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Key biological processes driving metastatic spread of pancreatic cancer as identified by multi-omics studies

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an extremely aggressive malignancy, characterized by a high metastatic burden, already at the time of diagnosis. The metastatic potential of PDAC is one of the main reasons for the poor outcome next to lack of significant improvement in effective treatments in the last decade. Key mutated driver genes, such as activating KRAS mutations, are concordantly expressed in primary and metastatic tumors. However, the biology behind the metastatic potential of PDAC is not fully understood. Recently, large-scale omic approaches have revealed new mechanisms by which PDAC cells gain their metastatic potency. In particular, genomic studies have shown that multiple heterogeneous subclones reside in the primary tumor with different metastatic potential. The development of metastases may be correlated to a more mesenchymal transcriptomic subtype. However, for cancer cells to survive in a distant organ, metastatic sites need to be modulated into pre-metastatic niches. Proteomic studies identified the influence of exosomes on the Kuppfer cells in the liver, which could function to prepare this tissue for metastatic colonization. Phosphoproteomics adds an extra layer to the established omic techniques by unravelling key functional signalling. Future studies integrating results from these large-scale omic approaches will hopefully improve PDAC prognosis through identification of new therapeutic targets and patient selection tools. In this article, we will review the current knowledge on the biology of PDAC metastasis unravelled by large scale multi-omic approaches.

Key words

Pancreatic ductal adenocarcinoma; metastasis; genetics; transcriptomics; proteomics

Introduction

The ability to spread and adapt to a hostile environment is one of the most dangerous characteristics cancer cells can acquire. This invasive feature encompasses multiple progression steps which start by acquiring the ability to migrate through and out of primary tissue, followed by invading and surviving in blood or lymphatic vessels, and finally establishing a new tumor microenvironment in the hostile receiver tissue[1]. Multiple mechanisms, e.g genetic instability and/or clonal expansion, explain the genetic evolution that tumors undergo to develop from a localized carcinoma into aggressive metastatic disease. Once these aggressive features are obtained, the survival of these patients reduces exponentially, and this is independent of the origin of the primary tumor. Metastases can develop very late after diagnosis implying a slow adaptation of a single cell in the distant microenvironment, but in some tumors, metastases develop synchronously during growth of the primary tumor[2]. This quick progression to metastatic disease is a key feature in pancreatic ductal adenocarcinoma (PDAC) patients, who often succumb from advanced local disease with widespread metastatic burden early after diagnosis[3].

Patients suffering from PDAC have a 5-year survival of 7.7%[4]. This disease is the fourth leading cause of cancer-related deaths in the USA and is projected to be the second cause of cancer-related deaths by 2030[5] due to an increasing incidence, lack of effective treatments, and a high metastatic propensity. The metastatic risk is highlighted by the fact that 91% of PDAC patients are diagnosed with (regional) metastatic disease[4]. This quick progression warrants new studies to understand the key processes that drive metastatic behavior.

Several studies investigated the effect of localization and size of metastases and the timing of migration. Rapid autopsy programs revealed that the majority of metastases are located in the liver (76-94%), peritoneum (41-56%), abdominal lymph nodes (LN) (41%) and the lungs (45-48%)[3,6–8] (Figure 1A). Moreover, these programs revealed the extent of metastatic disease in these patients, with on average 2.9 distant organs involved. Additionally, metastases occur mostly in a widespread fashion, with some patients harboring over 1000 metastases[7]. The size and the growth rate of metastases is inversely correlated to survival[3].

PDAC is characterized by an interactive microenvironment with up to 90% of the primary tumor consisting of a stromal compartment[9] (Figure 1B). Interestingly, liver metastases harbor pathological resemblance to the primary PDAC tumor with similar extracellular matrix (ECM) components[10]. Data suggest that PDAC cells can recruit local stromal cells and create an extracellular environment similar

to the primary tumor upon colonization of the receiving organ[10]. Using a fluorescent lineage tracing mouse model, Aiello et al.[11] showed that there is a correlation between the size of the liver metastasis and the recruitment of stroma. This model was previously used to show that epithelial-mesenchymal transition (EMT) proceeds metastasis and that PDAC cells retain their EMT state in circulation[12].To allow growth of the metastasis, PDAC cells need to return to their epithelial morphology after their previous mesenchymal state (Figure 1C). Upon multiplication of tumor cells, as early as in nano-metastases (2-10 cells), myofibroblasts were found to be in contact with tumor cells and ECM composition recapitulated that of the primary tumor[11]. This highlights the complex biology of PDAC and its metastatic features.

Large-scale omics have been performed in order to understand the biology of PDAC's aggressive behavior. Emerging insights from these studies can be used to elucidate the rapid progression to metastatic disease. In the following sections research involving genomic, transcriptomic and proteomic studies of PDAC and its metastases are reviewed.

Genetic events and clonal evolution of PDAC

Multiple genetic events are thought to occur before metastatic spread is initiated. Four commonly mutated genes characterize PDAC. The *KRAS* driver mutation is identified in more than 90% of the PDAC tumors[13]. Mutations at codon 12 (G12D or G12V) are most abundant and result in aberrant, persistent activation of the KRAS pathway. Inactivating mutations in TP53, CDKN2A and SMAD4 (DPC4) are also very common, occurring in 74%, 35% and 31% of all PDAC patients respectively[14]. However, the presence of these somatic common mutations in the primary tumor does not clearly correlate to patients with a very long survival (more than ten years after resection) [15]. Yachida et al.[6,16] performed comparative analysis of primary tumors wil matched metastatic tissue and showed that over 90% of tissues had concordant driver gene mutations between matched primary and metastatic material, indicating that these mutations are early events in PDAC tumorigenesis. These results were recently confirmed in another study, which showed low genetic heterogeneity between metastases and the primary tumor[17]. In contrast to very long survival times, the number of mutational driver genes in a tumor is correlated to the metastatic burden of the patients and disease free survival (DFS)[6]. Especially, mutations of TP53 and SMAD4 are associated with a higher metastatic burden and poor prognosis of PDAC patients[,6,7,10], compared to KRAS or CDKN2A mutations, which are associated with oligometastases. The correlation of driver gene mutations with metastatic burden was independent of tumor stage or grade. Of note, in 37% of the patients analyzed all four driver genes were mutated[6]. This indicates that some PDAC tumors do not follow the original progression model of sequential mutations in the four known driver genes during the

development from Pancreatic Intraepithelial Neoplasia (PanIN) precursor lesions to PDAC[19]. Most likely, a subset of tumors acquire mutations in a different order and do not need all four mutations for tumorigenesis. This finding was further highlighted by Notta et al.[20] who showed by whole genome sequencing that complex rearrangements in the genome of the tumors can take place by chromothripsis, where genomic rearrangements are clustered on a small number of chromosomes, and that 67% of analyzed tumors showed a non-conventional mutagenesis resulting in quick tumor progression and mutation of driver genes. This particular genetic rearrangement was also identified by Waddell et al.[14] and the mechanisms and its contributions to PDAC will need to be further explored.

To further elucidate the genetic aberrations driving this aggressive tumor, multiple other sequencing analyses of primary PDAC tumors have been performed. Jones et al.[21] sequenced protein-coding exon DNA from 24 tumors and showed an average of 63 mutations per tumor. Despite the identification of multiple mutations in this PDAC cohort, identical mutations in more than one patient were sparse. However, pathway analysis of this heterogeneous mutational landscape identified 12 aberrant pathways affected in PDAC tumorigenesis, which were altered in 67-100% of the tumors. Gene expression analysis confirmed differential expression of these pathways in tumors compared to normal epithelial pancreatic duct cells. These pathways include known driver pathways, such as KRAS signaling, but also highlighted pathways that function in tumor-stroma crosstalk like Hedgehog, TGF-β, integrin and WNT-NOTCH signaling.

Exome sequencing and copy number analysis established additional frequently mutated genes in the core affected pathways, and axon guidance was identified as a new aberrant pathway[22,23]. The importance of this pathway in PDAC biology was underlined by epigenetic genome-wide methylation analysis of 167 tumors, where axon guidance was identified as one of the most significant epigenetic deregulated pathway with the promotor regions of SLIT/ROBO signaling being hypermethylated[24]. The identification of axon guidance as a key pathway in PDAC is supportive of the fact that peri-neural invasion is a poor prognostic factor in PDAC[25]. Moreover, peri-neural growth and interaction of tumor cells with the nervous microenvironment has been shown to stimulate the migration of PDAC cells[26,27]. When surgical margins are clear, cell migration in the peri-pancreatic nerve system can indeed be a source for later recurrence and metastases[25].

