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The primary aim of *World Journal of Gastrointestinal Oncology* (*WJGO*, *World J Gastrointest Oncol*) is to provide scholars and readers from various fields of gastrointestinal oncology with a platform to publish high-quality basic and clinical research articles and communicate their research findings online.

WJGO mainly publishes articles reporting research results and findings obtained in the field of gastrointestinal oncology and covering a wide range of topics including liver cell adenoma, gastric neoplasms, appendiceal neoplasms, biliary tract neoplasms, hepatocellular carcinoma, pancreatic carcinoma, cecal neoplasms, colonic neoplasms, colorectal neoplasms, duodenal neoplasms, esophageal neoplasms, gallbladder neoplasms, *etc.*

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Liquid biopsy approach to pancreatic cancer

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Abstract

Pancreatic cancer (PC) continues to pose a major clinical challenge. There has been little improvement in patient survival over the past few decades, and it is projected to become the second leading cause of cancer mortality by 2030. The dismal 5-year survival rate of less than 10% after the diagnosis is attributable to the lack of early symptoms, the absence of specific biomarkers for an early diagnosis, and the inadequacy of available chemotherapies. Most patients are diagnosed when the disease has already metastasized and cannot be treated. Cancer interception is vital, actively intervening in the malignization process before the development of a full-blown advanced tumor. An early diagnosis of PC has a dramatic impact on the survival of patients, and improved techniques are urgently needed to detect and evaluate this disease at an early stage. It is difficult to obtain tissue biopsies from the pancreas due to its anatomical position; however, liquid biopsies are readily available and can provide useful information for the diagnosis, prognosis, stratification, and follow-up of patients with PC and for the design of individually tailored treatments. The aim of this review was to provide an update of the latest advances in knowledge on the application of carbohydrates, proteins, cell-free nucleic acids, circulating tumor cells, metabolome compounds, exosomes, and platelets in blood as potential biomarkers for PC, focusing on their clinical relevance and potential for improving patient

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Core Tip: Pancreatic cancer (PC) is still an especially lethal malignancy, with a 5-year survival rate below 10%. Liquid biopsies are a readily-available source of biomarkers to determine the situation of patients. This review summarizes the most recent published findings on the diagnostic, prognostic, and predictive potential for PC of biomarkers identified in liquid biopsies, discussing the strengths, limitations, and drawbacks of their clinical application. There remains a need to validate and verify the clinical value of liquid biopsies for PC in large-scale human trials using appropriate controls.

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INTRODUCTION

Despite recent advances in understanding the molecular events underlying pancreatic cancer (PC), it remains one of the deadliest malignancies[1]. In the absence of novel diagnostic methods and/or treatments, PC is expected to become the second leading cause of cancer-related deaths by 2030[2]. Complete surgical resection is a potentially curative treatment, but 80%-85% of patients present with non-resectable disease at their diagnosis due to local invasion or distant metastasis[3]. An early diagnosis is crucial but is highly challenging due to the lack of specific symptoms during initial stages of the disease. The etiology of PC is not well understood, and new prospective studies are needed to fully elucidate related factors[4]. The development of diagnostic, prognostic, and predictive biomarkers is urgently needed to improve the clinical management of PC[5].

An ideal tumor biomarker is both specific, *i.e.*, exclusive to the disease in question, and sensitive, detecting early cancer development and identifying all cases. It should have positive predictive value, enabling detection in the general population during very early stages, and a long lead time, sufficient to allow modification of the natural course of the disease. In addition, it should correlate with tumor stage and allow the monitoring of biomarker levels to detect early relapse. Finally, the biomarker should be easy to collect and involve a simple and economically feasible test[6]. Ideally, a diagnostic biomarker allows PC to be detected at an early stage, a prognostic biomarker provides information on the disease in untreated individuals, and a predictive biomarker yields information for personalizing treatment to obtain the best therapeutic response and deliver optimal patient care[7].

Liquid biopsy is a promising non-invasive matrix that has proven useful for the early diagnosis of various types of cancer. It offers a novel tool for monitoring tumor stage and the response to treatment throughout disease progression[8]. However, unlike in some other cancers, the clinical applicability and reliability of liquid biopsy results have yet to be established in PC[9]. The aim of this review was to provide an update on recent advances in the development of liquid biopsy biomarkers for PC and evaluate their potential clinical usefulness (Figure 1).

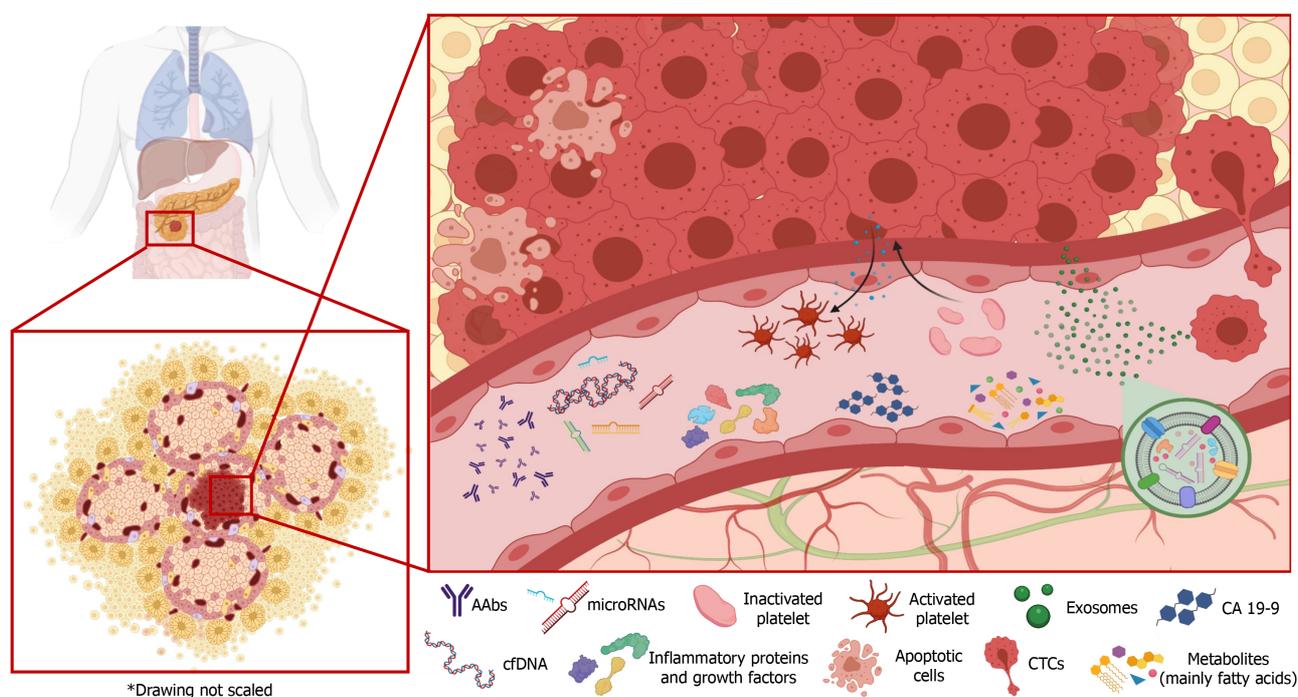


Figure 1 Analytes and biomarkers encompassed in liquid biopsy. Different types of tumor components can be detected in the peripheral blood of patients with pancreatic cancer. As mentioned in this review, these tumor analytes include circulating tumor cells, cell-free nucleic acids, proteins, metabolites, exosomes, and tumor-educated platelets. In general, these tumor-derived analytes are masked by a large amount of non-cancerous analytes shed by normal cells, which makes it difficult to isolate and detect the fraction that comes specifically from the tumor. In this scenario, the development of improved sensitive technologies has driven interest in this field allowing the use of liquid biopsies as a minimally invasive source of neoplastic material for molecular analysis. Each analyte has its own advantages and disadvantages and will provide different information. Depending on the type of study, the use of one or the other may be more interesting. In some cases, their mere isolation may already be informative or they may be used as a starting material for the detection of biomarkers. In general, the combination of several analytes improves the predictive capacity of each of them individually. Created with BioRender.com. AAbs: Autoantibodies; cfDNA: cell-free DNA; CTCs: Circulating tumor cells. "Created with BioRender.com".

