExpress databases, including 922 samples (320 controls and 602 cases). Differential gene enrichment analysis identified 1153 significant dysregulated genes in PDAC patients that contribute to a desmoplastic stroma and an immunosuppressive environment (the hallmarks of PDAC tumors). The results highlighted two gene signatures related to the immune and stromal environments that cluster PDAC patients in high- and low-risk groups, impacting patient stratification and therapeutic decision-making. Moreover, *HCP5, SLFN13, IRF9, IFIT2*, and *IFI35 immune genes* were related to prognosis value in PDAC patients, for the first time.

Keywords: pancreatic ductal adenocarcinoma, desmoplasia, immune system, heterogeneity cancer, biomarkers, molecular profile, meta-analysis, transcriptomics, prognosis, meta-analysis

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, representing over 80% of all diagnosed pancreatic neoplasms. This highly lethal cancer has a poor prognosis (five-year survival rate below 5%) and a median survival rate of fewer than six months [1]. While currently the seventh leading cause of cancer death worldwide [2], the yearly increase in incidence may make PDAC Europe's third leading cause of cancer death by 2025 [2]. The absence of reliable biomarkers for effective screening and early diagnosis at pre-symptomatic stages where treatments function most effectively represents a primary reason why most PDAC cases remain incurable. Currently, most patients present locally advanced (30%-35%) or metastatic (50%-55%) PDAC at diagnosis [3].

In advanced-stage PDAC patients, curative surgery remains impossible, and systemic therapeutic options (including immunotherapy) remain limited and ineffective [4]. Among solid tumors, PDAC represents an immunologically "cold" tumor characterized by sparse T-cell infiltration [5,6]; in contrast, immunologically "hot" tumors (such as melanoma) suffer from high neoantigen load and immune cell infiltration [7]. As distinctive features, PDAC tumors possess an extracellular matrix (ECM) composition and fibrotic stroma that make it highly desmoplastic and significantly influence immune responses [8]. PDAC cells strongly interact with the surrounding microenvironment, which includes components such as immune cells, cytokines, metabolites, fibroblasts, and hyaluronan. These interactions create a highly fibrotic and active organized stroma (desmoplastic stroma) and an immunosuppressive environment that makes PDAC invasive and highly resistant to immunotherapy [6,9]; therefore, characterization of the stroma and tumor immune microenvironment in PDAC patients represents a critical step in developing more effective therapeutic strategies.

This study aimed to understand the stroma and tumor immune microenvironment of PDAC patients by retrieving and analyzing transcriptomic data from twenty-one different studies (representing a population of 922 samples; 320 controls and 602 cases) from the Gene Expression Omnibus (GEO)-NCBI and ArrayExpress data repositories. Through a meta-analysis, we identified a series of gene signatures that may play a significant role in the therapeutic decision-making in PDAC patients, including 5 genes not previously related to PDAC survival. We also provide a friendly-user web tool with detailed and interactive visualization of our comprehensive meta-analysis results.

2. Materials and Methods

All bioinformatics and statistical analyses employed R software v.4.1.3 [10] (Supplementary Table S1 details R packages and versions).

2.1. Study search and selection

Publicly available datasets were collected from the GEO-NCBI [11] and ArrayExpress databases [12]. The data available in the Cancer Genome Atlas (TCGA) [13] were excluded from the original search with the purpose of using this dataset as an external cohort for survival analysis. Following the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines [14], a systematic search of published studies was conducted in 2021 (period: 2002-2021). Three researchers in the study conducted the literature search (C.P.C, L.F., and I.P.D.), and the consistency of the review and selection procedures used was evaluated and confirmed. A broad search was performed using the MeSH (Medical Subject Headings) thesaurus keyword "pancreatic cancer" after which stringent filters were applied. The final inclusion criteria were:

- Normal and PDAC samples available
- RNA samples extracted directly from human pancreas biopsies
- Patients had not undergone treatment before biopsy
- Sample size > 4 for PDAC and control groups

Finally, normalized gene expression from twenty-seven microarray studies (GSE86436, GSE71989, GSE62452, GSE62165, GSE60979, GSE56560, GSE55643, GSE46234, GSE43795, GSE43288, GSE41368, GSE32676, GSE28735, GSE27890, GSE22780, GSE19650, GSE18670, GSE16515, GSE15471, GSE1542, GSE11838, GSE102238, GSE101448, E-MTAB-3365, E-MTAB-1791, E-MEXP-950, and E-EMBL-6) and count matrices of two RNA-sequencing (RNA-seq) (GSE119794 and GSE136569) datasets were retrieved for further analysis.

2.2. Individual preprocessing and analysis

Datasets were individually analyzed in two steps: preprocessing and differential expression analysis.

The nomenclature of the clinical variables included in each study was standardized for data preprocessing, and then an exploratory analysis was performed. Prior to the exploratory analysis, RNA-seq raw count matrices were normalized using the trimmed mean of m-values from the edgeR package [15,16]. The normalization method performed by the original authors for each microarray dataset was assessed, and the matrices were log2-transformed when necessary. The exploratory analysis included expression boxplots, unsupervised clustering, and principal component analysis (PCA) to detect patterns of expression between samples and genes and the presence of batch effects in each study.

Differential gene expression analyses were performed in R using limma (v.3.48.3) [17], and a paired sample design was implemented in those datasets where applicable. Differentially-expressed genes were identified using p-values with Benjamini-Hochberg correction [18] for a false discovery rate (FDR) at a significance level of 0.05.

2.3. Gene expression meta-analysis

Gene expression analysis results were integrated into a meta-analysis using the DerSimonian & Laird random-effects model [19], considering individual study heterogeneity. This model considers the variability of individual studies by increasing the weights of studies with less variability when computing meta-analysis results.