The genomes of PDAC tumors contain certain types of chromosomal rearrangements. In a comparative analysis of primary tumor and metastatic tissue, genomic instability was shown to be very heterogeneous between patients. Intra-chromosomal rearrangements were more common than deviations between chromosomes. Interestingly, fold-back inversions were commonly present in

tumors[28]. Other genomic variation screens identified similar trends favoring intra-chromosomal rearrangements[29.14]. Four different genomic subtypes were identified, of which the locally rearranged type showed foci in possible target oncogenes, however with low penetrance in the whole patient group, and the unstable subtype was identified by wide-spread genomic instability, most likely due to DNA repair dysfunction[30]. Another study based on the mutational signatures also identified four subtypes, of which the DNA repair dysfunction signature was correlated with increased tumor immune response[31] (Figure 2A). The clinical value of these genomic subtypes still needs to be further explored. However, the genetic rearrangement seems to be relatively stable between the primary and metastatic tissue of patients, for example, fold-back inversions are identified in matched samples, indicating an early event in tumorigenesis[28,31].

The moment of dissemination of PDAC cells during tumorigenesis remains a point of discussion. The genetic and clonal evolutions, which are needed for the PDAC cells to gain metastatic capabilities, can be explained by multiple mechanisms. Campbell et al.[28] showed that most genetic structural aberrations of the primary tumor were present in metastases in their cohort of 13 PDAC patients, however, some patients showed genetic variations between different metastases. This proves that multiple subclones in the primary PDAC can establish different distant metastases. In particular, some mutations and rearrangements can enhance the metastatic capabilities of a clone to colonize a specific organ site. For example, two patients harboring lung metastases showed a more extensive evolution from the primary tumor than abdominal metastases, and contained MYC and CCNE1 mutations, possibly enhancing the lung-seeding capacity of these cells. Interestingly, in the study of Witkiewicz et al.[32], MYC amplification was correlated to poor survival, a correlation that will need to be further investigated in the perspective of metastasis.

By Sanger sequencing, Yachida et al.[16] recognized that mutations in metastases are most likely clonal, and that the genetic heterogeneity in different metastases results from subclones from the primary tumor. By computational modeling they estimated the average time of development from carcinoma *in situ* to gain of metastatic competence to be 6.8 years[16]. Another mathematical model based on clinical progression and autopsies, predicted metastasis most likely to be present at time of diagnosis. This risk is correlated to the size of the primary tumor, underlining the need for early detection and improved therapeutic options[3]. New models evaluating the genetically diverse subclones and their metastatic capability are needed to shed light on the exact timing of dissemination during carcinogenesis.

The clonal progression of these tumors has recently been further evaluated by a mouse model with confetti lineage labeling of tumorigenic cells[33]. By following the fluorescent cells, subclonal

heterogeneity of primary mouse PDAC tumors can be tracked in the metastatic sites. Interestingly, the majority of the abdominal metastases were polychromatic, indicating that they resulted from multiple subclones of the primary tumor. This polyclonality was mostly a result of two founder cells. Functional experiments validated that most likely, these metastases were formed from cell clusters rather than from seeding of multiple single cells. Remarkably, larger lung and liver metastases consisted mostly of monoclonal cells. This monoclonality was directly correlated to the size of the metastases, indicating a genetic advantage of a subclone after establishment of the distant tumor. This clonal progression from polyclonality to monoclonality in liver and lung metastases could be an explanation for the genetic heterogeneity found in some previous genomic studies[16], since upon monoclonality there is reduced resemblance to the primary tumor with multiple subclones.

Transcriptomics and gene expression of metastases

Although deep sequencing of the genome has identified multiple aberrantly regulated pathways contributing to pancreatic cancer, the mutational status does not explain the full spectrum of the aggressive PDAC phenotype. Since multiple cellular processes can influence gene expression, for example epigenetics, regulation by transcription factors, and cell-extrinsic factors, screening of differential expressed transcripts can deepen the understanding of tumor biology.

Efforts identifying prognostic important subtypes in PDAC resulted in multiple transcriptomic classifiers (Figure 2A); however, different approaches to the heterocellular consistency of PDAC have been used. Collisson et al.[34] were the first to describe three subtypes in PDAC, each with a different biology and prognosis. Their dataset consisted of 27 microdissected tumors to enrich for the epithelial compartment of PDAC. Of the three subtypes identified (quasi-mesenchymal (QM), exocrine and classical), the QM subtype was associated with the poorest prognosis, while patients identified retrospectively with the classical subtype showed relative good overall survival. The relatively higher expression of mesenchymal genes in the QM subtype very likely contributes to a higher occurrence of EMT and thus the ability to metastasize, leading to poor prognosis. However, the need for elaborate microdissection and extensive genetic analyses will hamper the clinical applicability of their classifier. A more feasible approach recently was suggested in a study which identified immunohistochemical classification markers (KRT81 and HNF1A). Interestingly, in this study the exocrine subtype was more resistant to paclitaxel treatment and tyrosine kinase inhibitors due to cytochrome P450 3A5 expression[35]. These results indicate that subtyping can have clinical applications. Another way to overcome the problem of microdissection, is bulk tumor analysis of high percentage epithelial tumors. Bailey et al.[23] identified four stable subtypes on an initial patient

dataset (n=96) consisting of tumors with minimally 40% epithelial cellularity. These subtypes were validated on a larger cohort with a more natural distribution of the stromal compartment. Their poor survival "squamous" subtype resembled the QM subtype previously described. Other subtypes identified were "pancreatic progenitor", "immunogenic" and "aberrantly differentiated endocrine exocrine", which partly overlapped with the previous classical and exocrine subtypes[34].

To account for the interaction between stromal and epithelial compartments and reduce the selection bias for only high epithelial tumors in analyses, Moffitt et al.[36] explored bioinformatics tools to dissect gene expression profiles of both. They identified two stromal subtypes, "activated" and "normal" stroma. These stromal subtypes were correlated to prognosis of PDAC patients, however their implication in metastases was not described and has not yet been further investigated. Interestingly, the stroma subtypes were relatively underrepresented in the metastases in their dataset, indicating less stroma signatures in metastases. This study also identified two prognostic PDAC tumor subgroups, basal-like and classical, which resemble in part two of the Collison subtypes (Figure 2A). The basal-like subtype was specifically enriched in metastatic tissue, implicating that transformation to a basal-like state is necessary for dissemination, or that some PDAC subtypes are more prone to metastasize. Interestingly, upon differential analysis of their classifier transcripts from matched primary and metastatic tissues, low heterogeneity was identified providing evidence that subtype specific gene expression is preserved in metastatic tissue.

Even though the computational dissection of bulk tumor identified plausible subtypes, consensus of all the different subtyping efforts is needed to further evaluate clinical relevance and utility. A large-scale laser microdissected dataset to experimentally prove gene expression profiles from different compartments of bulk tumor will help to improve subtyping these tumors and define consensus subtypes. The QM subtype was concordantly identified so far in the large-scale studies and is consistently correlated to poor outcome and increased metastatic potential. This finding is in line with transcriptomic analysis of other tumor types where the mesenchymal classification is the most aggressive subtype[37–39].

Gene expression profiling has been used to identify important genes and pathways in metastases. In a comparative analysis, genes with functions in cell proliferation, cell cycle regulation, were overexpressed in tumors with lymph node metastases. Moreover, apoptosis and cell motility were down regulated[40]. Stratford et al.[41] compared tissue from primary tumors with and without metastases and identified 6 genes related to metastases (FosB, KLF6, NFKBIZ, ATP4A, GSG1, SIGLEC11). These genes were prognostic for survival. Interestingly, another study with matched metastatic tissue from multiple locations did not show evident differential gene expression[42]. This

could be explained by the hypothesis that the primary tumor already acquired the metastatic abilities and that the majority of gene expression does not show substantial heterogeneity from the primary tumor. Finally, a recent study explored the metabolic differential expression of PDAC and its metastatic tumors. Compared to normal pancreatic tissue there was enrichment of aerobic glycolytic genes. The majority of metabolic genes were comparable between tumor sites. Specifically, glucose transporter (GLUT1) was overexpressed in all tumor sites. SLC2A2 was overexpressed in liver metastases and IDH3 was overexpressed in lung and lymph node metastases, indicating some differential energy acquiring process in different metastatic locations[43]. Since the RNA technology is advancing with increasing depth of analysis, future studies will define more differential gene expression profiles of subtypes and prognostic profiles for metastatic disease. This hopefully will lead to better patient staging and clinical management for subgroups of patients.

