



# Pancreatic Cancer Biomarkers: Oncogenic Mutations, Tissue and Liquid Biopsies, and Radiomics—A Review

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## Abstract

Pancreatic cancer is one of the most fatal malignancies, as approximately 80% of patients are at advanced stages by the time of diagnosis. The main reason for the poor overall survival is late diagnosis that is partially due to the lack of tools for early-stage detection. In addition, there are several challenges in evaluating response to treatment and predicting prognosis. In this article, we do a review of the most common pancreatic cancer biomarkers with emphasis in new and promising approaches. Liquid biopsies seem to have important clinical applications in early detection, screening, prognosis, and longitudinal monitoring of on-treatment patients. Together with biomarkers in imaging, can represent valuable alternative non-invasive tools in order to achieve a more effective management of pancreatic cancer patients.

**Keywords** Pancreatic cancer · Biomarkers · Oncogenic mutations · Liquid biopsies

## Introduction

Pancreatic cancer (PC) is a highly fatal malignancy, as approximately 80% of patients are unresectable or metastatic by the time of diagnosis [1]. Most PC are pancreatic ductal adenocarcinomas (PDAC), a malignancy of the exocrine pancreas that remains a disease with a very poor overall

5-year survival rate (9%, approximately) [2]. The main reason for the poor overall survival is late detection [3] due to a clinically silent disease with non-specific symptoms in its early stage [2, 4] and the lack of tools for early-stage detection [2]. The only curative treatment is complete surgical resection [2], when possible, thus it is extremely important to detect PDAC in early and resectable stages.

PDAC is a heterogeneous disease characterized by the accumulation of molecular and genetic abnormalities and activating mutations of the Kirsten rat sarcoma viral oncogene homolog (*KRAS* gene) are present in 90% of PDAC cases [2, 5]. Precursor lesions include pancreatic intraepithelial neoplasia (PanIN) and cystic neoplasms such as intraductal papillary mucinous neoplasms (IPMN) that are characterized by point mutations in *KRAS* [1].

Currently, there are several challenges in early diagnosis of PDAC, evaluating response to treatment and predicting prognosis. Because of the relative rarity of this disease, population-based screening is impractical but it may have a role in high-risk cohorts (patients with high-risk genetics, pancreatic cysts, diabetes, and chronic pancreatitis) [2]. It is mandatory and urgent to discover biomarkers to allow for early diagnosis of PDAC, to detect early recurrence with prognostic impact, and to tailor therapy. Also, biomarkers to assess the risk of progression and early transformation of IPMN are needed. Nowadays, biomarkers still have limited utility for detecting early-stage PDAC [6].

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In this article, we perform a review of the most common PC biomarkers with emphasis in new and promising approaches.

## Biomarkers

### Oncogenic Mutations

Around 5–10% of PDAC patients have germline mutations in known susceptibility genes [2]. PDAC development is characterized by an accumulation of multiple genetic alterations in four common genes: *KRAS*, tumor protein 53 (*TP53*), mothers against decapentaplegic homolog 4 (*SMAD4*), and cyclin-dependent kinase inhibitor 2A (*CDKN2A*), being mutations in *KRAS* an early event [2, 4]. Mutations in *KRAS* are very interesting since they have a dual role as prognostic for PDAC status and therapy decision. Modifications in G12 codon are the most frequent *KRAS* mutations (99%). In addition, patients with low *TP53* messenger RNA expression are associated with a poor prognosis and patients with regular *TP53* expression show a longer progression-free survival period, compared with the ones with complete *TP53* loss. On the other hand, *CDKN2A* deletion was reported in 50% of patients with PDAC and is associated with shorter overall survival (OS) rate. Studies also show that *SMAD4* genetic alterations are associated with a poor prognosis in these patients [2].

Hayashi et al. detected mutations in *KRAS* (96% of cases), *CDKN2A* (42%), *TP53* (13%), and *SMAD4* (7%). Among patients after a pancreatectomy followed by chemotherapy, the presence of up to 2 (fewer) mutated driver genes served in this study as an independent predictor of a better OS [Hazard ratio (HR) 0.20]. This shows that the number of mutated driver genes has a potential to be used as a prognostic biomarker for PDAC [7].

Holter et al. identified germline breast cancer susceptibility protein (*BRCA*) types 1 and 2 mutations in 4.6% of PDAC patients with incidental diagnosis (1% type 1 and 3.6% type 2). In this study, none of the mutation carriers met the criteria for familial PDAC and they suggested that these patients should be treated with platinum-based regimens (cisplatin) and poly ADP-ribose polymerase (PARP) inhibitors, because they increase the OS, according to the literature. *BRCA2* mutations can also be used as predictive biomarkers for increased sensitivity of PDAC to the application of DNA-intercalating agents [8].

To explore the spectrum of hereditary pancreatic cancer susceptibility, Slavin et al. evaluated germline DNA from PDAC patients in order to identify high-risk patients. In this study, 30% of the patients had a pathogenic or likely pathogenic variant probably related to their hereditary PDAC predisposition and 13% had mutations in genes associated

with well-known cancer syndromes, namely ataxia-telangiectasia mutated (*ATM*), *BRCA2*, MutS homolog 2 (*MSH2*), and MutS homolog 6 (*MSH6*). In addition, many had mutations in Fanconi anemia complex genes (*BRCA2*, Fanconi anemia complementation group F (*FANCF*), and Fanconi anemia complementation group M (*FANCM*)) [9].