A total of 24,365 genes were evaluated. P-values, FDR-corrected p-values, the logarithm of Fold Change (logFC), and 95% confidence intervals of logFC were calculated for each evaluated gene, and both funnel and forest plots were computed for each gene. These representations were assessed for possible biased results, where logFC represents the effect size of a function, and the standard error of the logFC serves as a study precision measure [20]. Genes were considered significant when FDR < 0.05, absolute logFC > 0.6, and were measured in at least eleven studies. Sensitivity analysis (leave-one-out cross-validation [21]) was conducted for each significant gene to verify alterations in the results owing to the inclusion of any study.

Statistically-significant results from the gene expression meta-analysis were functionally enriched by over-representation analysis (ORA) using clusterProfiler [22,23] and ReactomePA [24]. Gene Ontology (GO) terms [25,26] and Reactome pathway [27] enrichment were performed following this approach. Only those functions and pathways with more than ten differentially-expressed genes found in the gene set were considered. Functional enrichment was explored and visualized with the *rrvgo* package [28].

2.4. Web tool

To make the data and results of our research widely accessible, a web tool was developed using the shiny package in R. The tool was developed in a user-friendly manner, allowing users to navigate and interact with

the data. Users can then select different variables and parameters to visualize the data in numerous ways. The tool also includes interactive plots and tables to display the analysis results. The web tool is hosted on a secure server and is regularly maintained to ensure stability and performance. The source code for the tool is also publicly available and can be accessed through our GitHub repository: https://github.com/ipediez/ShinyReport.

2.5. Survival analysis

RNA-seq expression data and metadata from patients in the Pancreatic adenocarcinoma (PAAD) TCGA cohort were downloaded from cBioPortal [29]. Z-scores of RNA-seq expression were used for survival analysis. For each analyzed gene, samples were divided into two groups based on their expression levels. Samples with expression Z-scores below the lower quartile were classified as having low expression, whereas samples exceeding the upper quartile were classified as having high expression. Forty-five samples with high expression and forty-five samples with low expression were included in the survival analysis. Gene-wise Kaplan-Meier survival analysis compared the low- and high-expression groups. This method estimates the probability of survival over time based on the expression levels of the gene of interest. The log-rank test was used to compare the survival curves between distinct groups of samples.

For risk-score- based survival, genes were tagged as highly-expressed for a given sample when expression levels were above the upper quartile. Then, samples were clustered into "high-risk" and "low-risk" groups based on the number of highly expressed genes. The cutoff was set as the median of the highly-expressed genes in each sample. Furthermore, a proportional hazard model using Cox regression was implemented to study the impact of clinicopathological variables on survival and evaluate the contribution of the risk-score in a multivariate model.

3. Results

We performed a systematic review and differential gene expression analysis of PDAC transcriptomic studies from the GEO-NCBI [11] and ArrayExpress [12] databases to explore stroma and immune environments in PDAC patients. We then integrated the results of each differential gene expression analysis into a meta-analysis. The biological context of the meta-analysis results was explored via functional enrichment, using an ORA of GO terms and pathways (Figure 1). Finally, we conducted a survival analysis to explore the impact of specific candidate genes on patient outcomes.

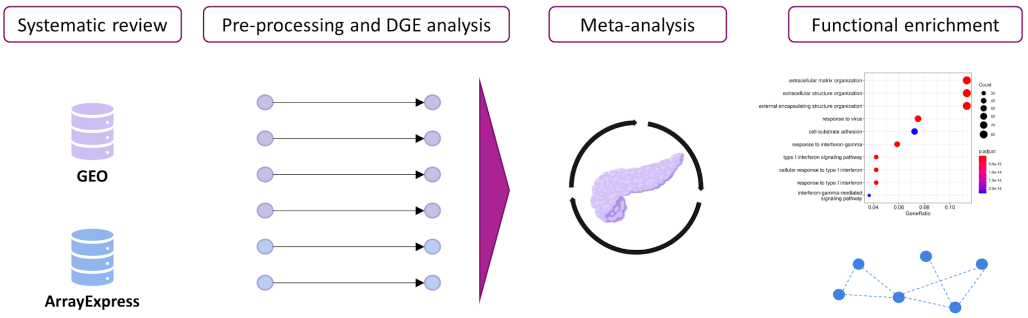

Figure 1. Workflow and analysis design. Relevant studies from the GEO-NCBI and ArrayExpress databases were retrieved, and data exploration and preprocessing were then performed. After DGE analysis, the results from different studies were integrated into a gene meta-analysis. Functional profiling methodologies were applied to explore the biological implications of the results.

3.1. Study search and selection of PDAC studies

The systematic review identified 143 non-duplicated studies. We searched for studies including RNA-seq samples of pancreatic cancer patients, and included in the exploratory analysis those studies that met the inclusion criteria: include PDAC and control samples, and include human primary tissue samples. Then, we excluded studies with samples from patients under cancer treatment and studies where sample size was less than 4 in the PDAC or the control group, resulting in a subset of twenty-nine studies (Figure 2). We discarded eight studies after exploratory analysis, giving a final set of twenty-one homogeneous and comparable studies for further analysis. The selected studies included 922 samples (320 controls and 602 cases). Although most studies did not include relevant sample metadata, we assessed clinical characteristics when available. Supplementary Tables S2 and S3 contain further information regarding the selected studies and clinicopathological characteristics of the study population.