Another important regulatory player in gene expression are microRNAs (miRNAs). These small non-coding RNAs, consisting of 20-23 nucleotides, regulate gene expression in the posttranslational phase by degrading their target messenger RNA (mRNA). This regulatory network is often deregulated in cancer, which results in aberrant expression of miRNAs that target oncogenes and tumor suppressor genes[44]. Many miRNAs have been correlated to poor prognosis[45,46] and metastatic potential[47–49] in PDAC. In a meta-analysis, Frampton et al.[46] analyzed combined data from differential expression profiles and validated onco-miRNA-21 as a prognostic marker in PDAC. MiRNAs can also be used predictive biomarkers. A panel of miRNAs were able to predict sensitivity of metastatic patients for lapatinib treatment. Onco-miRNA-221 was shown to influence sensitivity of PDAC cells i*n vitro*[50]. Integrative analysis combining mRNA and miRNA profiles can highlight their regulatory network. This was shown in a cohort of nine patients, where 3 miRNAs (miR-21, miR-23a, miR-27a) were identified as regulators for multiple known tumor suppressors[51]. Future large-scale efforts combining RNAseq and small RNAseq of primary and matched metastatic tumors will further deepen our knowledge of this regulatory network and its importance in metastases of PDAC.

Proteomics applied to PDAC to identify new biomarkers and protein subtypes

As outline above, PDAC is caused by alterations in DNA that yield altered gene products that make cells grow in an uncontrolled way and spread throughout the body. Comprehensive analysis of the alterations in each tumor's complete set of functionally relevant proteins, the proteome, can add a complementary layer of information that is expected to increase our understanding of how molecular changes interact to drive the disease.

In recent years, the field of proteomics has evolved from limited protein inventories to in-depth (close to) proteome-wide discovery due to massive improvements in mass spectrometry (MS)

technology and (bio)informatics tools. Together with robust relative protein quantitation based on label-free or stable isotope labeling, proteomics studies are increasingly being used in cancer research. Moreover, the integration of proteomic and genomic data by which MS/MS data is searched against customized databases of individual matched DNA/RNA sequence data, referred to as proteogenomics, has enabled a more comprehensive view of the molecular determinants that drive cancer than genomic analysis alone and may help to identify the most important targets for cancer detection and intervention. This was recently shown for colon, breast and ovarian cancer[52,53,54]. Importantly, from these studies it also became apparent that proteome profiling data can outperform transcriptome profiling data for co-expression based gene function[55], underlining the importance of proteomics in gene function and human disease studies.

Mass spectrometry based identification of proteins in complex biological samples such as tissues and biofluids has been performed to develop multiple cancer diagnostic applications. To identify protein biomarkers for non-invasive applications, proximal fluids that contain relatively high levels of tumor secreted proteins are an appealing biomarker source since they do not contain high levels of albumin that will mask low abundant biomarkers in blood-based screens. Another option to facilitate biomarker identification is to remove highly abundant proteins from blood. In PDAC, multiple studies have been performed in the recent years applying these techniques. A summary of the studies is described in Table 1[56–80]. We will describe some of the different approaches in more detail below.

Kosonam et al.[75] analyzed ascites from patients suffering from peritoneal metastases as proximal fluid. A total of 456 proteins were commonly identified in 3 patients. To further select possible PDAC specific biomarkers, this list was compared to known secreted PDAC proteins and previous ascites proteome analyses. This yielded a final possible new biomarker list of 12 proteins. Another study used the secreted proteins of PDAC cell lines to find common secreted proteins for early diagnostic possibilities[81] In an attempt to facilitate non-invasive biomarker screening, Radon et al.[65] screened urine of PDAC patients and patients suffering from chronic pancreatitis and healthy controls. By LC-MS/MS they identified multiple differentially secreted proteins. Interestingly, gender differences were significant and had to be taken into account in further analysis. Upon selection based on fold change and known PDAC expression, they reduced their list to 3 proteins (LYVE1, REG1A, and TFF1). These proteins showed very good sensitivity and specificity as a panel in a validation cohort of over 300 patients. These studies prove that multiple biofluids can be a source for protein biomarker identification. Future validation is needed to establish these proteins for clinical use.

Another way to implement protein biomarkers is to classify subtypes previously established by transcriptomics. Kuhlmann et al.[59] made use of primary cells lines representing three Collison

transcriptomic subtypes to detect subtype-specific protein biomarkers. Interestingly, in their cohort only the exocrine subtype had differential protein expression detectable on their cell surface or as secreted proteins. Further proteomic studies are needed to validate the transcriptomic subtypes in PDAC and their value or to establish whether proteome data may yield a different classification system. For the latter purpose, large-scale proteome profiling of clinical samples is needed as recently performed for other tumor types[82].

Identification of prognostic and metastatic protein markers in PDAC

To understand the variability in survival of PDAC patients, several studies have explored differential proteome landscapes of long versus short survival or metastatic versus non-metastatic disease. Matched formalin-fixed paraffin-embedded (FFPE) tissues from very long (more than ten year survival post-surgery) and short surviving patients were screened to understand the different underlying biology. In the short survival group, proteins associated with the cytoskeleton were increased as well as RNA processing / protein biosynthesis. This can point to higher motility and metastatic capability. Interestingly, one of the upregulated proteins identified was galectin-1 (LGALS1) which is mainly expressed in cancer-associated fibroblasts (CAF) in PDAC[83]. Knockdown of this gene reduced their ability to migrate and therefore to stimulate PDAC cells[84].
In another approach to understand the basic principles of metastatic capability, Naidoo et al.[85] analyzed seven primary PDAC samples and their LN metastases. This yielded 856 commonly expressed proteins, of which the majority clustered in the biological functions of cell proliferation and growth, cell death and cellular movement. Only a small subset of proteins was differentially regulated, implying again that malignant epithelial cells in LN metastases are not very different from primary tumor cells. One of the proteins differentially expressed was S100P, which was validated by IHC. This protein was previously identified as an important player in trans-endothelial migration of PDAC cells and upon knockdown, less migration into the vasculature and less metastases were seen in a fluorescent zebrafish model[86]. These results show that this protein could be an interesting target to inhibit migration of tumor cells, and moreover, that differential protein expression can lead to new targets and understanding of cancer biology.

Another factor thought to contribute to metastatic disease, is the population of cancer stem cells (CSC). These cells have, or have regained, the ability to self-renew and are recognized as important modulators of metastatic capabilities and chemoresistance[87]. In an effort to elucidate their biology, Brandi and collaborators[58,88] profiled the proteome and the secreted proteins of a CSC subpopulation in the PDAC cell line PANC1. These analyses showed that CSCs upregulated multiple metabolomic pathways. Moreover, CSC were relatively sensitive to metabolic inhibition by existing drugs[89]. Similar

metabolomics pathways were dysregulated in the secretome of CSCs[90]. Interestingly, the secreted proteins could be identified by ELISA assays in blood sample of PDAC patients, indicating their possible use as biomarkers (Table 1).

Exosome protein content and establishment of the metastatic niche

In recent years, we have come to understand that migration of cancer cells into the vasculature or lymph vessels by itself is not enough to establish metastasis. The hostile environment of the distant site requires certain changes that enable tumor cells to attach and thrive. Exosomes can be a player at the metastatic site to assist adhesion and growth of a tumor. Exosomes are small extracellular vesicles of endosomal origin that can carry nucleic acids and proteins, and have been shown to be able to influence the migratory capacity of tumor cells[91]. To further investigate how exosomes can influence the metastatic capability of tumor cells, Hoshino et al.[92] analyzed the organotropic preference of tumor exosomes. PDAC are known to migrate primarily to the liver, and indeed, exosomes from PDAC cell lines were tracked and located preferentially in the liver. Next to the uptake of PDAC exosomes in the liver, exosomes were shown to create a “metastatic niche”, which modulates the local microenvironment to enhance metastatic capabilities of tumor cells. To further understand why exosomes of certain tumor types have a partiality to specific distant sites, proteomic analysis showed differential integrin expression which could explain the organotropism. Integrin beta 5 and integrin alpha V were highly abundant in PDAC exosomes (Figure 1D). Knockdown or inhibition of this specific integrin complex resulted in reduced exosome uptake and less liver metastases. This shows that exosomes carrying specific protein content can modulate the metastatic capacity of PDAC cells. Studies have shown that PDAC exosomes are taken up by Kupffer cells in the liver predominantly[92,93]. Gene expression analysis of these cells after exposure to PDAC exosomes showed overexpression of the liver fibrosis pathway, indicating that by exosome interaction, fibrosis of the metastatic niche can be induced[93]. Moreover, upregulation of S100P was seen in Kupffer cells after treatment with PDAC exosomes, which as discussed previously, plays a role in transendothelial migration[92]. Further proteomic analysis of pancreatic cell line exosomes showed high expression of macrophage migration inhibitory factor (MIF) (Figure 1D). Knockdown of this protein in PDAC exosomes resulted in reduced metastatic burden in mice. Importantly, expression of this factor in exosomes could already be identified in early PanIn lesions in a genetic engineered mouse model of PDAC[93]. These studies indicate that preparation of the metastatic niche might already occur early during tumorigenesis. Another study extracting exosomes from human PDAC serum showed increased metastatic and EMT related proteins compared to healthy control exosomes. These PDAC exosomes stimulated migration of PDAC cells *in vitro*, supporting their pro-metastatic function[57]. Interestingly, several exosome proteins identified served as predictive biomarkers for treatment