CARBOHYDRATE ANTIGEN 19-9, OTHER CARBOHYDRATE MARKERS, AND CARCINOEMBRYONIC ANTIGEN

Carbohydrate antigen (CA) 19-9, an epitope of sialylated Lewis antigen, appears elevated in PC[10], and there have been numerous studies on its usefulness for screening, diagnostic, prognostic, and predictive purposes and for assessing resectability[11]. It has been documented as an early detector of PC[12], and it is the only biomarker approved for monitoring its progression and the therapeutic response[13]. However, some shortcomings have been reported, including poor sensitivity (69%-98%) and specificity (46%-98%) for PC diagnosis[14]. Furthermore, it is not tumor-specific, given that elevated concentrations of CA 19-9 have also been observed in other gastrointestinal malignancies (*e.g.*, colorectal cancer and gastric cancer) and benign diseases (*e.g.*, chronic pancreatitis (CP) and acute cholangitis)[15]. Finally, CA 19-9 is not expressed by around 10% of Caucasian individuals, even those with large PC tumors, because of their possession of the Lewis a-/b- genotype[16].

Other carbohydrate markers that have been related to PC include CA 50, CA 72-4, CA 125, and CA 242. However, these biomarkers have been little used in the clinical management of PC due to their low sensitivity and specificity. CA 242 contains a different sialylated carbohydrate type I chain to that in CA 19-9 and was found to offer lower sensitivity but higher specificity for PC[17]; however, elevated concentrations of CA 242 are found in other types of cancer (*e.g.*, cervical and lymphoma), in type 2 diabetes mellitus, and in coronary heart disease, suggesting an association between this biomarker and the presence of chronic disease in general[18]. CA 125, also known as mucin 16, has the same drawback, being most frequently used in the diagnosis of ovarian cancer[19], and altered CA 125 concentrations have been observed in a wide range of diseases, including heart failure[20], pulmonary disorders[21], deep endometriosis[22], and extrapulmonary tuberculosis[23].

There has been little published research on the clinical relevance of carcinoembryonic antigen (CEA) in PC, which has been widely used to diagnose colon cancer[24] and also appears elevated in around 60% of patients with PC. Its sensitivity to

diagnose PC is only around 37%, and elevated concentrations have been observed in patients with nonspecific colitis and in heavy smokers. For this reason, CEA has been less frequently utilized in comparison to CA 19-9. However, it may be useful to evaluate the effectiveness of treatment and to detect recurrence in patients with a diagnosis of PC[25]. It has been described as a better predictive biomarker than CA 19-9 in metastatic and locally advanced PC[26] but a worse predictive biomarker for survival and early recurrence[27]. Finally, alongside other carbohydrate biomarkers, CEA may be of value in the clinical management of Lewis-negative patients, achieving high specificity (98.0%) for the diagnosis of PC and being related to the metastatic status and response to therapy[28].

None of the above markers are sufficiently accurate when applied alone, and recent studies have focused on their combined use in panels. Notably, Zhang *et al*[29] carried out a meta-analysis on the capacity of CA 19-9, CA 242, and CEA to diagnose PC, selecting 21 studies that included 3497 participants. They obtained higher sensitivity with the combination of CA 19-9 plus CA 242 (89.95%) than with the separate application of CA 19-9 or CA 242.

More recently, Deng *et al*[30] correlated CA 19-9, CA 125, and CEA with multiple clinical factors in PC, relating their baseline levels to the primary tumor site, number of organ metastases, and liver metastases. They reported that the baseline neutrophil count, lactate dehydrogenase enzyme concentration, and CA 19-9 and CA 125 Levels were associated with overall survival (OS) and proposed them as potential prognostic factors.

This combination of CA 19-9, CA 125, and CEA has also been described as useful in the surgical setting. After a study of more than 1000 patients with PC, Xu *et al*[31] proposed the preoperative serum signature of CA 19-9 ≥ 1000 U/mL, CA 125⁺, and CEA⁺ to rule out pancreatotomy, because resected patients with this signature showed no survival advantage over those with locally advanced disease who did not undergo pancreatotomy.

In summary, despite the modest results obtained with CA 19-9 and its poor specificity, it remains the standard against which any new biomarker is compared for the clinical management of PC. Currently, no single carbohydrate marker or combination of markers appears to be useful for this purpose. The main limitations of research to date are the lack of standardization and the absence of validation studies in larger cohorts[32].

METABOLITES

Metabolomics is a powerful technology that analyzes the concentrations of low-molecular-weight molecules, also known as metabolites. An increase in sensitivity, resolution, reproducibility, and coverage has been achieved over the past few years through its combination with improved analytical techniques such as high-resolution mass spectrometry (HRMS) and nuclear magnetic resonance spectroscopy[33]. Metabolomic techniques are increasingly supported as an approach to the discovery of biomarkers in cancer, because they explore an intermediate stage between genotype and phenotype[34]. PC is characterized by a nutrition-deficient and hypoxic condition in which dense connective tissue and poor angiogenesis predominate[35]. In order to adapt to these conditions, PC cells reprogram their metabolism to enable their survival and the maintenance of elevated proliferation rates, through the so-called Warburg effect[36]. These changes lead to the presence of specific metabolites in the serum of patients with PC, and these can serve as biomarkers.

A wide range of studies have proposed the usefulness of metabolomics, in a targeted or untargeted approach, for the discovery of novel biomarkers for PC. Our research group recently demonstrated the potential of untargeted metabolomics by liquid chromatography coupled to HRMS as a diagnostic tool for PC. We proposed a multivariate model based on nine metabolites that discriminated between PC patients and healthy controls with an area under the ROC curve (AUC) of 0.992; all 60 healthy control samples were correctly classified, and only 4 of the 59 PC samples were misclassified[37].

In a similar study, comparing between 60 patients with PC and 60 healthy controls, Luo *et al*[38] proposed a panel of five differentially-expressed metabolites for the diagnosis of PC (β -sitosterol, sphinganine, creatine, inosine, and glycocholic acid). In comparison with the conventional biomarkers CA 125, CA 19-9, CA 242, and CEA, their panel proved more accurate and specific to diagnose PC. The same authors also proposed two more plasma biomarkers (succinic acid and gluconic acid) for the

diagnosis of PC progression and metastasis. They claimed that their novel biomarker panel could diagnose PC at an early stage; however, out of the 60 PC patients in their study, 24 were already in stage III/IV.

Recently, however, Moore *et al*[39] were critical of studies that compare patients in different stages of PC with healthy volunteers, describing this approach as inadequate to identify useful biomarkers for screening the general population. They described metabolomics as a useful tool to differentiate PC stages, which do not follow a linear stepwise progression. They applied this method in plasma samples from patients with neuroendocrine, intraductal papillary mucinous neoplasm (IPMN), localized PC, locally advanced PC, or metastatic PC. They described various combinations of metabolites associated with distinct PC stages, thereby facilitating differentiation between early and late PC, the follow-up of patients with pancreatic lesions, and the identification, in combination with CA 19-9 Levels, of patients with advanced disease who are candidates for resection.