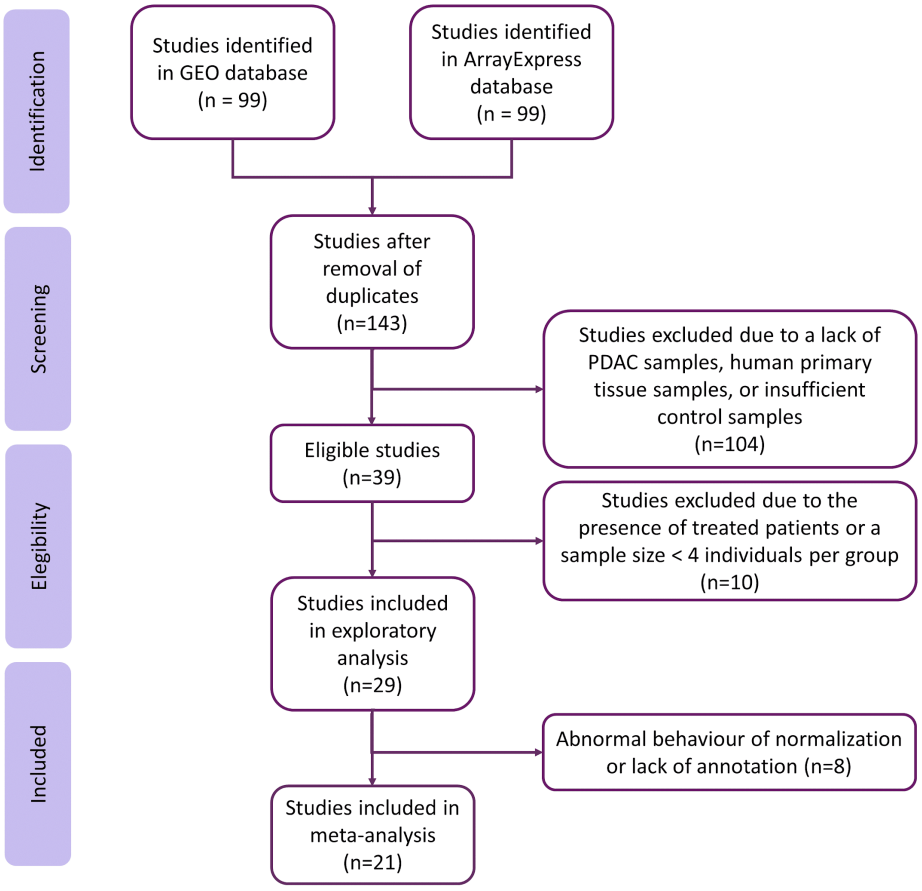

Figure 2. Flow of information through the distinct phases of the systematic review, following PRISMA Statement guidelines.

3.2. Data acquisition and preprocessing

As the normalized data derived from different platforms, we performed exploratory and processing steps on the dataset to ensure the comparability and integration of subsequent analyses. The exploratory analysis found abnormal normalization or a lack of annotation in eight studies, which we excluded from further analysis (listed in Supplementary Table S4).

3.3. Meta-analysis

We performed an independent differential gene expression analysis in each study and a functional meta-analysis for the 24,365 genes evaluated in the different datasets, including every gene found in at least two studies. We considered significant those results with an FDR < 0.05, an absolute logFC > 0.6, and evaluated in at least eleven studies; overall, 1153 genes accomplished these criteria (Figure 3, further detailed in Supplementary Table S4).

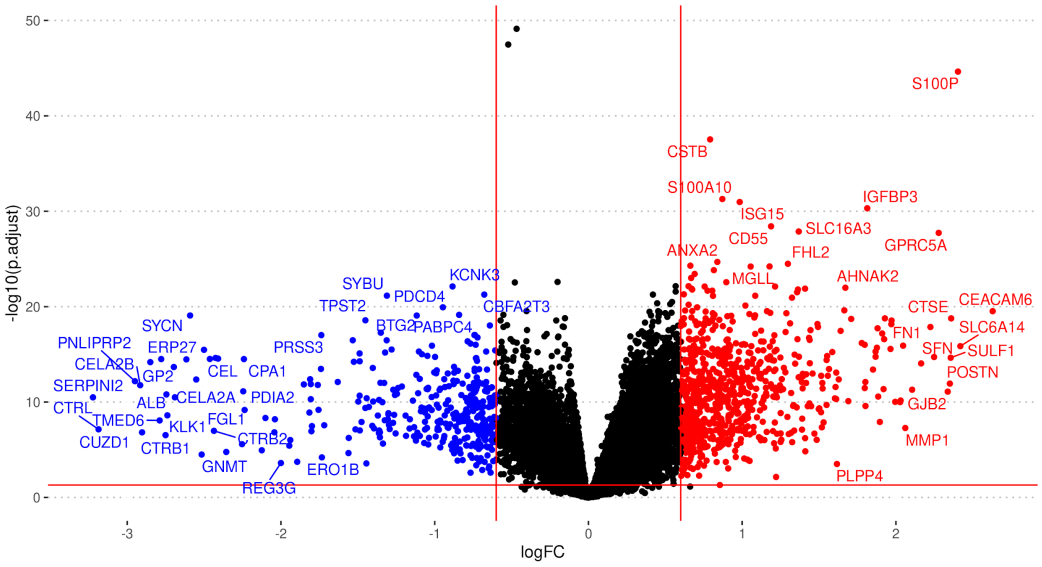

Figure 3. Volcano plot summarizing the gene expression meta-analysis. Significantly over-expressed genes shown in red, and significantly under-expressed genes shown in blue (FDR < 0.05, absolute logFC > 0.6). Only genes found in at least eleven studies shown.

We noted the presence of genes encoding ECM components (e.g., collagens, fibronectin, laminin, stratifin), proteoglycans (e.g., versican), cell adhesion molecules, integrins, matrix metallopeptidases, and additional peptidases and enzymes that impact mechano-contractility, epithelial tension, and stiffness of tumoral stroma, which can promote tumor progression and resistance to therapy (Figure 4). Table 1 displays the twenty genes with the highest and lowest logFC values from the meta-analysis; said genes mainly play roles in ECM remodeling, desmoplasia, metabolism, and the immune system. Supplementary Table S4 reports the complete list of significantly affected genes.