response. However, the regulation of molecular cargo of cancer-associated exosomes to influence metastatic capability is not fully understood. Recently, the protein myoferin was identified as a possible regulatory protein for the composition of the exosomal proteome[94]. Upon silencing of expression of myoferin in PDAC cancer cells, their exosomes were inhibited in their potency to initiate migration and proliferation. Quantitative proteomic analysis validated its role by showing down regulation of vesicle mediated transport proteins upon silencing. Interestingly, exosomes that influence tumor growth and metastasis can also be microenvironment derived. By analyzing the proteome and interactome of the stromal compartment of three PDAC tumors, Leca et al.[60] identified 11 cytoplasmic vesicle related proteins of which three (ANXA, LRP1 and TSP1) formed a complex in exosomes from CAFs. Functional validation proved these proteins to be important for tumor progression and liver metastasis. These studies show that exosomes from PDAC cells and its microenvironment can influence the metastatic niche with their protein content. Future proteomic studies will increase our understanding of the specific proteins important for this metastatic function of PDAC exosomes.

Phosphoproteomics to unravel pathways and the aggressive behavior of PDAC

Aberrantly activated pathways are common in cancer, and even though differential protein expression can identify a certain degree of pathway regulation, it cannot directly reveal inter- and intracellular signaling. Signaling is regulated via reversible phosphorylation of tyrosine, serine and theorine amino acids of proteins by kinases. Global phosphoproteomics can be used as a read-out for these phosphorylation events and give insight in the actual activation state of kinases in the cancer cell[95,96]. Britton et al.[97] compared the phosphoproteome of 12 PDAC tumors to normal pancreatic tissue. With this approach, they identified 2,101 phosphorylated proteins of which 152 were differentially phosphorylated. One of their top differential identified proteins was Mucin-1, a known PDAC oncoprotein involved in proliferation and metastases[98]. Moreover, pathways for tight junction, adherence junction, and focal adhesion signaling were significantly differentially phosphorylated in the cancer samples, which could explain the increase motility and intercellular communication. Finally, they identified multiple differential phosphorylated kinases, which could be possible drug targets. Upstream kinases in canonical cell signaling often harbor specific tyrosine phosphorylated residues. This trait can be exploited by enrichment of tyrosine phosphorylated proteins before MS/MS analysis. Harsha et al.[99] performed tyrosine phosphoproteomics on a hyperphosphorylated primary PDAC cell line and compared the identified phosphosites to an immortalized non-malignant pancreatic ductal cell line. They identified epithelial growth factor receptor as a target in this particular cell line. Functional validation by inhibition with tyrosine kinase inhibitor erlotinib showed significant inhibition of growth in their xenograft model. Similarly, a large-scale tyrosine

phosphoproteomic effort analyzed commercially available as well as primary cell lines, and identified different subtypes based on phosphorylation. One hyperphosphorylated subtype was identified, harboring deviant phosphorylation of multiple receptor tyrosine kinases. This subgroup was more sensitive to erlotinib treatment than the other subtypes[100]. However, erlotinib in clinical trials did not show significant survival benefit in unselected patient groups with advanced gemcitabine resistant disease[101], which possibly underlines that selection based on phosphorylation of marker proteins is needed. Remarkably, the phosphorylation subtypes identified did not correlate to known transcriptomic PDAC subtypes[100], emphasizing the complexity and non-linear cellular regulations from DNA, RNA , proteins and signaling. Moreover, as discussed before, PDAC is a tumor with multiple clones in the primary tumor and metastatic sites in which different kinases can be activated. Kim et al.[102] analyzed three primary cell lines of one patients' metastatic sites. In the normal proteome of these cell lines, 58% of the protein expression was similar. However, the phosphoproteome was highly variable between the different metastatic cell lines. This can indicate a dynamic state of phosphorylation, or differential aberrant activations after clonal evolution. It was found that AXL was phosphorylated in liver and lung metastatic cells, but not in peritoneal metastasis. This phosphorylation status was correlated to sensitivity of AXL inhibitors, proving this approach for treatment selection. On a side note, the finding of phosphotyrosine heterogeneity complicates a single drug regimen selection for these patients and underlines the difficulty of targeting this disease.

The phosphoproteomic approach can be used for identification of signaling that can explain some of the aggressive PDAC traits. By identifying an aberrant phosphosite of the kinase SGK223 via tyrosine screening, Tactatan et al.[103] explored the function of this kinase, which turned out important for STAT3 transcription and invasion and migration. Importantly, as discussed before, PDAC is a multicellular disease with interaction of stromal cells and tumor cells, which influences signaling. Tape et al.[104] have expanded our knowledge of this interaction by analyzing different signaling events upon co-cultures. They identified that only 7% of the tumor signaling is regulated by KRAS activation. Interestingly, the phosphoproteome was influenced on a similar level by CAF interaction as by KRAS, highlighting the influence of the microenvironment on PDAC. One of these stimulatory events is activation of the IGF1R/AXL-AKT axis. This crosstalk signaling can stimulate the tumor cells on a different level than tumor-tumor interaction. Future research of multi-cellular systems or whole tumors will elucidate the importance of activated pathways by the microenvironment and will guide towards new targeted therapies.

Future perspectives and concluding remarks

During the last decade, large-scale omic approaches have greatly expanded our knowledge of PDAC genetics and the resulting tumor biology. Subtyping at the transcriptome level has identified poor prognosis subtypes characterized by the expression of mesenchymal genes and other programs that contribute to poor outcome[23,34,36]. Whole genome analyses of these tumors have proven that carcinogenesis is not always a sequential progression of mutations[20]. Even though numerous mutations have been revealed by whole-exome sequencing, no commonly mutated genes other than the known four driver genes (KRAS, P53, CDKN2A, SMAD4) have been identified, but the mutational status of PDAC does seem to cluster around certain pathways[21,22]. Future analyses identifying the concordance of these pathways in metastases and new studies targeting these pathways are needed to validate the importance of these findings. Hopefully, this will result in clinical translation and applications. This can be in the form of new therapeutic targets and/or stratification tools that could be used to improve the use of currently available treatment modalities.

Following our understanding of the genomic rearrangements and gene expression that drive these tumors, analyses at the protein level are a logical next step to help understand the disease and improve survival of these patients. Proteome analysis adds another level to known genomic and transcriptomic data since it identifies the functional players in cell biology. For example, phosphoproteomics can give detailed insight in key signaling pathways that drive growth of tumors that might appear similar at the genetic level (Figure 2B). Large-scale profiling studies of well-characterized clinical cohorts are needed to reveal the proteome landscape of PDAC and exploit this functionally relevant information in a more comprehensive way than was possible to date.

At this moment, an integrated analysis of all levels of molecular profiling data is called for to improve our understanding of how they interact to contribute to the disease, and clinically compatible assays need to be developed in order to capitalize on these findings. For example, although transcriptomic subtypes show survival differences, the transition to clinical practice is not easy.. This is partly due to the number of genes in a classifier. Adding a proteomic analysis to matched samples of subtypes could identify the most differential proteins, which can be evaluated by standardized immunohistochemical techniques in pathology laboratories. Additionally, even though many analyses were performed with one particular technique, all the levels of these omics approaches are interwoven and influence each other. The connectivity of these data should be used to create multi-level biological networks that explain the acquired aggressive capacities more faithfully (Figure 2B).