### Tissue Biopsies and Surgical Specimens

Usually, definitive diagnosis and cancer-related molecular alterations are investigated using tumor samples from specimens from surgery or a biopsy. In the majority of cases (except for resectable tumors) a pathological confirmation of diagnosis is required. These biopsy specimens are generally obtained by endoscopic ultrasound—fine-needle aspiration (EUS-FNA), which only has a sensitivity of 75–94% and a specificity of 78–95% for pancreatic cancer. This technique may be inconclusive in up to 20% of early-stage pancreatic cancer cases, requiring repeated biopsies, which represent invasive procedures. In addition, analysis carried out on small samples do not reflect tumor complexity and cannot be applied to the assessment of drug sensitivity, which may change during treatment due to tumor evolution and clonal selection [10].

Because the oncogenic *KRAS* point mutation is frequent in PDAC, the identification of this gene mutation in tumor tissues may facilitate clinical diagnosis. *KRAS* mutation analysis, performed on EUS-FNA samples, appears to be highly accurate in differentiating benign from malignant pancreatic solid lesions. Combining these results with EUS-FNA cytopathology can greatly improve the sensitivity and accuracy of diagnoses and enhance the negative predictive value of cytopathology (from 67 to 88%) [11]. Biopsy-based genotyping is the principal method for classifying tumors for clinical decisions but it requires an invasive procedure and, therefore, is not used to guide treatment over time [2].

In addition to detecting *KRAS* mutations, obtaining biological material represents a unique opportunity to integrate other biomarkers. The evaluation of programmed cell death-ligand 1 (PD-L1) expression [12] and the measurement of v-raf murine sarcoma viral oncogene homolog B1 (*BRAF V600E*) [13] mutations has probably opened new possibilities of therapy, especially in patients not candidates for up-front surgery. In recent years, detection of *BRCA1/2* mutations has aroused interest, as it allows for the identification of hereditary cases [14] and paves the way for the use of PARP inhibitors as personalized therapy, with promising results [15, 16].

A minority of PC (up to 1.3%) belongs to the spectrum of Lynch Syndrome-associated cancer and are characterized by microsatellite instability (MSI) [17] that is easily identified by immunohistochemistry; when only IPMN-associated PC are considered, the percentage of cases rises to 6.9%.

In MSI PC, the amount of CD8 + T cells at the invasive front and the expression level of programmed cell death protein-1 (PD-1) and PD-L1 is higher than in patients with intact mismatch repair system [18]. Studies have shown that treatment based on pembrolizumab (PD-1 inhibitor) has been only successful in MSI-high (instability in at least two of the five microsatellites markers) PC patients [17].

Tumor–stromal interactions can also be important therapeutic targets. A dense and hyaline stroma is a characteristic of PC, which is thought to prevent circulating drugs from reaching the tumor cells, to inhibit the immune system (which is responsible to low responses to immunotherapy) [19], and to provide tumor cells with growth factors. Nevertheless, a strong desmoplastic reaction could also prevent tumor spread, encapsulating the tumor cells [20]. PC stem cells (PCSC) are enriched with the sonic hedgehog (SHH) pathway signaling which is one of the signaling pathways that induces of desmoplastic reaction in PDAC. Askan et al. [21] investigated the relationship between the PCSC markers and the tumor stroma and identified a relationship between PDAC stroma and PCSC markers with an increased incidence of loose stroma in PDAC expressing PCSC markers, particularly CD44. Patients with loose stroma had a worse clinical course in this cohort, with a higher rate of cumulative local recurrence.

### Liquid Biopsies

To overcome limitations of tissue biopsies, less invasive tools are needed to capture tumor heterogeneity, detect residual tumor, and recurrence and allow for real-time analyses of acquired molecular changes. According to the literature, liquid biopsies can have clinical applications in PDAC: early detection, screening and diagnosis, prognosis, longitudinal monitoring of on-treatment patients as a readout of therapeutic efficacy, and disease progression [2, 5]. Also, they are a potential substitute for the entire genome and analyze biomarkers in biofluids such as blood or saliva that can indicate the present state of the pancreatic tumor [2].

### Blood Biomarkers

Blood is likely the most accessible and available fluid in the human body. Some promising biomarkers for PC detection have been identified in the blood (see Table 1). However, there are no solid results and consensus on which circulating biomarker can and should be used in clinical practice. Main reasons for diverging data are, probably, the small sample size of the majority of studies, the enrollment of heterogeneous patients, and the use of different technological approaches [10]. It must be stated that, in PC, peripheral blood analysis remains dependent on the degree of tumor burden and the yield has been prohibitively low until the

disease is metastatic. Some studies suggest that specific targeting of vascular compartments may provide a higher yield of liquid biopsies [22]. Absolute numbers of circulating tumor cells (CTC) in portal vein were significantly higher than in peripheral circulation from patients with advanced PC [23], which shows the possible need to adapt the blood sampling methods for this purpose.

**Proteins** Carbohydrate antigen (CA) 19.9 is the most extensively validated PDAC biomarker and the only one used in clinical practice but limited due to false negatives and positives [24, 25]. It is reported to have a sensitivity of 68% and a specificity of 95% a year prior to PDAC diagnosis and a sensitivity of 53% 2 years prior [26]. Sensitivities reach 60% at 99% specificity within 0–6 months prior to diagnosis and 50% at 99% specificity for cases diagnosed with early-stage disease [25]. Its combination with CA 125 offers a sensitivity of 57.1% and specificity superior to 90% up to 1 year prior to the diagnosis [3]. On the other hand, comparison of resectable PDAC cases with chronic pancreatitis yields 46% sensitivity at 99% specificity and for patients with non-cancerous cysts, 30% sensitivity also at 99% specificity [25].