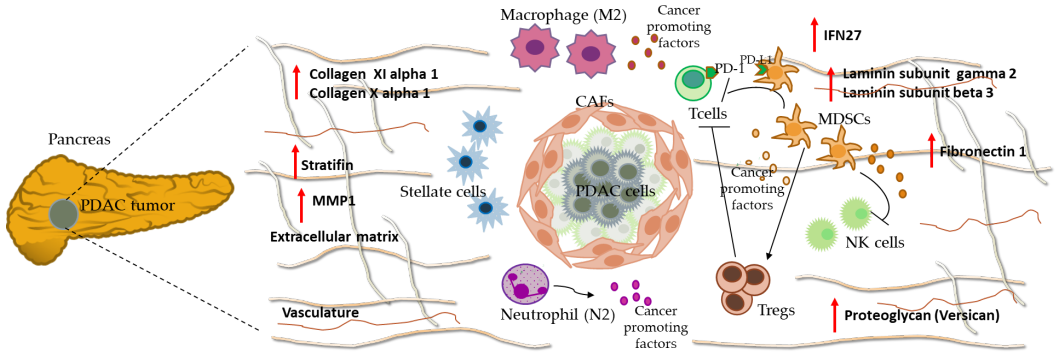

Figure 4. Overview of PDAC microenvironment. Meta-analysis results indicated an overexpression of several ECM components e.g., stratifin, fibronectin 1, different laminin subtypes (gamma2 and beta3), collagens and proteoglycans that characterize the dense and desmoplastic stroma of PDAC tumors. Also highlight the presence of immune components such as IFN27, which contribute to an increase of M2 macrophages and a decrease of CD8+ T cells. Therefore, the desmoplastic stroma and the immune system favor immune tolerance and poor prognosis in PDAC. IFN27: interferon alpha inducible protein, MMP1: matrix metallopeptidase 1, NK cells: natural killer cells, T cells: T effector lymphocytes, Tregs: T regulatory lymphocytes T.

Table 1. Top twenty genes up- and down-regulated in PDAC patients.

Gene Symbol	Gene Name	Expression Level	Function
CEACAM6	CEA cell adhesion molecule 6	UP	EMR
SLC6A14	Solute carrier family 6 member 14	UP	EMR
S100P	S100 calcium-binding protein P	UP	EMR
CTSE	Cathepsin E	UP	EMR
SULF1	Sulfatase 1	UP	EMR
POSTN	Periostin	UP	EMR
GJB2	Gap junction protein beta 2	UP	EMR
GPRC5A	G protein-coupled receptor class C group 5 member A	UP	EMR
SFN	Stratifin	UP	EMR
FN1	Fibronectin 1	UP	EMR
LAMC2	Laminin subunit gamma 2	UP	EMR
CEACAM5	CEA cell adhesion molecule 5	UP	EMR
MMP1	Matrix metallopeptidase 1	UP	EMR
COL11A1	Collagen type XI alpha 1 chain	UP	EMR
TSPAN1	Tetraspanin 1	UP	EMR
IFI27	Interferon alpha inducible Protein 27	UP	IS
CST1	Cystatin SN	UP	EMT
LAMB3	Laminin subunit beta 3	UP	EMR
COL10A1	Collagen type X alpha 1 chain	UP	EMR
VCAN	Versican	UP	EMR
CTRB2	Chymotrypsinogen B2	DOWN	EMR

PLA2G1B	Phospholipase A2 group IB	DOWN	Metabolism
CTRC	Chymotrypsin C	DOWN	EMR
GNMT	Glycine N-methyltransferase	DOWN	Metabolism
AQP8	Aquaporin 8	DOWN	H2O2 transport
SYCN	Syncolin	DOWN	Exocytosis
CPA2	Carboxypeptidase A2	DOWN	Metabolism
CELA2A	Chymotrypsin-like elastase 2A	DOWN	EMR
GP2	Glycoprotein 2	DOWN	Metabolism
KLK1	Kallikrein 1	DOWN	Serine protease
ALB	Albumin	DOWN	Oncotic pressure
CTRB1	Chymotrypsinogen B1	DOWN	EMR
ERP27	Endoplasmic reticulum protein 27	DOWN	Lipid and protein synthesis
TMED6	Transmembrane p24 trafficking protein 6	DOWN	Insulin secretion
PNLIPRP1	Pancreatic lipase-related protein 1	DOWN	Metabolism
CUZD1	CUB and zona pellucida like domain 1	DOWN	EMR and IS
CELA2B	Chymotrypsin-like elastase 2B	DOWN	EMR
PNLIPRP2	Pancreatic lipase-related protein 2	DOWN	Metabolism
CTRL	Chymotrypsin-like	DOWN	EMR
SERPINI2	Serpin family I member 2	DOWN	Protease inhibitor

EMR = ECM remodeling, IS = Immune system, EMT = Epithelial-mesenchymal transition

We performed ORA using GO biological process terms to identify the possible implications of the 1153 significantly differentially-expressed genes in PDAC samples. We considered only those biological processes with at least ten associated genes and an adjusted p-value under 0.05. We found 546 over-represented biological processes among the over-expressed genes and forty biological processes over-represented among the under-expressed genes (Supplementary Table S5). The ORA revealed the enrichment of terms related to the tumor microenvironment (Figure 4), with GO terms related to the immune system, cell adhesion, and ECM remodeling/degradation. Of note, additional over-represented functions had relevance to metastasis (vascularization, cell migration, collagen, mesenchymal transition, cell proliferation, and peptidyl modifications)[8,30].