Geiser *et al*[40] also applied metabolomics in a study of the progression from pancreatic cystic neoplasm (PCN) to PC. They proposed the combination of preoperative metabolomics with perioperative lipidomic analysis in plasma and cyst fluid from patients to support the differential diagnosis of PCN. Their integrated data modeling allowed them to discriminate between IPMN and serous cystic neoplasm with 100% accuracy and to discriminate between IPMN, and invasive cancer with an accuracy of 90.06%.

In a different approach, Zhang *et al*[41] studied alterations that may be discriminative for PC in 550 serum samples, analyzing the preoperative and postoperative metabolome in training cohorts. In combination with the clinical data of the patient, three discriminative metabolites (docosahexaenoic acid, lysoPC (14 : 0), and histidinyllysine) proved to be independent predictors of PC diagnosis. They found that the concentrations of these metabolites in postoperative patients were closer to those in healthy controls than in the paired preoperative PC group, and they obtained > 97% accuracy for this panel to differentiate between PC patients and healthy controls in an independent validation analysis.

Mayerle *et al*[42] undertook one of the largest metabolomic investigations in PC in order to obtain a metabolic profile that allowed PC to be distinguished from CP. In three consecutive studies, they enrolled 914 subjects in four groups (PC, CP, liver cirrhosis, healthy, and non-pancreatic disease) and were able to identify a biomarker signature composed of nine metabolites and CA 19-9. The authors stated that the clinical use of this panel would have improved the diagnosis and treatment stratification of PC in one-third of their patients in comparison to CA 19-9 alone, achieving an AUC of 0.94, with a sensitivity of 89.9% and specificity of 91.3% to distinguish between PC and CP.

Another study of major clinical interest demonstrated the value of serum metabolites in the differential diagnosis between PC and distal cholangiocarcinoma, which share many symptoms, are not easily distinguishable by imaging techniques, and require distinct management approaches. In this study, Macias *et al*[43] developed a logistic regression model based on a panel of nine metabolites and CA 19-9 that achieved an AUC value of 0.89 to discriminate between patients with these diseases.

The serum metabolome has also proven useful to predict recurrence in patients with resected PC. Rho *et al*[44] studied the preoperative serum metabolome of 57 patients undergoing pancreatectomy and developed a nomogram based on CA 19-9 alongside three metabolites, which showed a Harrell's concordance index of 0.823 and an AUC of 0.816 to predict 6 mo and 1-year cancer recurrence-free survival after surgery.

In summary, because the metabolome is the closest level to the phenotype, metabolomics is attracting increasing research interest as a promising technology to discover potential biomarkers for cancer diagnosis and for monitoring the metastatic status and therapeutic response of patients. However, most metabolomic studies have not been on a sufficiently large scale to confirm their clinical value[45]. Besides, a wide variety of analytical and clinical methodologies have been applied in metabolomic studies, limiting the reliability of their results. Efforts are currently focused on creating a database to gather data and protocols from these studies, including different biological samples and using different analytic techniques and various platforms[34].

CIRCULATING TUMOR CELLS

Circulating tumor cells (CTCs) are released by a tumor into the blood before it is detectable. The study of CTCs has major potential for the diagnosis and prognosis of

cancer in its earliest stages[46], and their levels have also been related to tumor aggressiveness and metastasization[47]. However, despite the clinical relevance of CTCs and abundant research on their role in other types of tumor, fewer data are available on their relation to PC[48].

Hugenschmidt *et al*[49] evaluated 242 preoperative samples from resectable PC patients and detected CTCs in 6.8% of high-risk patients and in 6.2% of patients with advanced disease, observing no CTCs in low-risk patients or in those with benign disease. The multivariate analysis showed that CTC status remained an independent prognostic factor after controlling for histological type, nodal status, and vascular infiltration. A worse outcome was observed in preoperative CTC-positive patients despite a successful tumor resection, and the authors proposed this methodology to guide treatment decisions in patients with PC.

Court *et al*[50] investigated the usefulness of CTCs as a preoperative biomarker to identify patients at high risk of occult metastatic PC. They identified CTCs in 78 of the 100 patients with PC but in none of the 26 with benign disease. The CTC count was also found to be correlated with higher PC stage and was described as a promising prognostic biomarker. In a prospective study of the capacity of CTCs to predict metastatic spread and survival, Effenberger *et al*[51] observed CTCs in 33.3% of a sample of 69 patients with PC, and reported significant progression-free survival (PFS) and OS in CTC-positive patients. The PFS was also significantly reduced in CTC-positive patients receiving chemotherapy, and CTC status was found to influence the outcome of patients with PC independently of other risk factors.

Further research is required not only to develop novel and more reliable technologies for CTC detection but also to identify new cellular markers for the accurate isolation of CTCs in patients with PC. Varillas *et al*[52] proposed a new microfluidic platform for the reliable isolation of CTCs and cancer stem cells (CSCs) in samples from these patients. It was tested in 24 patients with metastatic PC undergoing treatment, collecting 78 blood samples at different time points and finding that 84.4% were positive for CTCs and 70.8% for CSCs. The authors described the complementary assessment of CTCs and CSCs as useful to evaluate the response to treatment and guide therapeutic modifications.

Cell-surface vimentin was recently proposed by Wei *et al*[53] as a biomarker to isolate CTCs in patients with PC. They found that vimentin was highly expressed on the surface of mesenchymal-phenotype PC cells and detected both vimentin and CTCs in 76% of the patients. The authors obtained an AUC of 0.97 for the diagnosis of PC by combining vimentin and CTCs with CA 19-9. Vimentin + CTC counts were correlated with the change in tumor burden in patients undergoing resection, and significantly reduced CTC counts were observed after chemotherapy in responders to this treatment. Finally, higher CTC counts were correlated with a reduced recurrence-free survival period.

In conclusion, the study of CTCs appears to be a promising approach to the clinical management of PC, but a number of challenges remain. Relatively few studies have been performed, and most of them are at an experimental stage, using varied methodologies and small cohorts of patients. There is a need to develop a consensus on methodologies and protocols and to study larger cohorts in order to confirm the usefulness of CTCs as diagnostic markers for PC and as indicators of the prognosis and response to treatment, for which better results have been obtained[54].

CIRCULATING CELL-FREE DNA

Cell-free nucleic acids (cfNA) are nucleic acids (nuclear DNA, mitochondrial DNA, and various types of RNA molecule) present in biological fluids and independent of cells[55]. The cellular source of tumor-derived circulating nucleic acids remains controversial. Although most cell-free DNA (cfDNA) are derived from normal cells of the hematopoietic lineage[56], a small proportion has been related to tumors (primary or metastatic tumors or CTCs undergoing necrosis or apoptosis) and is designated circulating tumor DNA (ctDNA)[57]. Higher rates of necrosis and apoptosis during tumorigenesis have been associated with increased cfDNA concentrations. cfDNA concentrations reach a maximum of 100 ng/mL of blood in healthy individuals, but can be 4- fold to 40-fold higher in cancer patients[58]. cfDNA fragmentation follows a specific pattern of nucleosome distribution characteristic of caspase-dependent cleavage during apoptosis. Longer DNA fragments are considered to result from necrotic cell death[59]. However, there are subtle variations in the size of cfDNA depending on the method of isolation and on the tumor type and stage[60]. In this

way, cfDNA fragment size was reported to have prognostic value for patients with advanced PC, with an association between shorter cfDNA fragment size and worse OS [61]. cfDNA can also be released *via* NETosis, a unique form of cell death. Upon activation, neutrophils play a role in defense mechanisms by inducing phagocytosis and degranulation and releasing neutrophil extracellular traps (NETs) in response to inflammation, infection, or hypoxia. NETs are web-like networks of decondensed nuclear DNA with histones, granule proteins (*e.g.*, myeloperoxidase), and antimicrobial peptides [62]. The contribution of NETosis to the pool of cfDNA in PC is yet to be explored and may have therapeutic potential. This because PC is associated with one of the highest rates of venous thromboembolism (VTE) among cancers, significantly increasing the mortality [63], and neutrophils contribute to VTE in part by releasing NETs [64]. Administration of DNase I to degrade cfDNA (including NETs) was found to reduce venous thrombosis in tumor-bearing mice [65].