In symptomatic patients, CA 19.9 levels have a sensitivity and specificity of 79–81% and 82–90%, respectively. They are not useful as a screening marker due to low positive predictive value (0.5–0.9%) but, preoperatively, provide useful prognostic information as patients with normal levels have longer median survival compared to patients with elevated levels [24]. Adding a decrease in CA 19.9 levels ( $\geq 30\%$ ) to image assessment seems to improve resectability prediction following induction chemotherapy and is correlated with improved survival [27]. Overall, CA 19.9 has a mean sensitivity of 78% and mean specificity of 83% for diagnosis, 79% and 90% for prognosis, and 80% and 88% for predictive role, respectively [10].

As stated before, the use of this biomarker is limited by its low sensitivity and high rates of false negatives (about 5–10% have fucosyltransferase deficiency and do not produce CA 19.9—Lewis-negative phenotype) and false positives, particularly in patients with obstructive jaundice (10–60%) [24], being used for disease monitoring supportive to imaging, rather than early detection [2, 3, 10]. Therefore, additional biomarkers are needed to improve sensitivity while maintaining high specificity [25].

Carcinoembryonic antigen (CEA) is the second most common biomarker used for PDAC. Some studies have shown that the combination with other serum markers like CA 19.9 and CA 125 should increase the accuracy in differentiate pancreatic cancer patients from healthy controls [28, 29]. It has a sensitivity of only 44.2% and a specificity of 84.8% for diagnosis [30]. As a prognostic tool, increased levels are associated with a higher tumor burden and worse prognosis [31].

**Table 1** Blood biomarkers with possible clinical use in PDAC

Blood biomarkers	Potential clinical use in PDAC
<b>Proteins</b>	
CA 19.9	Prognosis Disease monitoring in clinical practice It is not used for diagnosis in clinical practice due to a low sensitivity (78%) and specificity (83%)
CEA	Prognosis
ADH and MIC-1	The addition of ADH and MIC-1 to CA 19.9 significantly improved the efficacy of diagnosis (AUC 0.89)
SPARC/Osteonectin	Detection of early stage (sensitivity 84.6%, specificity 87.5%)
Panel of TIMP1, LRG1 and CA 19.9	Detection of early stage (sensitivity 84.9%, specificity 95%, AUC 0.949)
IGFBP3	Detection of early stage (sensitivity 76.3%, specificity 70.7%); increased effectiveness as a compensatory biomarker for CA 19.9 (AUC 0.90)
apoII-ATQ/AT	Decline significantly in PDAC; combined with CA 19.9 as a sensitivity of 95.4% and specificity of 98.3%
<b>Metabolites</b>	
Five-metabolite panel (acetylspermidine, diacetylspermine, an indole derivative, and two lysophosphatidylcholines)	Detection of early stage: performance was significantly improved when combined with the previously validated protein panel of CA 19.9, LRG1, and TIMP1 (AUC 0.924)
<b>Circulating tumor DNA</b>	
<i>KRAS</i> mutant ctDNA	Diagnosis (sensitivity 47%, specificity 87%); combination with CA 19.9 had a sensitivity of 98% and specificity of 77% Disease monitoring in advanced PDAC patients under chemotherapy Prognosis
cfDNA hypermethylation	Diagnosis: ZNF154 cfDNA methylation discriminated cases from healthy donor plasma; integrated model of 5mC and 5hmC (AUC 0.997) Prognosis
<b>Circulating tumor cells</b>	
CK-20, CEA, C-MET, and the human telomerase reserve transcriptase mRNA expression	Diagnosis (CK-20: sensitivity 84%, specificity 93%; CEA: sensitivity 80%, specificity 100%; C-MET: sensitivity 80%, specificity 100%; human telomerase reverse transcriptase: sensitivity 100%, specificity 100%) Disease monitoring Prognosis
CEA mRNA expression	Prognosis
CTC numbers	Disease monitoring Prognosis
Circulating <i>miRNA</i>	See Table 2
<b>Circulating exosomes</b>	
Circulating exosomes positive for GPC1	Diagnosis Disease monitoring Prognosis
<i>KRAS</i> mutations from exosomes	Diagnosis: identified in 66.7%, 80%, and 85% of patients with localized, locally advanced, and metastatic disease, respectively Prognosis
MUC5AC	Diagnosis: can predict the presence of invasive carcinoma within IPMN (sensitivity 82%, specificity 100%)

CA 19.9 Carbohydrate antigen 19.9, CEA Carcinoembryonic antigen, AHD Alcohol dehydrogenase, MIC-1 Circulating macrophage inhibitory cytokine, SPARC Secreted protein acidic and rich in cysteine, TIMP1 tissue inhibitor of metalloproteinase-1, LRG1 leucine-rich alpha-2 glycoprotein 1, IGFBP3 Insulin-like growth factor-binding protein 3, apoII-ATQ/AT C-terminal truncations of the circulating apolipoprotein AII homo-dimer—ctDNA circulating tumor DNA, cfDNA cell-free DNA, 5mC 5-methylcytosine, 5hmC 5-hydroxymethylcytosine, GPC1 glypican-1

Alcohol dehydrogenase (ADH) and circulating macrophage inhibitory cytokine (MIC-1) were evaluated as early-stage diagnostic markers of PDAC in Egyptian patients, alone or in combination with CA 19.9. Their levels were significantly higher in PDAC patients than in healthy controls. MIC-1 had a 94% sensitivity and a 45% specificity, and ADH had a sensitivity of 62% and a specificity of 87.5%. The addition of ADH and MIC-1 to CA 19.9 significantly improved the efficacy of diagnosis with an area under the curve (AUC) of 0.89 [32].