3.3. Web tool

The web tool contains comprehensive information regarding the data and results of the meta-analysis of gene expression. The application includes tables and plots for the differential expression results of the twenty-one studies included in the meta-analysis and the meta-analysis results. Statistical indicators such as the log odds ratio, confidence intervals, and adjusted p-values are provided to estimate each study's global expression and specific contribution. This web resource is available in: https://bioinfo.cipf.es/MetaPDAC/

3.4. Immune system: a functional overview in PDAC

To focus our analysis on the tumor immune microenvironment, we extracted a consensus list of genes related to the immune system and inflammation from the NCBI and GO databases (mainly framed in the categories of HLA, interleukin, CD, interferon, chemokine, and S100 genes). Considering an FDR threshold of 0.05 and an absolute fold change greater than 0.6, we discovered the significant differential expression of 322 immune genes in our meta-analysis results. To explore the functional involvement of these results, we performed an ORA on

this group of genes, using GO biological process terms and Reactome pathways. We considered significant functional terms with at least ten associated genes and an adjusted p-value < 0.05. We discovered the over-representation of thirty-three GO terms and twenty-seven pathways among the over-expressed immune-related genes and none when analyzing the under-expressed genes. The enriched terms suggest increased activity of neutrophil-related immune response, negative regulation of cell killing, interferon signaling, and antigen presentation via major histocompatibility complex II.

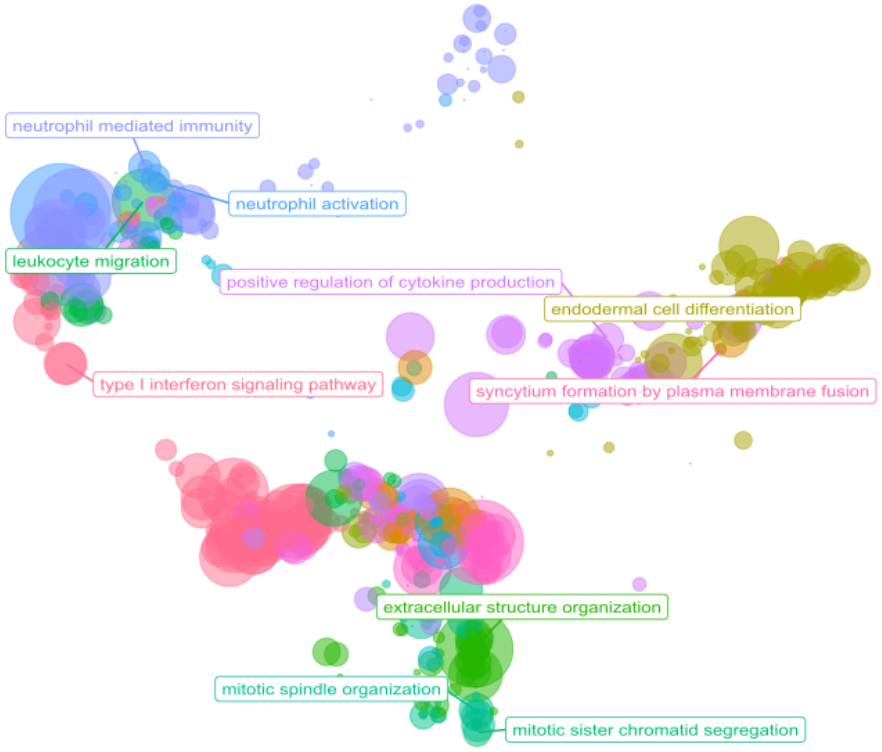

Figure 4. Scatter plot of ORA results. The scatterplot reports the GO biological process representative terms after redundancy reduction in a two-dimensional space derived from the semantic similarities between GO terms. The dot size represents the number of biological processes related to a GO term. The parent terms of the main clusters are labeled.

3.5. Immune and stromal survival signatures impact PDAC prognosis

We explored the 322 differentially-expressed immune-related genes and identified a set of seventy genes of particular interest in our experimental research (Table 3). We performed a survival analysis using the TCGA PAAD cohort for each of these genes and found statistically significant differences in twenty-eight genes (*IFI27, IL1R2, IL1RN, IL1RAP, IL18, IL22RA1, HCP5, SLFN13, CD58, CD109, IFI44L, IFI16, IFITM1, IFIT1, IFIT3, IRF9, IFIT2, IFI35, CXCL10, CXCL5, CXCL9, S100P, S100A6, S100A2, S100A16, S100A11, S100A14*, and *S100A10*), which shared a pattern: higher expression in patients correlated with lower survival. As far as we are aware, this is the first time that *HCP5, SLFN13, IRF9, IFIT2*, and *IFI35* are related to prognosis value in PDAC patients (Supplementary Figure S1).

Table 3. Subset of immune-related genes.

Functional Group	Genes
HLA	*HLA-F, HLA-DRB5, HLA-B, HLA-A,* ***HCP5****, HLA-DRA, HLA-DPA1, HLA-DQB1, HLA-DQA1, HLA-DMB, HLA-DRB1, HLA-G, HLA-DPB1, SLFN12,* ***SLFN13****, SLFN11*
Interleukin	***IL1R2****,* ***IL1RN****,* ***IL1RAP****, IL7R, IL2RG, IRAK3,* ***IL18****, LIF,* ***IL22RA1***
CD	***CD58****,* ***CD109****, CD52, CD53, CD74, CD14, CCDC80, CCDC141, CCDC69, DCDC2, PDCD4*
Interferon	***IFI27****,* ***IFI44L****, IFI6, STING1,* ***IFI16****,* ***IFITM1****, ISG20,* ***IFIT1****,* ***IFIT3****, IFITM2,* ***IRF9****,* ***IFIT2****, IFNGR2, IFITM3,* ***IFI35***
Chemokine	*CCL20, CCL18,* ***CXCL10****,* ***CXCL5****, CXCL8, CXCR4, CKLF,* ***CXCL9****, CXCL3, CXCL14, CXCL12*
S100	***S100P****,* ***S100A6****,* ***S100A2****,* ***S100A16****,* ***S100A11****, S100A4,* ***S100A14****, S100A10*