So far, treatment of PDAC patients with advanced disease still relies on cytotoxic agents[105]. Some survival improvement has been established during the last decade. Especially, neoadjuvant therapy can possibly make a difference in the treatment of this disease. Preliminary retrospective studies

show improved survival upon multi-regimen treatment preoperatively[106]. This improvement is in line with a computational analysis that calculated the effect of inhibition of proliferation to have a bigger impact on survival than just tumor bulk reduction by surgery[3]. One of the presumed mechanisms underlying the benefit of the neoadjuvant approach is the avoidance of development of genetic heterogeneity of subclones and its associated resistance, with progression of the cancer and dissemination of different metastases. Large randomized clinical trials are needed to show the survival benefit of neoadjuvant treatment, preferably in collaboration with biomarker discovery studies to understand resistance and improve patient selection.

Another future treatment perspective will be immunotherapy. As discussed before, exosomes can influence the metastatic niche and influence local immune response against PDAC cells[93]. PDAC has immune-evasive capacities but certain genetic subtypes do initiate a more immunogenic response, which is translated from the primary site to the metastatic tumor[31]. Interestingly, Steele et al[107] identified CXCR2 as an immune modulator which after inhibition induced an enhanced T-cell response and reduced metastatic burden. The promising data of patients with mismatch-repair deficient colorectal cancer responding to immunotherapy might also be relevant for a small number of patients with pancreatic cancer[108]. These studies indicate that next to tumor targeting, the microenvironment and immune response of primary, as well as metastatic PDAC, will need to be further explored to change the prognosis of these patients.

Future studies, including proteomics studies, should also identify novel biomarkers in order to select the group of patients who may gain the most benefit of cancer immunotherapy, as well as implement the design of novel clinical trials designs that allow tumor sample collection in order to understand the mechanism of action and resistance of PDAC (and its metastasis) to (immune)therapy. Biomarker-based selective clinical trials for targeted therapy are indeed incorporated into many ongoing trials, raising hope that future studies and treatments can be given more efficiently. A new clinical study by the Pancreatic Cancer Action Network will make use of molecular profiling of PDAC patients to select patients for specific tracks in their clinical trial[109]. Moreover, in the near future readout of aberrantly activated kinases identified by phosphoproteomics will hopefully aid in the stratification of patients for targeted therapy.

Finally, in a possible and desirable future, with the availability of genome/proteome-wide screening platforms at reasonable costs, a thorough omic analysis of both the tumor and the metastastic specimens in conjunction with user-friendly computational tools will help clinicians to identify the most appropriate drug regimen to be administered to the patient. Hopefully, this approach will

become a strategic companion for patient stratification and optimization of currently available cytotoxic treatments as well as novel anticancer drugs in clinical development.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Figures Legends

Figure 1: PDAC, its microenvironment and its route of metastasis

Figure 1A: Common metastatic localizations of PDAC. PDAC has some preferential metastatic localizations. Liver and peritoneal metastases are most common. The data from this figure is adapted from studies based on rapid autopsy programs[3,6–8].

Figure 1B: PDAC and its microenvironment. Pancreatic ductal adenocarcinoma (PDAC) arises from the pancreas. This tumor is characterized by a dense stromal reaction, consisting of a desmoplastic reaction with extra-cellular matrix (ECM), cancer-associated fibroblast (CAF), tumor cells and immune cells. Both PDAC and CAFs can release exosomes.

Figure 1C: Multiclonal PDAC, clonal expansion and EMT. The primary tumor of PDAC exists of multiple subclones. Not all subclones are capable of dissemination. Upon acquirement of genetic aberrations and epithelial-mesenchymal-transition (EMT), PDAC cells start to migrate into the vasculature. Dissemination mainly occurs polyclonal and while intravascular, these PDAC cells remain the mesenchymal state. Upon establishment of metastases, PDAC cells return towards their epithelial state and recruit local myofibroblast to gain a stromal reaction. Depending on their location, multiple metastatic clones will grow, or one of the subclones will take over and reduce polyclonality upon growth of the metastasis, like for example in the liver.

Figure 1D: Metastatic niche development in the liver. Exosomes released by PDAC cells can enter the vessels. Due to specific integrin complex expression (Integrin beta 5 and Integrin alpha V), they preferentially locate into the liver. There, they are taken up by Kuppfer cells, which in turn react by increased TGF-β signaling, and upon release of macrophage migratory inhibitory factor (MIF) by the exosomes, an immune-evasive response is initiated. This modeling of the liver results in creation of a metastatic niche and can be followed by liver metastases.

Figure 2: Multilayer omic approaches in PDAC

Figure 2A: Subtyping in PDAC. Multiple genomic and transcriptomic analyses have been performed to define subtypes in PDAC. Two studies looked at genomic subtypes, of which only the unstable subtype and mismatch repair (MMR) / double stranded break repair (DSB) group overlap partly. Transcriptomic studies recognized some similarities, especially in a mesenchymal subtype. Future consensus subtyping is needed to establish the subtypes relevant for clinicians.

Figure 2B: The multi omics approach. Multiple layers of the cell can be investigated into depth by genomic sequencing, RNAseq, proteomics and phosphoproteomics. These different layers all have

their own advantage. The big data acquired from genomic studies is more extensive with genomic analysis than proteomics. However, phosphoproteomics and other post-translational modifications can create very large datasets with over 200 000 different modifications. Integrated analaysis will help to identify the most important aberrant pathways and networks that are important for PDAC.