The presence of cfDNA in human blood was first described by Mandel and Métais in 1940 [66]. Since then, researchers have compared mutations in tissue with modifications in soluble cfDNA. Mutant *K-RAS* has been studied in depth as one of the key driver genes in PC. A longitudinal study of PC patients undergoing surgery/chemotherapy found that pre-therapeutic levels or early changes in mutant circulating *K-ras* levels predicted the response to treatment of patients with advanced PC receiving chemotherapy [67]. They observed an improved prognosis when no *K-ras* ctDNA was detected within one year post-surgery, describing an improved therapeutic response to first-line chemotherapy when *K-ras* ctDNA levels were absent or reduced at six months of treatment [68]. Accordingly, changes in *K-ras* ctDNA status can yield critical information for predicting the response to treatment.

ctDNA not only contains the same mutations as tumor cells but also shows the same epigenetic patterns (DNA methylation, histone modification, and chromatin remodeling), which have been associated with gene expression and tumor phenotypes [69]. One of the most widely studied epigenetic modifications is DNA methylation, *i.e.*, the covalent addition of a methyl group to cytosines by DNA methyltransferases, generating 5-methylcytosine (5mC). Methylation commonly translates into repression of gene transcription. Conversely, oxidation of 5mC by ten-eleven translocation protein produces DNA demethylation (5hmC), which is generally associated with transcriptional activation [70]. Hence, changes in DNA methylation patterns play an important role in cancer, leading to the silencing of tumor suppressor genes or activation of oncogenes. Patients with PC have higher levels of hypermethylated genes in their cfDNA. A method based on methyl-CpG (cytosine nucleotide followed by a guanine nucleotide) binding (MBD) protein coupled with digital PCR was recently used to develop a panel of five DNA methylation markers (*ADAMTS2*, *HOXA1*, *PCDH10*, *SEMA5A*, and *SPSB4*) identified in fine-needle aspiration samples and paired blood samples. PC patients with methylation markers and/or *KRAS* mutation in cfDNA had a higher frequency of liver metastases, indicating a worse prognosis [71]. Methylated DNA immunoprecipitation coupled with high-throughput sequencing (MeDIP-seq) has been applied as an alternative method for the genome-wide detection of cfDNA methylation profiling. Li *et al.* [72] used this technique to compare differentially methylated regions of cfDNA in PC patients and healthy controls and constructed a diagnostic prediction model that included eight differentially methylated markers (*MAPT*, *SIX3*, *MIR663*, *EPB41L3*, *FAM150A*, *TRIM73*, *LOC100128977*, and *LOC100130148*) for potential application in the non-invasive diagnosis of PC.

Global DNA hypomethylation and a reduction in 5hmC levels are also frequently observed in cancer. A different hydroxymethyl profile was recently described in PC patients in comparison to cancer-free individuals. Genes with the most altered hydroxymethylated profile were previously implicated in pancreas development (*GATA4*, *GATA6*, *PROX1*, *ONECUT1*, and *MEIS2*) or PC (*YAP1*, *TEAD*, *PROX1*, *ONECUT2*, *ONECUT1*, *IGF1*, *NFAT*, and *FOXA*). This epigenetic fingerprint of hydroxymethylated genes proved capable of differentiating between PC and healthy samples [73]. As previously commented, the individual contribution of a single gene may not be able to capture the complex biology of this disease. The combination of 5mC and 5hmC profiling improved the diagnostic power, especially the predictive sensitivity in early-stage PC samples [74]. Very recently, a new blood test named *Cancerseek* addressed this issue by combining protein biomarkers with genetic biomarkers to increase the sensitivity; they were able not only to identify the presence of relatively early cancers but also to localize the organ of origin of eight common solid tumor types, including PC [75].

The utilization of epigenetic modifications has advantages over techniques based on genetic differences. The cfDNA sequence is identical to that of the genomic DNA, making it impossible to identify the tissue of origin. In contrast, the DNA methylation profile is highly tissue-specific, allowing organ-specific disease to be monitored. In addition, epigenetic modifications show a greater consistency in cancer in comparison to genetic changes[69].

In summary, cfDNA can be detected at relatively early stages of tumor development and is therefore useful for the early diagnosis of cancer, especially in occult organs such as the pancreas[76]. Given the short lifespan of cfDNA, ctDNA can be considered a more applicable, sensitive, and specific biomarker not only to diagnose cancer but also to monitor the tumor and response to therapy. Detection of ctDNA variants before and after anti-cancer therapy can yield information about the effectiveness of treatments and about tumor dynamics during treatment, facilitating the design of individualized treatments[77]. One cfDNA-based liquid biopsy test has been approved by the U.S. Food and Drug Administration to detect epidermal growth factor receptor (EGFR) mutations in the ctDNA of patients with non-small cell lung cancer who are candidates for targeted therapy with erlotinib and osimertinib[78]. Sixteen liquid biopsy-based clinical trials on diagnostic biomarkers for PC are under way, combining cfDNA analyses in blood and other body fluids such as intra-cystic fluid.

CIRCULATING MICRORNA

Another cfNA subset, composed of circulating microRNAs (miRNA), has recently become a new approach to liquid biopsy. miRNAs are short (19–24 nucleotides in length) non-coding RNAs that regulate messenger RNA (mRNA) or protein levels by promoting mRNA degradation or decreasing protein translation through an improper binding at 3-untranslated region. miRNAs can be synthesized by virtually all of the cells in the body. Many of them are ubiquitously expressed but others are tissue-specific, depending on the transcriptional and post-transcriptional regulation of miRNA precursors within the cell[79]. miRNAs can be released into the extracellular circulation and blood by passive or active secretion. Some miRNAs have been found packaged in exosomes derived from multivesicular bodies (see next section). Others may be exported in the presence of RNA-binding proteins, which give stability to the RNA molecule and make it more resistant to RNase-mediated degradation. This stability represents a major advantage for the handling and processing of samples, because miRNA can resist degradation at room temperature for up to 4 d, during boiling and multiple freeze-thaw cycles, and at high or low pH values. Whether the miRNA is predominantly exosomal or vesicle-free depends on the miRNA itself, the cell type from which it originates, and/or other factors affecting miRNA secretion in individuals[80].