Secreted protein acidic and rich in cysteine (SPARC) or Osteonectin is a secreted, phosphorylated calcium-binding glycoprotein, which plays a central role in various processes during the development of pancreatic adenocarcinoma. It has a sensitivity of 84.6% and a specificity of 87.5% for detection of early-stage PDAC, as shown by Papapanagioutou et al. [33].

A model of tissue inhibitor of metalloproteinase-1 (TIMP1), leucine-rich alpha-2 glycoprotein 1 (LRG1), and CA 19.9 granted a AUC of 0.949, reaching sensitivities of 84.9% at 95% specificity in discriminating early-stage PDAC vs healthy subjects. The performance of this biomarker panel was statistically significantly improved compared with CA 19.9 alone [6]. For pre-diagnostic cases below cut-off value for CA 19.9, the combination with LRG1 and TIMP1 yielded a statically significant increment of 13.2% in sensitivity at 99% specificity in identifying PDAC cases diagnosed within 1 year of blood collection [25].

Insulin-like growth factor-binding protein 2 (IGFBP2) was reported to have a sensitivity of 68.4% with a specificity of 67.7% for detection of early-stage PDAC, while IGFBP3 has slightly higher values of 76.3% and 70.7%, respectively. Based on this study, these proteins may serve as compensatory biomarkers for CA 19.9, because using this combination resulted in an increased effectiveness of detection, with an AUC of 0.90 [34].

Circulating apolipoprotein AII (apoAII) isoforms apoAII-ATQ/AT (C-terminal truncations of the apoAII homo-dimer) decline significantly in PDAC. AUC values of apoAII-ATQ/AT to detect early-stage PDAC are higher than CA 19.9 values in a study by Honda and colleagues. The sensitivity of this protein combined with CA 19.9 was 95.4% with a specificity of 98.3% [35].

An *in vitro* study by Liu and colleagues confirmed, through biological analysis and cytological validation, that circulating chaperonin-containing T-complex protein 8 (CCT8) is a new predictive biomarker for PDAC. The results suggest that knockdown of CCT8 could suppress the metastatic phenotype of PDAC cell lines [36].

Regarding circulating antibodies, some were identified in PDAC patients. Most single autoantibodies have a low sensitivity (<50%) but a high specificity (>90%) [37, 38].

**Metabolites** A five-metabolite panel in combination with CA 19.9, TIMP1, and LRG1 exhibited substantially improved performance in the detection of early-stage PDAC compared with a protein panel alone, according to a study by Fahrman et al. [39]. These five metabolites (acetylspermidine, diacetylspermine, an indole derivate, and two lysophosphatidylcholines) yielded AUC ranging from 0.726 to 0.842, and the combined model yielded an AUC of 0.892 (sensitivity of 66.7% and specificity of 95%). Performance was significantly improved by combining the metabolite panel with the previously validated protein biomarkers panel that included CA 19.9, LRG1, and TIMP1, with an AUC of 0.924 ( $p=0.02$ ), which also outperformed CA 19.9 alone.

This panel could be used to target high-risk patients, such as people older than 50 years that recently developed diabetes or with a family history of PDAC. It will also be important to discover how effectively the metabolite and protein panels can distinguish PDAC from non-cancerous pancreatic cysts [40].

**Genetic and Epigenetic Circulating Tumor DNA** *KRAS* mutation has been found in circulating tumor DNA (ctDNA), CTC, and in cargo from isolated exosomes [2]. CtDNA released from necrotic or apoptotic cells of primary tumors, metastasis, and CTC can reveal genetic and epigenetic alterations with tumor-specific and individual mutation and methylation profiles [41, 42]. Usually, ctDNA is detectable as small fragments with the length of 170–180 base pairs [42]. Its concentration is very low and varies considerably among individuals, ranging from 1 to 100 ng/ml, also depending on the type and dimension of the tumor burden [43], but even if sensitivity and specificity are low, tumor DNA can be characterized by detecting somatic mutations localized in the genome of cancer cells only [44]. Several technologies are available to analyze ctDNA mutations: real-time reverse transcriptase polymerase chain reaction (RT-PCR), automatic sequencing, mass spectrometry genotyping, next-generation sequencing (NGS), and digital PCR platforms, such as digital droplet PCR (ddPCR) [10]. The Wong group has developed the electric field-induced release and measurement (EFIRM) technology to capture and monitor in biofluids key oncogene mutations in cancer patients. It is an electrochemical (EC) platform integrating sensitive and specific multiplex assays and is based on the principle that nucleic acid hybridization can be facilitated through applying electric fields selectively. The total detection time is 30 min and requires only 20–40  $\mu$ L of plasma or saliva for direct ctDNA detection [2]. Definitive validation of this technique will allow for its use in clinical practice.