Genes in bold possess statistically significant differences in the survival analysis

We analyzed genes that displayed statistical significance as a "signature," dividing the samples into high-risk and low-risk groups based on the number of highly-expressed genes (above the upper quartile). We set the median (six highly-expressed genes) as the cutoff value to divide the samples into groups. Interestingly, patients in the high-risk group possessed shorter survival times than those in the low-risk group (p-value < 0.0001, Figure 6A). Furthermore, we studied the effect of this signature in a multivariate Cox model also including age, alcoholic history, presence of chronic pancreatitis, diabetes diagnostic, tumor grade, AJCC classification of metastatic tumor and residual tumor as covariates. The proposed signature was the only variable with p-value < 0.05, and showed a hazard ratio of 2.36 (Supplementary Figure S2). We then analyzed the co-occurrence of highly-expressed genes in samples, finding two main co-occurrence groups that related to high-risk patients: i) the interferon gene family (IFN genes) and ii) the S100 and IL genes (*S100A14, S100A16, S100A6, S100A11, IL1R2, IL1RN, S100P*) (Figure 6B).

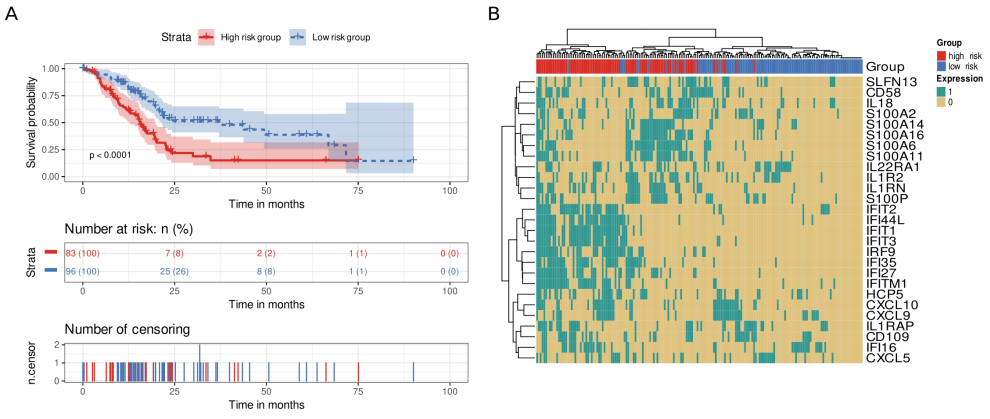

Figure 6. Survival analysis of immune system genes. A twenty-eight gene signature clustered patients into high-risk or low-risk groups based on the number of highly-expressed signature genes in their transcriptomic profile. Patients with at least six highly-expressed genes were classified as high-risk, whereas those with five or fewer were classified as low-risk. (**A**) Kaplan-Meier curve. Patients from the high-risk group (red) had shorter survival times than patients from the low-risk group (blue). Below, the number of still alive patients and percentage in each group at 0, 25, 50, 75, and 100 months, and the

censored events. (**B**) Heatmap demonstrating the patterns of high expression between genes and samples. Gene expression was coded as 1 for a sample above the upper quartile.

To explore how a desmoplastic environment can affect patient survival, we employed an homologous approach using genes related to ECM remodeling (Table 1). We discovered eleven genes whose survival analysis showed statistically significant differences (*CEACAM5*, *CEACAM6*, *FN1*, *GJB2*, *GPRC5A*, *LAMB3*, *LAMC2*, *SFN*, *SLC6A14*, *TSPAN1*, and *VCAN*). Again, we divided samples into high-risk and low-risk groups using the median of the number of highly-expressed genes as the cutoff value (median = 3). Patients with high expression in three or more genes from the signature presented lower survival times than those with fewer highly-expressed genes (p-value = 0.00012, Figure 7A). Of note, we distinguished a cluster of co-occurrence of patients with high levels of *GJB2*, *FN1*, and *VCAN* at the same time.

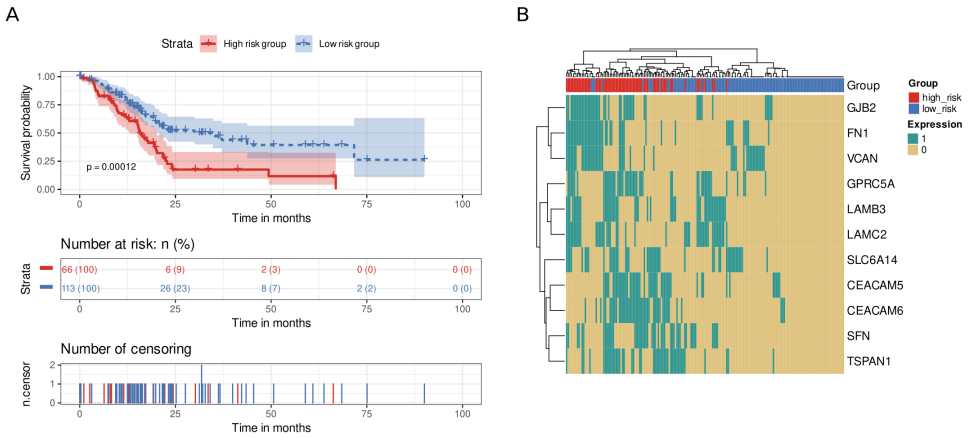

Figure 7. Survival analysis of ECM remodeling genes. An eleven-gene signature clustered patients into high-risk or low-risk groups based on the number of highly-expressed signature genes in their transcriptomic profile. Patients with at least three highly-expressed genes were classified as high-risk, whereas those with five or fewer were classified as low-risk. (**A**) Kaplan-Meier curve. Patients from the high-risk group (red) had shorter survival times than patients from the low-risk group (blue). Below, the number of still alive patients and percentage in each group at 0, 25, 50, 75, and 100 months, and the censored events. (**B**) Heatmap demonstrating the patterns of high expression between genes and samples. Gene expression was coded as 1 for a sample above the upper quartile.