Figure 1A. Common metastatic localizations of PDAC

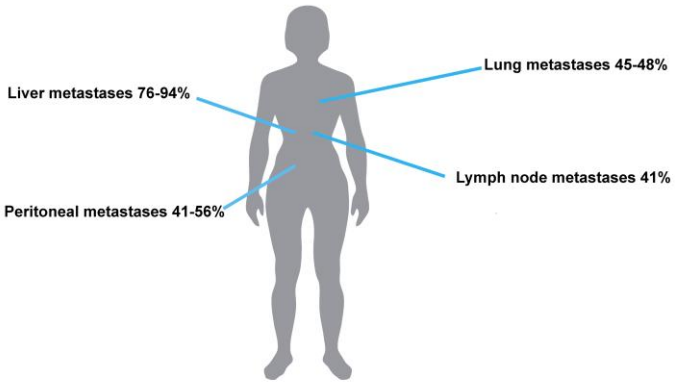

Figure 1B. PDAC and its microenvironment

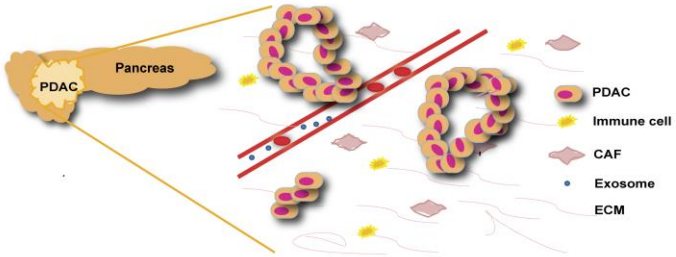

Figure 1C. Multiclonal PDAC, EMT and colonization

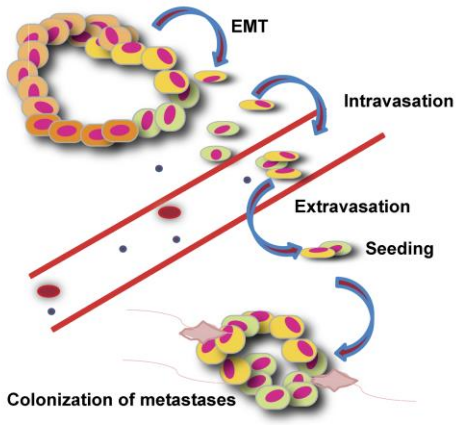

Figure 1D. Metastatic niche establisment in the liver

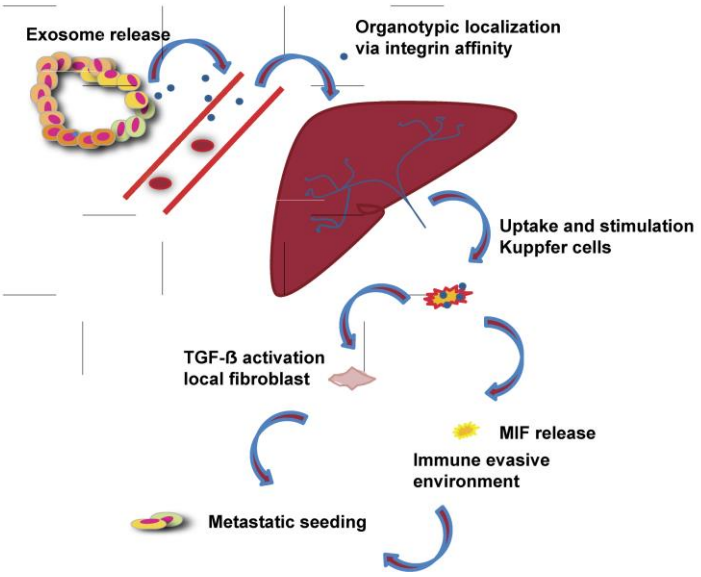

Figure 2A. Subtyping of PDAC

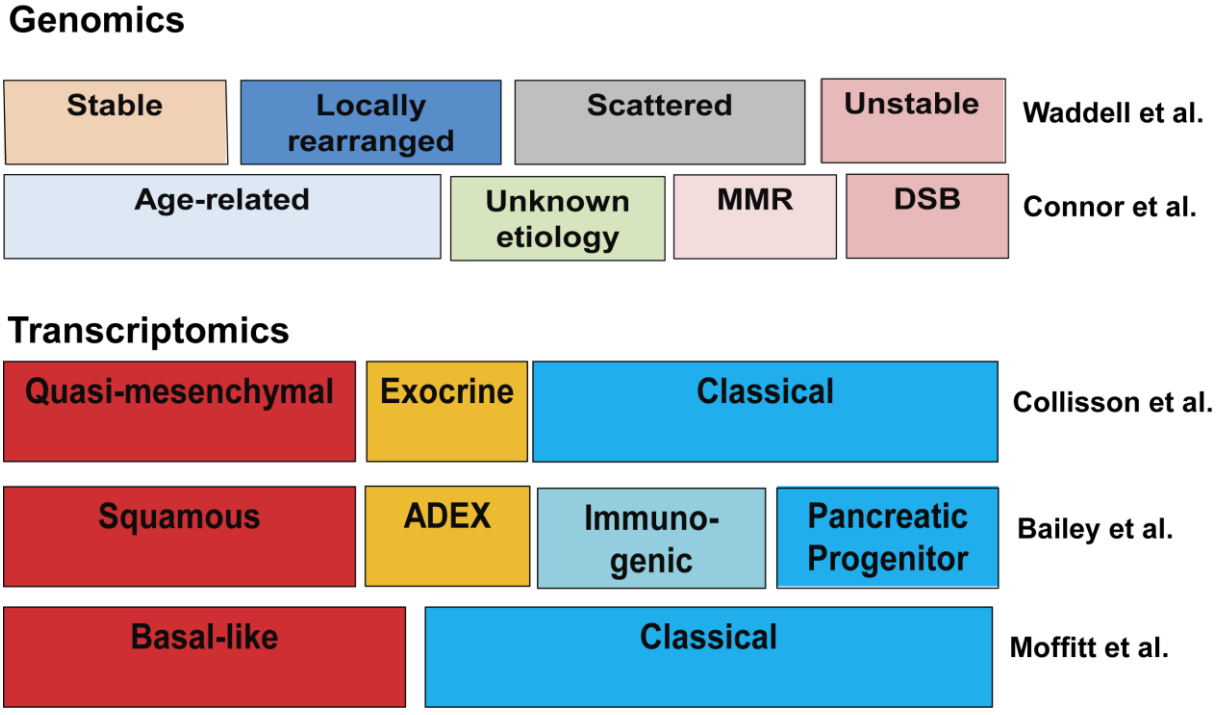

Figure 2B. The multi-omics approach and its application

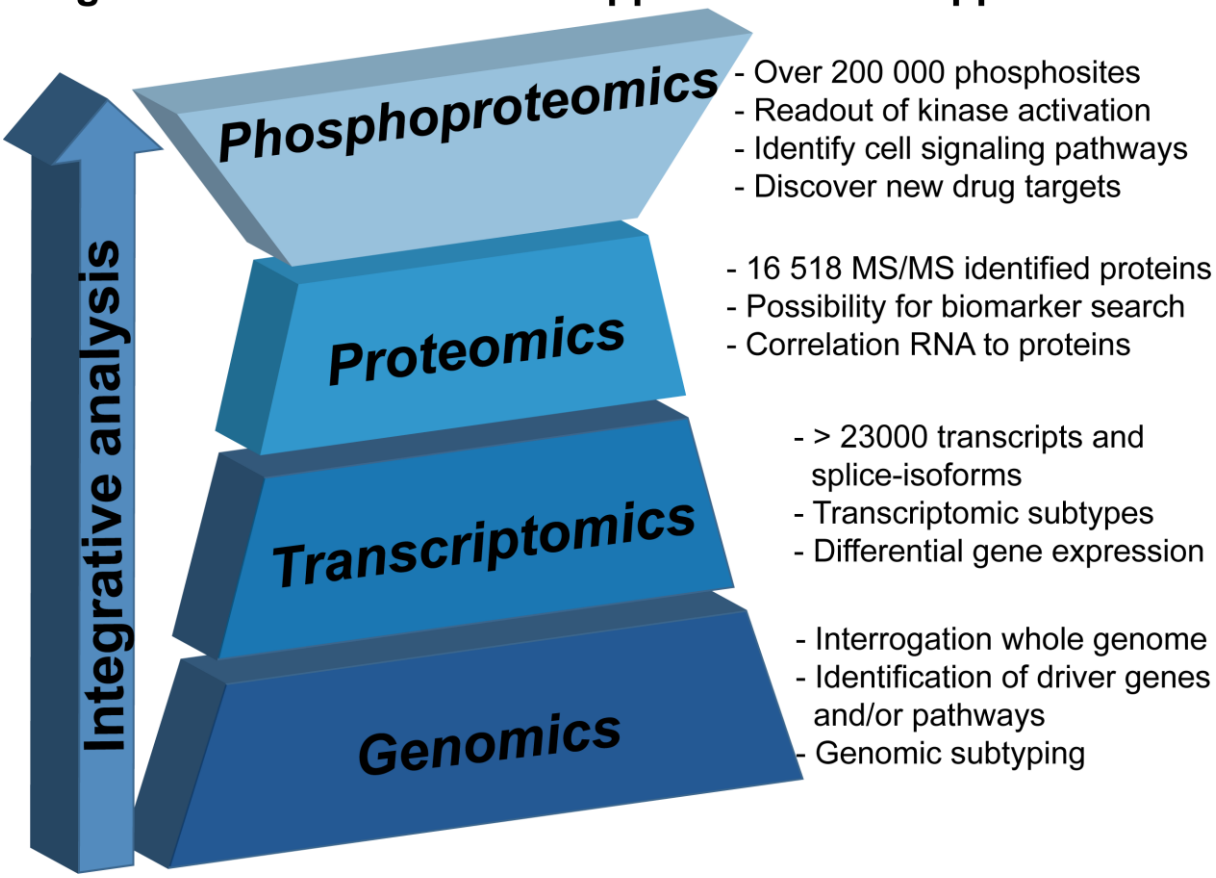

Table 1: PDAC biomarker discovery by proteomics

Abbreviations in Table 1: Cancer stem cell (CSC), Enzyme-linked immunosorbent assay (ELISA), Endoscopic retrograde cholangiopancreaticography (ERCP), Intraductal papillary mucinous neoplasm (IPMN), Isobaric tag for relative and absolute quantitation (iTRAQ), Liquid chromatography tandem mass spectrometry (LC-MS/MS), Mucinous cystic neoplasm (MCN), Multiple reaction monitoring (MRM), Pancreatic intraepithelial neoplasia (PanIN), Pancreatic ductal adenocarcinoma (PDAC), Reverse phase protein lysate microarray (RPPA), Serous cystic neoplasm (SCN), Selected reaction monitoring (SRM), Tandem mass tagged (TMT), not applicable (NA)

Author	Year	Journal	Aim of the biomarker	Method of discovery proteomics	Proteomic platform and number of IDs	Original material and enrichment method of biofluid	Discovery screen groups	Validation method	Validation group	Possible new biomarker proteins	Findings
Drabik et al. [56]	2017	Journal of Proteome Research	Diagnostic	nano LC-MS/MS	Amazon ETD mass spectrometer (Bruker Daltonics)	Immune depleted serum	PDAC (n=76)	NA	NA	HPT, LIFR, CE350, VP13A	221 glycosylated proteins were identified
					2286 proteins identified	Glycosylation enriched proteins	Controls (n=26)				Four were identified in over 50% of the patients
					221 glycosylated proteins						Future validation is needed to establish their clinical value