The ideal liquid biopsy should capture the signature of ctDNA concordant with tissue biopsy genotyping. Studies have shown a concordance of 48% to 100% in PDAC. This



heterogeneity of results emphasizes the inadequacy of some of the technologies used [2]. Actually, detection of ctDNA can be challenging because ctDNA abundance is considered very low (< 1% in many cases) in total cell-free DNA (cfDNA) [45]. The low diagnostic sensitivity of ctDNA tests in carcinomas could also be a major reason for discordant tissue and ctDNA genotyping results. However, the first FDA-approved application in 2018 for cfDNA assay in routine clinical practice could demonstrate high concordance between plasma and tumor tissue genotyping for early detection of specific epidermal growth factor receptor (EGFR) mutation and therapy stratification in advanced non-small cell lung cancer patients [41, 46].

Regarding diagnosis, ctDNA analysis seems to have a limited use in PDAC patients, particularly due to the low abundance of detectable ctDNA in early stages [10]. Maire et al. reported a sensitivity of 47% and a specificity of 87% of ctDNA *KRAS* mutations for the diagnosis of PDAC. The combination of these mutations and CA 19.9 had a sensitivity of 98% and a specificity of 77%, respectively [47]. On the other hand, CA 19.9 seems to be superior to ctDNA for detection of PDAC in low burden disease [48]. In patients with chronic pancreatitis, four out of four patients who also had ctDNA *KRAS* mutations were diagnosed with PDAC during follow-up [49].

In 2019, plasma cfDNA profiling in 38 patients with advanced PDAC receiving neoadjuvant chemotherapy with FOLFIRINOX demonstrated that 65.8% of them had at least one common driver gene alteration in *KRAS*, *TP53*, *SMAD4*, or *CDKN2A* in high concordance with corresponding tumor tissue [50]. The dynamics of total cfDNA concentration correlated positively with tumor burden after chemotherapy and seems a good tool for early response prediction and therapy surveillance in patients with advanced PDAC [41]. Serial plasma testing of *KRAS* mutant ctDNA in advanced PDAC patients receiving chemotherapy seems to allow better monitoring than CA 19.9 kinetics [51]. As *KRAS* is the best characterized tumor-related gene in PDAC, with mutation appearance in early stages, *KRAS* mutant ctDNA seems to represent a promising biomarker and therapeutic target of PDAC. Chen et al. reached a 93.7% detection rate of *KRAS* mutant ctDNA that correlated with time to progression and OS of 189 patients with unresectable PDAC [52]. In metastatic cases, absence of *KRAS* mutant ctDNA is shown to be significantly associated with a survival benefit of 37.5 versus 8 months ( $p < 0.004$ ) [53]. PDAC patients with *KRAS* mutant ctDNA are more likely to relapse after curative surgery than those without it, with disease-free survival of 6.1 versus 16.1 months and OS of 13.6 versus 27.6 months ( $p < 0.001$ ) [54–56]. Berger et al. dynamically monitored the most frequently ctDNA-mutated genes using NGS and ddPCR. TP53 and *KRAS* mutation levels were significantly decreased

during treatment and increased during tumor progression, correlating with progression-free survival [57].

DNA promoter hypermethylation is a hallmark of cancer. It is a mechanism of early carcinogenesis, which can use inactivation of tumor suppressor genes [58]. Methylation analyses of ctDNA that reveal epigenetic alterations also reflect the heterogeneity in PDAC patients. CfDNA promoter hypermethylation in plasma or serum has the potential to be prognostic markers for PDAC, as they can be detected in all stages of PDAC, and differentiate them [59]. A prediction model was developed by Henriksen et al., based on plasma-derived cfDNA hypermethylation of a large gene panel that enables the stratification of patients into risk groups according to survival. Also, they found that hypermethylation of more than ten genes in plasma-derived cfDNA is an independent risk factor of poor OS in patients with PDAC [60]. Additionally, further methylation analyses of ctDNA in a prospective study were able to differentiate PDAC from chronic pancreatitis and healthy controls. A diagnostic prediction model had an AUC of 0.86 (sensitivity 76%, specificity of 83%), independent of cancer stage [58]. A study by Miller and colleagues assessed ZNF154 methylation in patient plasma as a multicancer marker in liquid biopsies. ZNF154 cfDNA methylation discriminated cases from healthy donor plasma samples in minimal plasma volumes and outperformed *KRAS* mutation frequency in pancreatic cancer [61].

Cao et al. developed a non-invasive approach combining epigenetic biomarkers for detecting PDAC. They demonstrated that both 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) biomarkers in cfDNA are effective in PDAC detection and an integrated model of both presents increased diagnostic power (AUC 0.997, sensitivity 0.938, specificity 0.955). Also, a diagnosis score calculated with the 5hmC model can distinguish stage I from stage II to IV patients [62].

In summary, ctDNA is a useful tool to detect genetic alterations in PDAC patients. It seems also useful to monitor treatment and disease progression and as a prognostic biomarker in these patients, although confirmatory studies are needed.

**Circulating Tumor Cells** CTC are released into the bloodstream through shedding from the primary tumor and can disseminate into blood vessels invading the tissue stroma [10]. Overall, methods for detecting CTC are highly specific but sensitivity is not high because of the low number of captured CTC [2]. Moreover, PDAC is characterized by a low CTC detection rate compared with other solid tumors [10], but, according to the literature, they can be used as markers of PDAC at any stage, for diagnosis, monitoring of treatment response [2, 63], and also for prognosis [64]. CTC analysis seems to have sufficient and high sensitivity (70%) to detect stage I and II PDAC [63, 65, 66].