Interestingly, miRNAs of body fluids tend to be positively correlated with various human tissues[81]. Expression patterns of miRNAs (miRNA signatures) are unique to individual tissues and differ between cancer and normal tissues. Some miRNAs are overexpressed or downregulated exclusively or preferentially in certain cancer types. The fact that miRNAs are present in various body fluids, are stable, and may reflect the pathophysiologic condition of the tissue of origin has attracted interest in them as a promising group of biomarkers. It has been reported that miRNAs can be detected in the serum of cancer patients decades before clinical manifestation of their malignancy [82]. Besides serving as cancer biomarkers, tumor-derived miRNAs can act as intercellular signaling molecules modulating non-tumor cells to the benefit of the tumor[83]. miRNAs play a major role in carcinogenesis as either tumor suppressor miRNAs or oncogenic miRNAs (also termed oncomiRs). Deregulation of any miRNA type contributes to the development of tumors. Several miRNAs are known to be tumor suppressors, and their downregulation is implicated in the initiation and progression of PC. Wang *et al*[84] selected a panel of four miRNAs known to be overexpressed in PCs (miR-21, miR-210, miR-155, and miR-196a) in order to evaluate their usefulness as blood-based biomarkers of the disease. They found that miR-21 inhibition reduces proliferation, migration, and invasion and delays the progression of pancreatic intraepithelial neoplasia (PanIN) to PC in mouse models[85].

Vila-Navarro *et al*[86] very recently identified a panel of 14 circulating miRNAs (let-7e-5p, let-7f-5p, miR-103a-3p, miR-151a-5p, miR-151b, miR-16-5p, miR-181a-5p, miR-192-5p, miR-21-5p, miR-221-3p, miR-23a-3p, miR-320a, miR-33a-3p, and miR-93-5p) that are significantly overexpressed in the plasma of patients with PC and IPMN in comparison to healthy individuals. They described them as potential noninvasive

biomarkers to indicate a neoplastic process in the pancreas. Two miRNAs, miR-181b-5p and miR-548d-3p, were significantly upregulated in PC plasma but not in pre-malignant IPMNs and therefore represent biomarkers of the malignant transformation of IPMNs. It is also very important in the clinical setting to discriminate between CP and other pancreatic diseases such as neoplasia and cancer. Consequently, some researchers have focused on miRNA profiles for their differential diagnosis. Cao *et al* [87] established a panel of three miRNAs (miR-486-5p, miR-126-3p, and miR-106b-3p) that demonstrated high accuracy to distinguish between PC and CP, showing an AUC value of 0.891 and higher diagnosis accuracy in comparison to CA19-9 (AUC = 0.775). Another study also reported that miR-486-5p yielded diagnostic values comparable to CA19-9 for differentiating patients with PC from healthy controls and patients with CP [88].

Mazza *et al*[89] studied three miRNAs overexpressed in patients with PC (miR-122-5p, miR-1273g-3p, and miR-6126). They obtained greater power to distinguish PC patients from healthy individuals by using plasma miR-1273g-3p levels in combination with CA 19-9 than by considering CA 19-9 alone, achieving a gain in sensitivity and negative predictive value and, therefore, a low false-negative rate. A role was proposed for these miRNAs in predicting the clinical outcomes of PC patients. Thus, higher miR-1273g-3p levels were associated with a more advanced tumor stage, and increased miR-122-5p expression emerged as an independent negative prognostic factor. In another study, the expression of miR-181b, miR-196a, and miR-210 was significantly upregulated in PC patients, and the diagnostic value for PC of each individual miRNA was increased when combined with CA19-9[90]. This panel was also associated with the prognosis, given that the expression of miR-181b, miR-196a, and miR-210 was correlated with lymph node metastasis, clinical stage, and vascular invasion. In another study, a panel of miR-99a-5p, miR-200c-3p, and miR-365a-3p proved able to discriminate PC patients with a poor post-resection outcome from those with longer survival[91].

Gemcitabine resistance has also been associated with a modulation in miRNA expression. MiR-125a-3p is a tumor suppressor found to be downregulated in plasma samples from patients with PC[92]. *In vitro* studies showed that miR-125a-3p can directly inhibit the expression of Fyn, thereby promoting the epithelial-to-mesenchymal process, and that Fyn overexpression can partially reverse miR-125a-3p-mediated chemoresistance to gemcitabine[93]. Numerous miRNAs have been associated with resistance to chemotherapy; however, most results have been obtained in *in vitro* studies, and the potential role of these miRNAs as blood-based prognostic biomarkers has yet to be validated.

As stated above, PC cannot be considered as a single disease but rather a combination of different subtypes. Kandimalla *et al*[94] identified a panel of nine miRNAs that were significantly upregulated (miR-205-5p and -934) or downregulated (miR-192-5p, 194-5p, 194-3p, 215-5p, 375-3p, 552-3p, and 1251-5p) in PC molecular (squamous and quasi-mesenchymal) subtypes associated with poor survival outcomes. They first analyzed the International Cancer Consortium dataset and The Cancer Genome Atlas dataset to identify nine tissue miRNAs specifically deregulated in both PC subtypes and observed that six of them had similar expression profiles in serum specimens. The five-year OS rate was higher in PC patients with high-risk scores for these miRNAs in serum than in those with low-risk scores. These findings confirm the usefulness of this miRNA profile to identify poor molecular subtypes and high-risk PC patients and to predict their prognosis.

It was also demonstrated that a panel of seven miRNAs (miR-486-5p, miR-106b-5p, let-7i-5p, let-7g-5p, miR-144-3p, miR-19a-3p, and miR-103a-3p) could estimate the risk of future VTE in patients with PC at their diagnosis. These miRNAs are implicated in the PC pathway and in complement and coagulation cascades[95].

Bioinformatic tools allow the integration of different PC-related serum miRNA expression profiles to determine the most relevant miRNA signatures. In this way, Shams *et al*[92] constructed a novel miRNA-mRNA regulatory network for PC. They proposed a panel of downregulated miRNAs (miR-125b-1-3p, miR-125a-3p, miR-92a-5p, miR-4530, miR-6893-5p, and miR-4476) and found that a combination of miR-125a-3p, miR-92a-5p, and miR-4530 was the most promising panel to differentiate PC patients from healthy controls, with an AUC of 0.95, sensitivity of 0.98, and specificity of 0.97. Among other upregulated miRNAs (miR-1469, miR-1246, miR-5100, miR-8073, miR-642b-3p, and miR-663a), miR-1469 was found to be the most powerful individual marker for PC diagnosis.

Five of the aforementioned genes (miR-125a-3p, miR-6893-5p, miR-125b-1-3p, miR-1469, and miR-4476) were also selected by Yan *et al*[96] for inclusion in a model with another eight miRNAs (miR-6075, miR-6836-3, miR-6729-5, miR-575, miR-204-3, miR-

6820-5, miR-4294, and miR-4792), and this 13-miRNA panel accurately differentiated between PC patients and healthy individuals. However, none of these models have been clinically validated, and further research is needed before their application can be recommended.

Most miRNAs that have been identified as cancer biomarkers have failed to advance from the experimental stage to a clinical trial. This is largely attributable to the wide variability of results and the lack of reproducibility. Further standardization and technological improvements are needed before miRNAs can be used to obtain an accurate and reliable cancer diagnosis in the clinical setting[97]. In this regard, elucidation of the miRNA expression profile of individual tumors could improve diagnostic accuracy, treatment selection, and prognosis prediction.