An et al. [57]	2017	Journal of Proteome Research	Diagnostic and predictive	iTRAQ labelled LC-MS/MS	Orbitrap	Exosomes isolated from serum	PDAC (n=10) pre/during/posttreatment	NA	NA	CD71, LYSC, OBSL1, PLF4, VIM	Eight proteins showed changes upon treatment and response
					1630 proteins identified					PARVB, MARE2, 1B51	These proteins could be possible predictive biomarkers for PDAC
							Healthy controls (n=5)				
Brandi et al. [58]	2016	Journal of Proteomics	Diagnostic	iTRAQ labelled LC-MS/MS	Triple TOF 5600 (AB Sciex)	Secretome from subclone CSCs cell line	PANC1 cell line	Western blot	PDAC (n=100)	MARCKS, CP	43 proteins were significantly differentially secreted by CSCs
					2045 proteins identified		PANC1 CSC subclone	ELISA on serum	Healthy controls (n=20)		After western blot validation, three proteins were

											further analyzed
											Ceruloplamin and MARCKS were validated as possible new biomarkers
Kuhlman et al. [59]	2016	Pancreas	Subtype specific	Label-free LC-MS/MS	MALDI TOF/TOF 5800 (Sciex)	Secretome and surface protein enriched proteome	Exocrine cell line (n=4)	IHC	NA	CDH17, LGALS4	Several proteins were identified as commonly secreted and present in PDAC cell lines
					3288 identified proteins	of primary cell lines	Quasi-mesenchymal cell line (n=4)	RNA		PCDH1, LCN2	PCDH1 and LCN2 were the most promising as possible pan-PDAC markers
							Classical cell line (n=4)				CDH17 and LGALS4 were

											exocrine specific
							Healthy cell line (n=2)				No specific proteins for the other subtypes were identified
Leca et al. [60]	2016	Journal of Clinical Investigation	Diagnostic and prognostic	Label-free LC-MS/MS	Orbitrap mass spectrometer (LTQ Orbitrap Velos; Thermo Fisher Scientific)	Tumor and stromal laser microdissected tissue	PDAC (n=4)	Extracellular vesicle detection	PDAC (n=108)	ANXA6	ANXA6 positive extracellular vesicles could be detected in patient sera
					2077 proteins identified			ANXA6 positive in serum	Healthy controls (n=30)		These vesicles are a possible new biomarker
									Benigne diseases (n=14)		Low levels of these vesicles is correlated to better survival
									Other tumors (n=11)		
Lin et al. [61]	2016	Medicine	Diagnostic	iTRAQ labelled	Triple TOF 4600 mass	Serum depleted of	PDAC (n=24)	ELISA	PDAC (n=54)	APOA-I, TF	APOA-I and TF were

				LC-MS/MS	spectrometer	14 highly abundant proteins					significantly downregulated in PDAC serum
					406 proteins identified		Healthy control (n=12)		Healthy control (n=24)		These findings were also found in CA19.9 negative patients
											Combination of these proteins with CA19.9 could be a new screening method
Sogawa et al. [62]	2016	British Journal of Cancer	Diagnostic	TMT-labelled LC-MS/MS	LTQ-Orbitrap XL (Thermo Scientific)	Pre- and postoperative serum	PDAC (n=3)	ELISA	PDAC (n=66)	C4BPA	20 proteins were identified with 2-fold decrease after surgery
					302 proteins identified with unique	Enriched for glycoproteins			Healthy control (n=40)		C4BPA was validated as possible new

					peptide sequences						biomarker for PDAC
									Pancreatitis (n=20)		Especially the combination with CA19.9 can be valuable
									Other tumors (n=80)		
Yoneyama et al. [63]	2016	PLOS one	Diagnostic	RPPA	For validation SMR:	Plasma	PDAC (n=164)	SRM/MRM LC-MS/MS	PDAC (n=139)	AK3L1, ANXA6, AP3B1, ATP6S1, C2	Multiple proteins were identified as possible new biomarkers in the screen
					ionization-triple quadrupole mass spectrometer (QTRAP5500; AB SCIEX)		Healthy (n=106)		Healthy controls(n=98)	CD82, CKS1B, CKS2, CSPG2, CYCS	IGFBP2 and IGFBP3 were validated as new combination biomarkers with CA19.9
							Other tumors (n=68)		Other tumors (n=287)	EVI1, HMGB2, HYOU1,	

										IGFBP2, MMP9	
							Other pancreas related diseases (n=10)		Other pancreas related diseases (n=53)	MST4, MYBL2, PI3, PPM1B, RNASE1	
										RNASET2, STMN1, VRK2	
Kim et al. [64]	2015	Journal of proteome research	Diagnostic for (pre)malignant tumors	MRM LC-MS/MS	6490 triple-quadrupole mass spectrometer	Plasma	IPMN (n=34)	MRM LC-MS/MS	IPMN (n=50)	LDHB, TXN, THBS1, IGFBP2, IGFBP3	260 proteins were analyzed based on datamining
							Healthy control (n=25)		Healthy controls (n=50)	LRG1, PPBP, KLKB1, C5, AGT, CPN2	29 were significantly differently identified by MRM
							Benign pancreatic diseases (n=25)				A final six proteins panel (IGFBP3, PPBP, C5, CPN2, IGFBP2, and LDHB) could
											discriminate IPMN from

											healthy controls
Radon et al. [65]	2015	Clinical Cancer Research	Diagnostic	Label-free LC-MS/MS	LTQ Orbitrap XL tandem mass spectrometer (ThermoFisher)	Urine	PDAC (n=6)	ELISA	PDAC (n=192)	LYVE1, REG1A, TFF1	A three protein biomarker panel showed promising results as non-invasive test for PDAC
					1500 proteins were identified		Healthy control (n=6)		Healthy controls (n=87)		This panel could also distinguish early stages (stage I-II)
							Chronic pancreatitis (n=6)		Chronic pancreatitis (n=92)		
									Other gastrointestinal tumors (n=66)		
									Premalignant lesions (n=33)		
Chen et al. [66]	2014	Pancreas	Diagnostic and prognostic	Label-free LC-MS/MS	QSTAR XL (Applied Biosystems	Pancreatic juice collected during	PDAC (n=13)	NA	NA	S1008A, S1009A	501 proteins were identified in

					/MDS Sciex)	surgery from the main duct					the pancreatic juice
					503 proteins were identified		Malignant progressed precursor lesion (n=4)			MUC1, MUC5AC, MUC5B	Two biomarker groups were differential, Mucins and the S100 family
							Premalignant lesions (n=2)				Mucin were possible diagnostic biomarkers for PDAC and IPMN
							Healthy controls (n=3)				High expression of S1008A and S1009A were prognostic for poor PDAC survival
							Other tumors (n=5)				
Navaneethan et al. [67]	2014	Gastroenterology report	Diagnostic	Label-free LC-MS/MS	Finnigan LTQ-Obitrap Elite hybrid	Bile collected during ERCP	PDAC (n=10)	NA	NA	FGB, C4BPA, ALB, GC, KNG1, A2M	18 proteins were more abundant and 30 proteins

					mass spectrometer system						were less abundant in cancer bile
					459 proteins identified with at least 2 peptides		Other tumors (n=3)			HPR, FGG, C3, MPO, AHSG, CP	Validation is needed to evaluate their diagnostic potential
							Primary sclerosis (n=6)			APOA1, FGA, C4A, A1BG, ITIH4, APOB	
							Benign strictures (n=5)				
Nie et al. [68]	2014	Journal of Proteome Research	Diagnostic	TMT-labelled LC-MS/MS	Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific)	Serum depleted of 14 highly abundant proteins	PDAC (n=26)	SRM and ELISA	PDAC (n=27)	Single amino acid variation (SAAV)	5 SAAV peptides were found differentially expressed in PDAC serum
						Focus on variant peptides	Healthy controls (n=27)		Chronic pancreatitis (n=20)	AACT, THBS1	SAAV of serotransferrin was validated by SRM

							Other pancreas related diseases (n=88)		Healthy controls (n=17)		SAAV in combination with AACT and THBS1 showed high discriminative power
Nie et al. [69]	2014	Journal of Proteome Research	Diagnostic	Label-free and TMT-labelled	Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) for TMT-labelled LC-MS/MS	Serum depleted of 14 highly abundant proteins	PDAC (n=37)	ELISA & Lectin ELISA	PDAC (n=34)	AACT, LRG, A1AT, LUM, A1AG2,ITIH3, AGEL, A1AG1	A total of 37 differential proteins were identified in 2 discovery screens
				LC-MS/MS	LTQ mass spectrometer (Thermo Finnigan) for label-free analysis	Enriched for glycoproteins	Healthy (n=30)		Healthy controls (n=30)	C1NH, APOA4, KLKB1, C9, C2, HPT, WDR96	Six proteins were validated (AACT, LRG, LUM, A1AT, HPT, THBS1)
					354 glycoproteins identified		Other pancreas related diseases (n=112)		Other pancreas related diseases (n=112)	Q9NYV6, LYAM1, TSP1, ITIH4, CRP, TTHY, A2MG	A biomarker panel (AACT, THBS1,