Zhou and colleagues, in 2011, used a multi-marker approach as an indicator for CTC by immunomagnetic/RT-PCR. By combining mRNA expression analyses of CK-20, CEA, C-MET, and the human telomerase reverse transcriptase, they were able to distinguish PDAC patients from benign controls (CK-20: sensitivity 84% and specificity 93%; CEA: sensitivity 80% and specificity 100%; C-MET: sensitivity 80% and specificity 100%; human telomerase reverse transcriptase: sensitivity 100% and specificity 100%). mRNA expression levels of CK-20, CEA, and C-MET were significantly higher during late stages (III and IV) than earlier stages, suggesting that assessment of CTC could be useful in monitoring disease progression [67]. Another study that used RT-PCR to evaluate CEA mRNA expression as a molecular marker for CTC in the peripheral blood of PDAC patients undergoing curative surgery demonstrated that CTC-positive patients had shorter progression-free survival (PFS) compared with negative ones (66 versus 138 days, respectively) [68]. Other studies also revealed an association with shorter PFS and an association with shorter OS for CTC-positive patients [64]. In a study by Okubo et al., the incidence of CTC positivity at 3 months after beginning of treatments in patients with progressive disease and stable disease or partial response were 45.4% and 24.1%, respectively, showing that CTC numbers represent a useful tool for predicting therapeutic responses to chemotherapy among patients with advanced pancreatic cancer [69].

The CTC-based CellSearch© system detects PDAC in up to 48% of patients in cohorts that include at least 53% of patients with metastatic or locally advanced disease, but even when all the patients had advanced disease, rates of CTC detection were not observed to increase [2, 11]. Magnetic bead enrichment tends to cause cell destruction and requires a certain level of expression of tumor-specific antigens [like epithelial cell adhesion molecule (Ep-CAM)] to maintain cells in the magnetic support [10]. This may be

responsible for the low CTC detection rate in PDAC patients (56%), probably due to the heterogeneous expression of Ep-CAM [70]. Alternatively, the isolation by size of epithelial tumor cells method (ISET) presents a higher detection rate of 93% [2, 71] and Ko et al. invented a microchip platform that combines fast, magnetic micropore-based negative immunomagnetic selection with rapid on-chip in situ RNA profiling of whole blood in PDAC patients, even in those with very low number of CTC [2, 72]. In conclusion, clinical relevance of CTC is still controversial but it seems like a promising biomarker for diagnosis, prognosis, and treatment response monitoring in PDAC patients.

**Circulating miRNA** Circulating miRNA can also be used as biomarkers for pancreatic cancer detection, differential diagnosis, predicting response to treatment, and assessing prognosis [2]. Table 2 shows circulating miRNA for PDAC and their clinical application.

**Circulating Exosomes** Exosomes are 30–150-nm extracellular vesicles of endocytic origin that contain nucleic acids, proteins, and lipids and facilitate communication between cells and the establishment of pre-metastatic niches [2, 4]. These nucleic acids derived from exosomes have been reported as high-quality DNA material that is protected from degradation while circulating [5]. Several methods are available to isolate exosomes from biological fluids, including ultracentrifugation (considered gold standard), filtration, polymer-based precipitation, chromatography, and immunological separation, but this isolation is still challenging because of complexity of biological samples, contamination from other extracellular vesicles, and exosome heterogeneity [82].

Although the mechanism is still not clear, exosomes in body fluids are believed to be related to cancer development [2]. A panel of pancreatic cancer-initiating cell protein

**Table 2** Circulating miRNA with possible clinical use in PDAC [3]

Circulating miRNA	Potential clinical use in PDAC
miR-21 [73, 74]	Diagnosis (sensitivity 0.90, specificity 0.72, AUC 0.91) Prognosis
miR-25 [75]	Diagnosis (AUC 0.915)
miR-196a and miR-196b (increased) [76]	Differential diagnosis (between PDAC and multifocal PanIN-2/3 vs PanIN-1, pancreatic neuroendocrine tumors, chronic pancreatitis, or healthy patients)
miR-223 [77]	Differential diagnosis (between benign IPMN and malignant IPMN)
miR-744 (increased) [78]	Poor prognosis (metastases, recurrence, chemotherapy resistance); postoperative period
miR-373-3p (decreased) [79]	Poor prognosis (metastases, recurrence, chemotherapy resistance); postoperative period
miR-18a (increased) [79]	Poor prognosis (recurrence); postoperative period
miR-196a and miR-196b (decreased) [76]	Postoperative prognosis
miR-221 (decreased) [80]	Postoperative prognosis
miR-483-3p (decreased) [81]	Postoperative prognosis

markers (CD44v6, tetraspanin-8, epithelial cell adhesion molecule, and CD104) and miRNA (miR-1246, miR-4644, miR-3976, and miR-4306) were significantly upregulated in most of serum exosomes in these patients, but not in healthy controls and patients with non-malignant diseases [83]. Melo et al. showed that circulating exosomes positive for glypican-1 (GPC1) distinguished early- and late-stage PDAC patients from healthy controls and patients with benign pancreatic diseases, with absolute specificity and sensitivity. In addition, levels of these exosomes correlate with tumor burden and survival of pre- and post-surgical patients [84]. A more recent study showed that exosomal miRNA levels are superior to exosomal GPC1 or serum CA 19.9 levels in diagnosing PDAC patients and differentiating malignant disease from chronic pancreatitis. Elevated exosomal miRNA levels decrease to normal values within 24 h following PDAC resection, by contrast with GPC1 [85].