EXOSOMES

Virtually all types of cells are able to release phospholipid bilayer-enclosed extracellular vesicles (EVs). Their diameter ranges from 30 nm to 5000 nm, and they are mostly classified as exosomes (30–120 nm in diameter), microvesicles (MVs, also known as ectosomes, or microparticles, 100–1000 nm), or apoptotic bodies (ranging from 800 to 5000 nm)[98]. Beyond their role in the elimination of cellular waste, EVs are potent vehicles of cell-to-cell communication. They transport all types of biomolecule (proteins, lipids, and nucleic acids) under both physiological and pathological conditions. EVs can use their specific cargo to modulate the physiology of recipient cells, including processes that are deregulated in human cancers (*e.g.*, proliferation, angiogenesis, and apoptosis)[99]. Encapsulation of the cargo in a membrane gives it a higher stability, longer half-life, greater resistance to degradation, and an increased capacity to travel long distances in comparison to free proteins, lipids, and nucleic acids in the cytoplasm. Moreover, this specific cargo provides a rich source of biomarkers that can be found in all body fluids[100]. EVs are formed by distinct mechanisms. MV biogenesis occurs through direct outward blebbing and pinching of the plasma membrane, releasing the nascent MV into the extracellular space. Exosomes originate from inward blebbing and budding (invagination) of the plasma membrane to form the early endosome that forms the late endosome after a series of modifications, and is secreted upon fusion with the cell surface[101]. Ultimately, the different particles are differentiated by their size, biogenesis, surface markers, and cargo content.

PC is characterized by a complex microenvironment with constant interaction between PC cells and stromal cells such as fibroblasts, myofibroblasts, stellate cells, vascular endothelial cells, and immune cells, which are all surrounded by an abundant fibrotic extracellular matrix that serves as a shield[102]. These interactions are decisive steps in tumor progression and are at least in part mediated by EVs[103], giving exosomes a key role in PC.

The cargos of exosomes largely comprise proteins, RNAs, and lipids (ExoCarta-Exosome dataset), and these molecules can all be used as diagnostic, predictive, and/or prognostic markers for PC. Table 1 summarizes the latest panels described in the literature regarding exosome's cargo[104–113]. The fact that almost all cells can release exosomes into the peripheral circulation is one of the major drawbacks of their utilization as tumor surrogates. The dilution of tumor exosomes with non-cancer cell-derived exosomes means that blood samples must be enriched in tumor-specific exosomes. Some of the surface proteins described in the exosomes have been used for that purpose[114]. One of the most studied components of the exosomes are the miRNA, but there are other types of RNA molecules that have been explored as biomarkers for PC. Interestingly, while extracellular circulating levels of some miRNA did not differ when comparing PC patients and healthy individuals, exo-miRNAs did. Consequently, some researchers have pointed to miRNA encapsulated in exosomes as a more useful source of biomarkers[115]. Although other biofluids are sources of exosomes, authors advised against evaluating pancreatic juice from patients with IPMN, because exosomes could not be reliably extracted due to the high viscosity of the mucin content[116]. Although most of the studies have focused on exo-miRNAs profiling, whole transcriptome analysis has identified novel disease biomarkers. Besides, very recently circular RNA (circRNA) has been recently recognized as a novel class of highly stable noncoding RNA species that is abundant in exosomes and that are generated from the ligation of exons, introns, or both. CircRNAs can function by binding to miRNAs as sponges and suppress miRNA activity. As miRNAs are known to alter the development and progression of cancer, circRNAs may offer a novel

Table 1 Recent exosomal cargos described in diagnosis/prognosis of pancreatic cancer

Proteins	Source	Outcome	Utility	Ref.
CLDN4, EPCAM, CD151, LGALS3BP, HIST2H2BE, and HIST2H2BF	Blood	Higher KRAS mutation in exosomes	Protein panel PC exosome-enriched samples	Castillo <i>et al</i> [114]
TAAAs	Plasma	Higher levels of circulating Ig-bound exosomes in PC patients than in healthy individuals	Circulating AAbs against TAAAs to discern between PC, CHP and healthy individuals	Capello <i>et al</i> [104]
CEACAM 1/5 and tenascin C	Pancreatic duct fluid	Upregulation in PC	Distinguish patients with PC/IPMN or CHP	Zheng <i>et al</i> [105]
RNA				
exo-miR155	Plasma Tissue	GEM-resistant cells secreted higher levels of exo-miR-155 miR-155 expression level induced exosome secretion	Predict resistance to Gemcitabine. Novel therapeutic target	Mikamori <i>et al</i> [106]
exo-miR-10b, miR-21, miR-30c, miR-181a and miR-let7a	Serum	Upregulation in exo-miR-10b, miR-21, miR-30c, miR-181a; Repression in miR-let7a	Distinguish patients with CP/CHP or other pancreatic disease; PC resection monitoring	Lai <i>et al</i> [107]
exo-miR-451a	Serum	Upregulation in stage II PC with recurrence after surgery	Biomarkerofrecurrence	Takahasi <i>et al</i> [108]
exo-miR-191, miR-21 and miR-451	Serum	Dysregulated in IPMN and PC patients	Diagnosticbiomarkers	Goto <i>et al</i> [115]
exo-miR-21 and exo-miR-155	Pancreatic juice	Upregulation in PC patients compared with CHP	Diagnostic of early-stage PC	Nakamura <i>et al</i> [116]
miR-4525, miR-451a, and miR-21	PVB	Upregulation of the panel	Identify patients at high risk for recurrence and poor survival after PC resection	Kawamura <i>et al</i> [109]
mRNA				
MMP8, TBX3, PDX1, CTSL, SIGLEC15, IL32, SIGLEC11, DCN, HOXA5, KLRB1	Serum	MMP8, TBX3, PDX1, CTSL, SIGLEC15 overexpression in PC/IPMN <i>vs</i> healthy; IL32, SIGLEC11, DCN repression in PC <i>vs</i> IPMN and healthy; HOXA5, KLRB1 repression in PC/IPMN <i>vs</i> healthy	Identify different subtypes	Kumar <i>et al</i> [110]
CCDC88A, ARF6, VAV3, and WASF2	Serum	Upregulation between PC and control patients	PC risk and PC diagnosis	Kitagawa <i>et al</i> [111]
circRNA				
circPDE8A; circIARS	Tumor tissues Plasma	Upregulation in PC	Predictlivermetastasis, vascular invasion, and tumor-node-metastasis (TNM) stage and por postoperativesurvival time	Li <i>et al</i> [112]
circPC	Tumor tissues Serum	Upregulation in PC	Diagnostic biomarker	Seimiya <i>et al</i> [113]

PC: Pancreatic cancer; TAAAs: Tumor-associated antigens; AAbs: Autoantibodies; CHP: Chronic pancreatitis; IPMN: Intraductal papillary mucinous neoplasm; GEM: Gemcitabine; PVB: Portal vein blood.

diagnostic and prognostic biomarker for cancer[117]. Most of the research carried out analyzing circRNA have been performed in tissue samples[118] and their potential as blood-based biomarkers for PC is unexplored.

Based on their role as biomolecule carriers and because they are non-immunogenic particles, exosomes have been proposed as new disease therapy strategies not without complications and challenges mainly regarding regulatory production guidelines, production of sufficient number of safe, high quality and efficient exosomes. A better understanding of the molecular and cellular processes regulating exosome biogenesis is expected to increase technological advances and potential clinical applications[119, 120]. Furthermore, the pathways on which any therapeutic miRNA functions would need to be extensively defined, to avoid undesirable off-target effects[121]. A major drawback is that currently there is no state-of-the-art technology to isolate EVs, for either therapeutic application or basic research which can impose lack of reproducibility. Exosome-based therapies are being tested. For PC, a phase I study is being developed by the University of Texas M.D. Anderson Cancer Centre in Houston, Texas (NCT03608631) to deliver KrasG12D siRNA (given the drug loading capacity of

exosomes) to treat metastatic pancreas cancer with KrasG12D mutation. Patients will receive the exosomes intravenously and outcomes will be evaluated over time[119].