											HPT) and CA 19−9 showed a high diagnostic potential
										FETUA, CNDP1, PLTP, NOE1, RAD50, DPP4, ACE, UBN1	Obstructive jaundice was found to be a confoundin g factor
										PEDF, CAD21, PGRP2, TTHY, AFAM, JADE2, CBPB2	
Lukic et al. [70]	20 13	Biochimica et Biophysica Acta	Diagnosti c	iTRAQ LC-MS/MS	LTQ Orbitrap velos from Thermo Electron (San Jose, CA)	Bile	PDAC (n=1)	Wester n Blot	PDAC (n=4)	RAC1, OLFM4, SDCB2	104 proteins were differentiall y secreted in bile
					1318 proteins were identified, 796 were identified with at least 2 peptides		Cholangiocarcino ma (n=1)		Cholangiocar cinoma (n=4)		Three proteins were further evaluated and could be validated by western blot

									Chronic pancreatitis (n=3)		
									Benign stenosis (n=2)		
Porterfield et al. [71]	2013	Journal of Proteome Research	Diagnostic of (pre)malignant tumors	Label-free LC-MS/MS	)LTQ Orbitrap XL instrument (Thermo Fisher Scientific)	Pancreatic juice	PDAC (n=3)	Western Blot	Unknown	AMYP, PRSS1, REG1A, REG3A, REG1B	Several proteins were differentially expressed
					368 unique proteins were identified by 1995 corresponding peptides		Chronic pancreatitis (n=3)			CCDC132, phospholipase A2, and elastase 2B	Further validation is needed to identify the value of these new possible biomarkers
							Premalignant lesion, IPMN (n=3)				
							Healthy (n=3)				
Kosanam et al. [72]	2013	Molecular and Cellular Proteomics	Diagnostic	Label-free LC-MS/MS	LTQ-Orbitrap XL mass spectrometer (Thermo	PDAC tissue & ascites	PDAC and adjacent benign tissue (n=4)	ELISA	PDAC (n=20)	DSP, LAMC2, GP73, DSG2, TSPAN1,	16 possible protein biomarkers were identified

					Fisher Scientific)					MSLN, ALPPL	
					2190 nonredundant proteins identified		Ascites (n = unknown)		Benigne disease (n=20)	CDH17, MUC13, S100A14, FXYD3, AF6, CLEC13B	2 were validated significantly different (LAMC2 and DSP)
										TSPAN8, TPBG, MUC4	LAMC2 showed best diagnostic potential in combination with CA19.9
Wehr et al. [73]	2012	Journal of Proteome Research	Diagnostic	LC-MRM/MS	TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Scientific)	Serum depleted from the 14 highest abundant proteins	PDAC (n=20)			Cystatin M, Vilin-2, IGFB7	72 proteins were quantified, of which three were differentially expressed
							Healthy control (n=20)				

Cuoghi et al. [74]	2011	Journal of Proteome Research	Diagnostic for (pre)malignant tumors	Label-free LC-MS/MS	LTQ Orbitrap mass spectrometer (Thermo Electron)	Immunodepleted cyst fluid	MCN (n=2)	Western blot	NA	MUC18, OLFM4	29 proteins were differentially expressed in premalignant versus no-malignancy related cysts
					220 to 727 proteins identified per sample		SCN (n=2)				2 proteins were validated to be present in the cysts by IHC
							pseudocyst (n=1)				
							IPMN (n=1)				
							Neuroendocrine tumor (n=2)				
Kosanam et al. [75]	2011	Proteomics	Diagnostic	Multiple fractionation followed	LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific)	Ascites	PDAC (n=3)	*In Silico* datamining	NA	POSTN, APOL1, LUM, DSP, NRP1, HSPG2, LAMC1	After comparison to publicly available datasets, 20 proteins were identified as possible biomarkers

				by label-free LC-MS/MS	816 proteins were identified					TCN1, SAA2, MMP2, PRSS2, GC, SPP1, NCAM1, CSF1R	Future validation is necessary
										JUP, TPI1, ECMM1, PLA2G7, STMN1	
Makawita et al. [76]	2011	Molecular and Cellular Proteomics	Diagnostic	Label-free LC-MS/MS	LTQ-Orbitrap XL hybrid mass spec-trometer (Thermo Fisher Scientific)	Secretome cell lines	PDAC cell lines (n=6)	ELISA plasma	PDAC (n=20)	CPA1, PRSS1, CPA1, CPA2, GP2, REG1A, CTRC, CPB1	15 proteins were identified commonly in the secretome and pancreatic juice
					3479 non-redundant proteins were identified	Pancreatic juice	Healthy control cell line (n=1)		Healthy Control (n=20)	GP2, PNLIP, SYCN, REG1B, CLPS, SPINK1, PLA2G1B	5 proteins were validated as possible biomarkers in plasma (AGR2, OLFM4, SYCN,

											COL6A1, PIGR)
							Pancreatic juice (n=6)				Combination of either of these proteins with CA19.9 improves its discriminative potential
Matsubara et al. [77]	2011	Cancer Epidemiology, Biomarkers & Prevention	Diagnostic	Label-free LC-MS / 2-DICAL	(ESI-Q-TOF) mass spectrometer (Q-Tof Ultima)	Plasma depleted of high-molecular weight proteins	PDAC (n=21)	Western Blot	PDAC (n=140)	CXCL7	10 proteins were differentially expressed
					53,009 independent MS peaks were identified		Healthy control (n=21)	RPPA microarray	Healthy control (n=87)		CXCL7 was downregulated in cancer patients and patients with chronic pancreatitis
									Chronic pancreatitis (n=10)		Combination with CA19.9 improved sensitivity

											and specificity
Pan et al. [78]	2011	Journal of Proteome Research	Diagnostic	Labelled LC-MS/MS	LTQ-Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific)	Pooled plasma depleted of 7 highest abundant proteins	PDAC (n=5)	ELISA serum	Unknown	APOE, C4BPB, CCL14, CCL5, CD14, DEFA1, FCGR3A	23 proteins were identified differential in the analysis PDAC vs chronic pancreatits or benign
					A total of 1423241 and 1023439 MS/MS spectra were identified		Chronic pancreatitis (n=5)			FCGR3B, ICAM1, IGF2, LBP, LPA, LRG1, LTBP2, MBL2	TIMP1, ICAM1, THBS1, CCL5, LBP and PPBP were evaluated as possible biomarkers
							Healthy control (n=5)			PF4, PF4V1, PPBP, SHBG, SPINK1, TFPI, THBS1, VCAM1	TIMP1 and ICAM1 showed promising results
											AZGP1 might be

											possible discriminating biomarker for chronic pancreatitis
Chen et al. [79]	2010	Molecular Cancer	Diagnostic	iTRAQ LC-MS/MS	Platform not stated	Pancreatic juice	Benign disease pooled sample (n=5)	Western Blot	PDAC (n=8)	AGR2, HIST1H2B, LYZ, MUC5AC, CA2, KLK1, ANXA5	20 proteins were differentially secreted in high-grade premalignant lesions
							PanIN 3 (n=3)	ELISA	Benign disease (n=18)	ACTB, SERPINA1, KRT8, PRSS1, YWHAE, PPIA, PSIP1	AGR2 was validated as possible biomarker in pancreatic juice for (pre)malignant lesions
									Premalignant disease (n=25)	AKR1B10, CLIC1, C2, HNRNPA2B1	Interestingly, the secreted protein levels in pancreatic juice did not correlate to

											serum levels
Matsubara et al. [80]	2010	Molecular and Cellular Proteomics	Predictive	Label-free LC-MS / 2-DICAL	Electrospray ionization quadrupole time-of-flight mass spectrometer (Q-Tof Ultima)	Serum and plasma	PDAC poor survival (n=29)	RPPA microarray	PDAC (n=304)	AAT, AACT	2 MS peaks were differentially identified (alpha1-antitrypsin & alpha1-antichymotrypsin)
							PDAC long survivial (n=31)				These proteins were validated as prognostic biomarkers of survival
											However, they were not predictive in this cohort for gemcitabine efficiency