*KRAS* mutations from exosomes outperformed assessment from ctDNA in detection of PDAC and predicting disease progression [5, 86]. It also showed good correlation with progression-free survival in patients with metastatic disease [83]. They were identified in 66.7%, 80%, and 85% of patients with localized, locally advanced, and metastatic disease, respectively. In comparison, *KRAS* cell-free DNA mutations were identified in 45.5%, 30.8%, and 57.9% of these PDAC patients [86]. Same authors also observed that a higher mutant allelic fraction (MAF) of *KRAS* in exosomal DNA (exoDNA) was associated with a poor prognosis in patients with localized disease (MAF < 1%: 441 days versus MAF > 1%: 127 days;  $p=0.031$ ). Also, *KRAS* mutations rate are reduced after resection compared with localized pre-resected patients (5 versus 66.7%) [86]. In general, the diagnostic performance of exoDNA ranges between 35 and 69% [5, 86]. Despite its good prognostic value, exoDNA based on detection of mutant *KRAS* may have a limited use for diagnosis of PDAC because of a high rate of false positives [86].

Bernard et al. isolated ctDNA and exoDNA in blood samples of patients with localized and metastatic PDAC and determined whether MAF of *KRAS* was associated with survival outcomes [5]. In the mentioned study, *KRAS* MAF circulating is higher in exoDNA and significantly higher in baseline metastatic samples than those with localized disease, cystic lesions and, finally, non-neoplastic pancreatic diseases. Concordance with *KRAS* detection in surgical tissue was 95.5% for exoDNA and 68.2% for ctDNA. Longitudinal assessment of exosomal *KRAS* MAF levels in localized PDAC patients correlates with response to neoadjuvant chemotherapy and surgical resectability. In metastatic patients: high levels of *KRAS* MAF in liquid biopsies is associated with increased tumor burden and reduced survival, exoDNA and ctDNA predict survival in these treatment-naïve patients, and plasma peaks in exoDNA *KRAS* MAF seem to precede disease progression [4, 5].

It was reported that tumor-derived exosomes with organ specificity were preferentially taken up by cells and prepared the metastatic niche [87]. Expression of integrins on exosomes exhibits a pattern reflecting organ specificity, as shown by Hoshino and colleagues. The expression of  $\alpha 6\beta 4$  and  $\alpha 6\beta 1$  integrins on tumor-derived exosomes was associated with lung metastasis, while  $\alpha v\beta 5$  integrins were associated with liver metastasis [88].

Regarding intraductal papillary mucinous neoplasms (IPMN), a study showed that MUC5AC in circulating extracellular vesicles can predict the presence of invasive carcinoma within IPMN [89].

In summary, results of these studies suggest a new source of circulating nucleic acids, protected from degradation by encapsulation. However, a minority of healthy controls can show mutant *KRAS* in exoDNA, which means that detection of PDAC cannot be assumed. As for prognostic and longitudinal monitoring, validation studies are needed.

### Urine Biomarkers

Urine samples seem to also play a role in liquid biopsies for detection of PDAC. A three-marker panel in urine of PDAC (LYVE, REG1A, and TFF1) was studied and it was able to discriminate early PDAC from healthy controls with an 80% sensitivity at a 76.9 specificity (AUC 0.926) [90]. A more recent case–control study by the same author showed that the substitution of REG1A with REG1B enhanced the performance of the panel to detect resectable PDAC. AUC improved to 0.936 with sensitivity and specificity > 85% [91]. Another study evaluated miRNA biomarkers in urine. The combination of miR-143 with miR-30e discriminated between PDAC stage I patients from healthy controls with a sensitivity of 83.3% and a specificity of 96.2% [92] and panel of three urine metabolites (trigonelline, hippurate, and myoinositol) was able to stratify patients with good or poor prognosis based on OS. PDAC patients with abnormal levels of two or more metabolites in their urine demonstrated significantly lower survival [93].

### Salivary Biomarkers

Saliva seems to be another potential source of biomarkers, although with low sensitivity and specificity. Four salivary mRNA biomarkers (*KRAS*, *MBD3L2*, *ACRV1*, and *CDKL3*) had a sensitivity of 71% and a specificity of 69% for differentiating PDAC patients from healthy controls. A study by Liu et al. identified 29 additional biomarkers with 92% sensitivity at a 100% specificity for detection of PDAC [94].

EFIRM technique confirmed that tumor-shed exosomes are detected in saliva, in addition to blood. Affinity-CD63 (exosomal-specific membrane protein marker)-based magnetic beads were used to extract the exosomes from



biological samples [2, 95]. In a pancreatic cancer animal model, EFIRM was used to study how exosomes, harboring mutated genes, can travel from the tumor through blood and saliva [96]. This study with an animal model showed that pancreatic cancer-specific salivary transcriptomic biomarkers were present. Moreover, they demonstrated that inhibiting exosome secretion by PDAC disrupted the panel of discriminating salivary biomarkers.

### Pancreatic Juice Biomarkers

The blood is considered the most common body fluid source for liquid biopsies. However, pancreatic juice (PJ) seems to also be an effective source for detecting PDAC [87], as well as mucinous lesions content.