INFLAMMATORY AND GROWTH FACTORS

Cytokines participate in intercellular communications and are highly relevant to tumor growth and metastasization[122,123]. They modulate cells, the extracellular matrix, and immune system communications in patients with PC[124-126]. In addition, the overexpression of growth factors such as insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor-alpha and beta (TGF α and TGF β), fibroblast growth factor (FGF), and an elevated expression of their cancer cell surface receptors have been reported in many gastrointestinal cancers, including PC. All of these factors trigger an intracellular signaling cascade that leads to cell proliferation, invasion, and survival. Overexpression of these proliferation factors is generally associated with a poor prognosis[125,127].

Another factor that influences the aggressiveness[125] and resistance to treatment [128-130] of PC is the specific microenvironment of the tumor, where its inflammatory nature is reflected. The PC microenvironment is characterized by hypoxia and an increased desmoplasia[131,132]. The stromal network comprises a large amount of extracellular matrix produced by pancreatic stellate cells (PSCs) and cancer-associated fibroblasts (CAFs). Various types of cell can be found in this matrix, including endothelial cells, tumor-associated macrophages, Th cells, myeloid-derived suppressor cells, dendritic cells, and natural killer cells[133]. These can all release pro-inflammatory cytokines, growth factors, and other local mediators that increase the aggressiveness of the tumor[134], as well as immunosuppressive cytokines[135]. Taken together, these factors and the physical barrier of the stromal network can prevent penetration of PCs by the immune system or treatments[129].

Growth factors reported to regulate desmoplasia and cellular growth in PC include EGF, IGF, FGF, TGF α , TGF β , and hepatocyte growth factor (HGF)[136]. Overexpression of EGFR has been described in 30%-95% of PC cases and is associated with a poor prognosis[137]. Its overexpression is moderate in CP and is not detected in healthy peritumoral tissue, suggesting its involvement in the malignant transformation of pancreatic lesions[138].

IGF and IGF receptor are expressed on pancreatic cells, and their activation has been implicated in cell proliferation, invasion, and survival. The binding proteins (IGFBPs) that can inhibit IGF-1 have also attracted research interest as possible prognostic markers for numerous diseases, including PC[138,139]. IGFBP2 overexpression was observed in PanIN lesions, suggesting its possible usefulness as a marker of early disease[140].

TGF is a key regulator of cell growth and apoptosis in PC[141], and alterations in its signaling have been associated with early and advanced stages of gastrointestinal cancer, especially in PC[142]. Elevated TGF β was not detected in pancreatic lesions by some authors[143], whereas TGF β -1, TGF β -2, and TGF β -3 isoforms were correlated with more advanced cancer PC stage and worse survival outcomes[144].

No association has been found between FGF and PC development, but the FGF receptor (FGF-R) has been correlated with very advanced stages when the tumor size is large. Consequently, the overexpression of FGF-R may be a marker of short-term survival[145].

The HGF/c-MET signaling pathway participates in desmoplasia regulation because it is involved in communications among PSCs[144]. It has been reported that the HGF/c-MET signaling pathway is upregulated in PC. The main producers of HGF are CAFs, promoting the malignant transformation of pancreatic cells[146].

Evidence has recently emerged on the diagnostic potential of pS2 peptide, which belongs to a newly described family of trefoil-shaped growth factors. Its expression was observed in more than 50% of PC cases but was not detected in CP[147].

As previously noted, the relationship of PC with inflammatory processes is well documented[148], and inflammation-associated conditions such as obesity or diabetes are major risk factors for PC[149]. Cytokines are the main inflammatory mediators, and some have been associated with a reduced OS in patients with PC, including interleukin (IL)-6, IL-8, macrophage inhibitory cytokine-1, oncostatin M (OSM), IL-10, IL-11, leukemia inhibitory factor, cardiotrophin-like cytokine, ciliary neurotrophic factor, cardiotrophin-1, neuropoietin, IL-27, and IL-3[150,151]. Serum IL-6 Levels are increased in PC patients and are even higher in those with advanced metastatic PC [150]. In general, elevated cytokine expression has been correlated with cachexia,

performance status, and survival in patients with PC[151]. The immunosuppressive cytokine IL-10 has also been implicated in immune system evasion in cases of PC[152].

Although each individual cytokine is of limited value as a biomarker, panels of cytokines have proven useful to predict a poor prognosis in PC, including the combination of B7-1/CD80, EG-VEGF/PK1, IL-29, NRG1-beta1/HRG1-beta1, and PD-ECGF, which may therefore represent novel therapeutic targets for this disease[153]. A panel composed of FGF-10/KGF-2, I-TAC/CXCL11, OSM, osteoactivin/glycoprotein nonmetastatic melanoma protein B, and SCF has also been proposed as a diagnostic biomarker[154].

AUTOANTIBODIES

Responses of the immune system to cancer, especially the humoral response, have been widely investigated[155,156], and the immune system is known to generate autoantibodies (AABs) against tumor antigens (TA)[157]. Tumor cells produce proteins altered by mutations, misfolding, or post-translational modifications such as glycosylation or simple overexpression. The immune system produces AABs against these new TAs, although the underlying mechanism of action has not been fully elucidated[158]. Considerable research has addressed the question why some patients develop a humoral response against TAs but others do not[159].

AAb production is an early event in tumor development, enhancing their potential value as tumor markers. In addition, they survive for a long time period without degradation and in a higher concentration than antigens TAs, with an amplified signal [160]. The “cancer seroma” has been described as the repertoire of antigens recognized by the humoral immune system[161], and research efforts have focused on the search for AAb or antibody signatures with predictive value as cancer biomarkers when considered alone or in combination with others[160].

As many as 124 AABs have been associated with PC but often in only one study with no replication, and 86% of them offered a sensitivity lower than 50%[158]. Four of these AABs met sensitivity and specificity criteria: anti-CLP peptide, anti-Mesothelin, anti-Ezrin, and anti-ENOA1,2[162,163].

The low prevalence of PC means that biomarkers should be highly specific[164]. It is difficult to properly correlate AABs with PC for diagnostic purposes because of the low immune response to this cancer, attributable to the protection offered by the stromal network and immunosuppressive mechanisms[159-161]. Additional research efforts are therefore warranted to systematize the study of tumor stage and to improve detection methodologies[159].

Other AABs have been studied, including MUC1[165], p53, calreticulin isoform 1 or 2, and Rad51[157]; however, their detection is not always linked to a poor prognosis, as in the case of AABs against MUC-1[165]. Moreover, they are not only detected in PC but also in other types of tumor (*e.g.*, MUC-1, mesothelin, ezrin, ENOA) or in other pancreatic diseases such as CP (*e.g.*, p53, ENO, or ANX), resulting in a low specificity [158].

Some AABs have been related to tumor stage[162], showing higher sensitivity for late stages[158]. For instance, EZRIN, detected in mouse models of PanIN lesions, only meets sensitivity criteria at advanced stages of cancer in humans, although it has been suggested as an early marker[162]. Other AABs associated with early lesions include VCL, PDC&I, hnRNPL, VIM, K2C8, or ANXA[166].

A further concern is the low capacity of AABs to differentiate between PC and other inflammatory diseases, especially CP or autoimmune pancreatitis, which are responsible for a large production of AABs. In this regard, some authors have proposed a panel of PP1R15A, CYP3A5, and WDR45 antigens to discriminate between PC and benign inflammatory disease[164].

Some researchers have suggested that AABs do not yield reliable information on the localization of cancers and may best serve to screen patients for the presence of cancer in general, followed by more specific diagnostic measures when a positive result is obtained[158].

CIRCULATING PLATELETS

Platelets are enucleated cells released as cytoplasmic bodies from megakaryocytes in bone and lung[167]. They have attracted research interest for the diagnosis and follow-up of tumors[168,169] due to the biochemical and physiological alterations undergone

during disease[170]. The short half-life (5-10d) of platelets represents a further advantage, allowing the disease to be monitored in almost real time[171].