4. Discussion

Using a comprehensive meta-analysis, we explored the immune environment and desmoplastic stroma of PDAC tumors to contribute to a deeper understanding of tumorigenesis and the design of effective therapeutic strategies such as immunotherapies. ECM components from the desmoplastic stroma tightly interact with the immune environment and contribute to immune evasion by modulating immune cell infiltration, thus influencing cell proliferation, tumor progression, and overall survival [31,32]. Meta-analysis and ORA results characterized differences in the gene-expression landscape of PDAC tumors and identified more than 1,000 dysregulated genes, most of them with immune system- and desmoplasia-related roles. We discovered thirty-nine genes (twenty-eight immune-related genes and eleven stroma-related genes) that impact PDAC patient survival.

Among the top forty dysregulated genes (Table 1), we observed the upregulation of collagens (*COL11A1* and *COL10A1*), which influence immune infiltration and chemoresistance and confer poor prognosis [33–35]. PDAC

patients also presented with upregulated periostin expressio*n*, which has been linked to shorter overall survival [36], and *cystatin SN*, which contributes to pancreatic cancer cell proliferation and may represent a potential biomarker for the early detection of pancreatic cancer [37]. Stratifin and matrix metallopeptidase 1 also appeared upregulated in PDAC patients, stratifin stimulates matrix metallopeptidase 1 expression in fibroblasts contributing to remodel ECM [38]. Increased expression of fibronectin in PDAC stroma has also been reported. The observed upregulation of *cathepsin E* and *sulfatase 1* expression in the PDAC microenvironment might also benefit the development of therapeutic strategies with polymer drug conjugates, since they may contribute to the drug release [39–41].

Analysis of the top forty dysregulated genes also provided evidence for the downregulation of genes coding for proteolytic enzymes released by the pancreas (e.g., *chymotrypsin, chymotrypsinogen, lipases,* and *phospholipases*). Pancreatic cancer cells express around 20% of chymotrypsin C normal cells expression, with this enzyme participating in cancer cell apoptosis and migration [42]. A recent report suggested that a combination of trypsinogen and chymotrypsinogen displayed anti-tumorigenic potential [43].

Focusing on the immune environment, PDAC tumors develop a wide range of mechanisms to evade the immune system (e.g., low expression of HLA antigens, immunosuppressive signals that inhibit natural killer and T cell functions, and the presence of immunosuppressive cells). This creates an immunotolerant environment in which the immune system of PDAC patients does not robustly recognize and target cancer cells [44]. We explored the expression of seventy genes of particular interest, including those from the HLA, interleukin, CD, interferon, chemokine, and S100 categories. Survival analysis of these genes in the TCGA PAAD cohort identified a twenty-eight immune-related gene signature with a prognostic value that clusters PDAC patients into high-risk and low-risk groups.

The proposed signature possessed significance in univariate and multivariate Cox models with clinicopathological variables, significantly adding statistical power to the survival analysis. This signature could aid the stratification of patients who could benefit from immunotherapeutic strategies, given that it could contribute to distinguishing "cold" PDAC tumors (characterized by the low presence of T cells (CD8+) and natural killer cells, high presence of immunosuppressive cell populations, poor prognosis and response to immunotherapy) from "hot tumors" (with an opposite profile) [45,46]. We uncovered two high gene-expression co-occurrence patterns, one composed of IFN genes and the other of S100/IL genes. The IFN signaling pathways participate in PDAC development, while over-expression of S100 genes blocks the infiltration and cytotoxic activity of CD8+ T cells and the low expression of IL1RN and IL1R2 has been associated with increase survival in PDAC patients [47–49].

To the best of our knowledge, this is the first report of data suggesting a link between the *HCP5, SLFN13, IRF9, IFIT2,* and *IFI35* immune genes and PDAC prognosis, presenting discriminatory power to cluster PDAC patients. The remaining genes of the immune gene signature have been individually associated with PDAC or other cancers, with data suggesting that their overexpression could impact diagnosis, prognosis, and response to treatment [50–55]; however, we report that a joint gene expression signature of these genes impacts PDAC patient survival.

Focusing on the PDAC stroma, altered genes include several types of collagens, fibronectins, and proteolytic enzymes such as metalloproteases and peptidases (Table 1, Supplementary Table S4), which significantly contribute to ECM composition and stromal remodeling and support desmoplasia and immunosuppression [56]. Survival analysis of significantly dysregulated stromal gene expression from the meta-analysis of the TCGA PAAD cohort revealed a gene signature with prognostic capacity that clustered PDAC patients into high-risk and low-risk groups. We observed a co-occurrence pattern in high-risk patients, indicating a subgroup of PDAC patients with high expression of *GJB2, FN1,* and *VCAN* genes. These results indicate stromal heterogeneity in PDAC [57] and the need to characterize it to stratify patients.