Kisiel et al. reported that methylation signature in pancreatic juice enables differentiating PDAC from normal or chronic pancreatitis [97]. Yu et al. performed a genetic analysis using digital NGS to detect PDAC using PJ collected from the duodenum. Mutant DNA concentrations are significantly higher in PDAC than in IPMN. Also, *TP53* and/or *SMAD4* mutations were commonly detected in PDAC and not in controls and, in few cases, were detected in PJ collected more than 1 year before diagnosis with PDAC. Although specificity was high (100%), sensitivity was low (32%) [98].

Pancreatic cystic neoplasms are heterogeneous groups with varying risks of malignancy [99]. Elevated CEA is a marker that distinguishes mucinous from non-mucinous cysts, but not malignant from benign [100]. *GNAS* (guanine nucleotide binding protein, alpha stimulating) mutations are found exclusively in IPMN, among the various pancreatic neoplasms but not all IPMN harbor *GNAS* mutation. It is more frequently observed in intestinal IPMN than in the non-intestinal sub-types. This mutation, usually detected in codon 201 in several cancers, leads to sustained activation of G $\alpha$ s (Gs alpha subunit) and an increased level of cAMP, which activates protein kinase A and subsequent cancer-promoting activities. *GNAS* mutation samples have potential clinical applications: differentiating invasive IPMN from concomitant PDAC using resect specimens, assessing clonality of recurrent IPMN in the remnant pancreas using resected specimens, and differentiating pancreatic cystic lesion using cystic fluid by EUS-FNA [101]. If a pancreatic carcinoma has *GNAS* mutation, it probably derived from IPMN [102]. On the contrary, it has been shown that concomitant PDAC tends to develop in the pancreas with IPMN with wild-type *GNAS*, irrespective of IPMN subtype (branch duct or main duct) [101].

As previously mentioned, PDAC usually exhibit *KRAS* mutations, which are considered the major oncological driver. This mutation is considered to be an early event in IPMN malignant transformation [103]. In this regard,

detection of *KRAS* mutations in PJ or cystic content could be a valuable tool for diagnostic assistance. It could also allow for cystic lesions follow-up and possible early detection of cancerization, in patients without worrisome features [100], although the procedure for collecting these fluids is not easy and risk free.

### Biomarkers in Imaging

Radiomics information can serve as a non-invasive method for primary tumor assessment and to predict the treatment response and prognosis of patients with PDAC. In this context, multiple studies have been conducted preoperatively to evaluate the biology of tumors, most of them performed on CT examination [104].

A study in resectable PDAC showed that CT-derived texture feature of dissimilarity and inverse difference normalized are associated with OS [105]. Yun et al., in a study with resectable pancreatic head cancer patients, found that lower standard deviation and contrast and higher correlation with lower average value representing homogeneous texture were significantly associated with poorer disease-free survival (DFS) and the presence of lymph node metastasis [106]. Chakraborty et al. also showed that texture analysis can be used to quantify heterogeneity in CT images to accurately predict 2-year survival in patients with PC [107]. The same results on CT texture features and their association with OS were achieved by Attiyeh et al. that presented two survival prediction models [108]. However, another study found that the texture signature was not associated with the DFS but that tumors that are more hypoattenuating on the portal-venous phase of a CT scan are potentially more aggressive with higher tumor grade, greater lymph node invasion, and shorter DFS [109].

Considering unresectable PDAC patients who underwent chemotherapy, Cheng et al. showed that pre-treatment CT quantitative imaging biomarkers from texture analysis were associated with DFS (tumor standard deviation (SD) and skewness measured with spatial scaling factor (SSF)=3 and tumor SD measured with SSF=4) and OS (tumor SD measured with SSF=3) [110]. Another study using PET-CT data found that the higher entropy was independently associated with worse survival, while total lesion glycolysis was not an independent prognostic factor [111].

Evaluating patients' response to treatment, including neoadjuvant chemotherapy, remains a challenge. A study by Kim et al. analyzed contrast-enhanced CT images of PDAC patients after neoadjuvant therapy [112]. They found significantly lower subtracted value surface area, higher subtracter values of gray-level co-occurrence matrices (GLCM) inverse difference moment (IDM), and GLCM contrast for predicting the R0 resection. Also, higher subtracted entropy and

lower subtracted GLCM entropy are important parameters in predicting the longer OS rate.

Radiomics analysis can also be used for predicting the risk for postoperative pancreatic fistula (POPF) that is one of the most harmful complications after pancreatic resection [104]. For example, Zhang et al. developed and validated a radiomics-based formula for predicting POPF in patients undergoing pancreaticoduodenectomy (the Rad-score, AUC of 0.8248) [113].

## Conclusion

Tools currently available to screen high-risk patients for PDAC seem to be not adequate, whereas liquid biopsies are the emerging technology that have the potential to transform their management [7]. However, further research is needed to validate these findings and introduce them in our daily practice. An efficient non-invasive tool to screen high-risk patients will significantly facilitate decision-making for further diagnosis and therapeutic approaches, overcoming the problem of tumor heterogeneity [2].

Liquid biopsies and radiomics biomarkers can represent alternative and non-invasive tools for diagnostic and prognostic purposes and for predicting and monitor response to treatment and disease progression, in order to achieve a more effective management of PDAC patients. They are relatively new opportunities to provide insights into the biological and clinical characteristics of a neoplastic disease, and their integration in the management of these patients should be extremely valuable, although validation studies are needed.

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## Declarations

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