With respect to other dysregulated genes, the upregulation of *CEACAM5* and *CEACAM6* represents an early event in pancreatic carcinogenesis, with these genes candidates for immunotherapies [58–60]. Furthermore, laminins *LAMBC2* and *LAMB3* support cancer progression and resistance to gemcitabine - one of the main chemotherapeutics used in PDAC patients [61,62]. In general, the association with poor prognosis of the stroma signature is consistent with the one described in previous studies for each gene: *CEACAM5* [63], *CEACAM6* [64], *FN1* [65], *GJB2* [66], *GPRC5A* [67], *LAMB3* [68,69], *LAMC2* [68,69], *SFN* [70], *SLC6A14* [71], *TSPAN1* [72], *VCAN* [65].

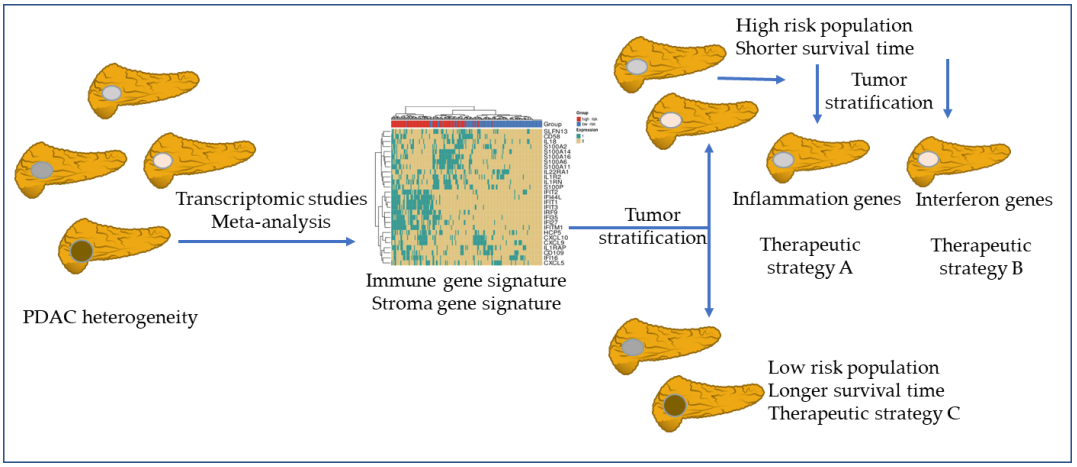

Figure 9. Patient stratification based on PDAC molecular features. The meta-analysis from transcriptomic studies allows a better understanding of the PDAC environment. In this study, the found gene signatures might contribute to the stratification of PDAC patients. In a first step the immune or the stroma gene signatures can divide patients into high and low risk populations. After, and focus on the immune signature co-occurrence, patients could be divided in those with a more S100/IL genes profile and in those with a more IFN expressed genes. The knowledge of these molecular features of PDAC tumors may guide the design of more effective therapeutic strategies.

With respect to other similar approaches, we are aware of two additional studies that integrated expression datasets to explore the nature of the PDAC in-depth: Gooneskere and colleagues, which integrated six PDAC and three other pancreatic carcinomas datasets [73], and Irigoyen and colleagues, which integrated two peripheral blood datasets [74]. Both approaches integrate different datasets at the gene level to increase the number of samples and perform a unique DGE analysis. In contrast, our approach analyzed each dataset independently and then integrated the results, evaluating their robustness. From the experimental design point of view, both studies differ greatly from ours, since Grooneskere *et al.* is not specifically focused on PDAC, and Irigoyen *et al.* does not analyze pancreatic tissue. From the methodological point of view, our study contributes to a more profound and robust analysis of the PDAC expression landscape by integrating data after DGE analysis has been performed, thus avoiding the necessity to control heterogeneity among studies and retaining the full potential of biological differences.

A potential limitation of our study has been the relative heterogeneity in sample sizes and sequencing platforms used. The meta-analysis methodology, which integrates data groups and provides results with higher statistical power and precision [75,76], addresses this issue by comparing each study independently and combining the results. A lack of clinical and/or molecular information in most studies, such as survival time, stage condition, or molecular pattern, represented an additional limitation. We employed TCGA data for survival analysis, but additional analyses should integrate other covariates of interest in the study.

Finally, we provided an interactive web tool that allows users to explore our results, facilitating the accessibility, transparency, and reusability of our research. Overall, the web tool provides a detailed and interactive visualization of the meta-analysis results, allowing users to further explore and understand the gene expression patterns identified in the studies. Other functionalities include the capability to customize and filter the data to further investigate specific aspects of the analysis in more detail. In this manner, we aim to align our

research with the FAIR principles to share our data in a way that can be of further use to the scientific community studying this aggressive and lethal tumor.

Conclusions

Therapeutic strategies to overcome the immune microenvironment and the desmoplastic stroma barriers remain limited and generally unsuccessful. This study performs a comprehensive transcriptional signature of the molecular PDAC environment. The identified immune and stroma genes signatures provide new insights into the potential therapeutic targets for this deadly disease that can stratify, in part, its heterogeneity. Further studies are needed to validate these findings and explore the potential of targeting the immune and stroma microenvironments as a treatment strategy for PDAC. Finally, we highlight the importance of sharing data and using open platforms to improve the effectiveness and performance of science.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Software version; Table S2: Dataset inclusion; Table S3: Clinical characteristics: Table S4: Gene meta-analysis results. Table S5: ORA results. Table S6: NCBI and GO Immune system genes.

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Data Availability Statement: Publicly available datasets were analyzed in this study. Data is openly available in GEO and ArrayExpress with the following accession numbers: GSE71989, GSE62452, GSE62165, GSE60979, GSE56560, GSE55643, GSE43795, GSE41368, GSE32676, GSE28735, GSE22780, GSE18670, GSE16515, GSE15471, GSE1542, GSE11838, GSE101448, GSE119794, GSE136569, E-MEXP-950, and E-EMBL-6.

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