It has been reported that interaction between platelets and cancer produces platelet hyperactivity, increasing the number of young platelets in cancer patients and reducing their count during post-treatment remission[172]. Cancer patients are known to be in a state of hypercoagulation and to have altered platelets, increasing the risk of thromboembolic disease. It has been proposed that metastasis, immune evasion, and/or angiogenesis in the tumor environment are favored by relationships among the blood coagulation system, platelet functions, and the spread of cancer through the blood[173].

VTE has been observed in around 60% of patients with PC[173], in part related to tissue factor (TF) expression[174]. TF is expressed during malignant transformation to PC, showing four-fold higher levels in cancer, endothelial, inflammatory, and stromal cells[174]. EVs produced by cancer cells to communicate with endothelial cells also exert pro-thrombotic effects, indirectly inducing the over-activation of platelets and the hypercoagulability state that characterizes PC[175]. Platelets are also activated through interaction with mucins released into the circulation by the tumor[176]. Inhibition of fibrinolysis has also been described, and all of these factors can produce a systemic hypercoagulable state in these patients[177].

A direct relationship has been established between tumor stage and platelet count. Chen *et al*[178] recently described for the first time a relationship between platelet count and the post-treatment survival of patients with PC, correlating the platelet count with the concentration of CA19-9. The prognostic value of the pre-treatment platelet/Lymphocyte ratio (PLR) has also been studied in PC, but the results have not been conclusive[179]. Although some authors related this ratio to a poor prognosis, more recent studies associated a high PLR with a better OS rate[179,180].

The activation of platelets increases their size, and the mean platelet volume (MPV) has been studied as an indicator of their activation[181]. Changes in MPV have been detected in various types of cancer[182], and its increase has been associated with worse survival and the development of liver metastases in patients with PC[183].

In a process of mutual communication, tumor cells transfer biomolecules to platelets, generating so-called tumor-educated platelets (TEPs)[169,170] that have been implicated in the progression and spread of solid tumors. Resulting alterations in the composition of platelets generate a dynamic flow of biomolecule exchange in the tumor microenvironment that can be used as a tumor biomarker[170,184,185]. Platelets physiologically interchange RNA and proteins with other platelets, immune system cells, endothelial cells, and tumor cells[171]. The composition of platelets can change in two ways. On one hand, interactions of platelets with the tumor environment produce changes in RNA maturation (mRNA and miRNA from platelets) and their transduction to proteins[186]. On the other hand, their composition can be modified by the direct transfer of biomolecules through tumor MVs. In this way, miRNAs in platelet-derived MVs can regulate the gene expression of tumor cells that phagocytose them. The mechanisms underlying the bi-directional selection and internalization of vesicles remain unknown[171]. Both of the above mechanisms generate a dynamic flow of biomolecules between platelets and tumor cells that can provide complete and updated information on the tumor in both space and time, given that platelets are renewed after around one week. TEPs are therefore a promising tool for the constant monitoring of cancer, especially for cancers of difficult access such as PC.

As noted above, this interchange of biomolecules involves RNA, miRNA from platelets, and proteins, which are all potential cancer markers. First, the characteristic profiles or signature of tumor RNA can offer specific information on the presence, localization, and molecular features of a cancer[171]. For instance, mRNA onco-signatures in TEPs from PC allowed differentiation between patients with PADC and other types of cancer and even between PC patients with KRAS mutant tumor and those with KRAS wild-type tumor[169]. RNA in TEPs can derive from the tumor itself or from an alteration in RNA processing (*e.g.*, intron retention in mRNA), resulting in RNA patterns that differ from those in normal platelets[187]. With regard to miRNA, which is highly abundant in platelets[188], isomiRs generated by failures in miRNA maturation or modification of its ends are valuable biomarkers of cancer[189]. Finally, platelets contain a large number of proteins with biological functions derived from their synthesis or from the circulation. Data on proteins in platelets from the tumor environment may therefore be useful as cancer markers[186].

In 2015, Best *et al*[169] were the first to use mRNA profiles of TEPs in the diagnosis of cancer. They developed two diagnostic algorithms based on TEP RNA sequencing data that were able to detect the presence of cancer with a sensitivity of 97%, specificity of 94%, and accuracy of 96%. These algorithms also obtained an overall

accuracy of 71% for an organ-specific cancer diagnosis in patients with breast cancer, colorectal cancer, glioblastoma, hepatobiliary cancer, lung cancer, or PC. The detection rate of these algorithms was 50%-62% for PC and 71% for gastric cancer. With the information generated by this study, Zhang *et al*[190] developed an algorithm for the analysis of gene expression profiles in blood. The following 18 genes distinguished the above cancer subtypes and healthy samples: ribosomal protein S20; ribosomal protein lateral stalk subunit P2; T cell receptor beta constant 2; ribosomal protein SA; ribosomal protein S11; ribosomal protein L11; ribosomal protein S16; CD3g molecule; Ras homolog family member H; CD27 molecule; ribosomal protein L27; ribosomal protein L9; ribosomal protein L31; ATM serine/threonine kinase; FAU, ubiquitin-like and ribosomal protein S30 fusion; ribosomal protein L34; and ribosomal protein L6.

Mantini *et al*[186] conducted a wide study designed to integrate and compare miRNA, mRNA, and protein omics data between platelets from patients with PC and from those with benign disease. Interestingly, SPARC protein was downregulated in platelets from PC patients but was negatively correlated with miR-17-3p, miR-29a-3p, miR-22-3p, and miR-221-5p. These miRNAs are associated with the overexpression of classical pathways in PC such as ErbB, PI3K-Akt, and mTOR while SPARC is a negative regulator of proliferation, adhesion, and angiogenesis. In addition, platelets from PC patients contained numerous miRNAs related to mRNA splicing, but the amounts of mature mRNA were low. As a possible explanation, the authors proposed that these miRNAs and proteins were exogenous to the platelets and derived from the tumor.

Analysis of the contents of platelets appears to be a promising tool for the *in-situ* diagnosis of PC and for tracking TEPs in liquid biopsies as a potential biomarker of early-stage PC. However, as in the case of ctDNA, its utilization and accuracy must be optimized to allow determination of the cancer subtype[169].

CONCLUSION

As highlighted throughout this review, liquid biopsies offer major opportunities to improve the screening, treatment guidance, and follow-up of cancer patients in general and patients with PC in particular. This is extremely important for patients with PC because there can be a time lapse of up to 20 years between the first appearance of lesions and development of the disease, providing a wide time window for preventative interventions. Among other advantages, the liquid biopsy is a non-invasive and readily-accessible source of information, and samples can be taken as often as needed to monitor the progress of the patient. Liquid biopsies can be used to obtain an early diagnosis, to estimate the risk of relapse or metastatic progression, and to follow up the therapeutic response in real time, allowing futile treatments to be suspended or avoided. Nevertheless, this approach has some limitations, including an inadequate sensitivity due to a low concentration of biosources such as cfDNA or CTCs in liquid biopsies. A further challenge is posed by the lack of standardization and the absence of broadly accepted standard operating procedures for liquid biopsy evaluation. Different isolation methodologies have yielded sensitivity values ranging between 0.27 and 0.95, giving a pooled sensitivity of only 0.64. The final result is influenced by the matrix (blood or serum), time interval between sample collection and processing, type of tube used, centrifugation parameters, and sample handling conditions, among other factors. This may explain the enormous number of molecular signatures described by different research groups. Considerable research efforts are required to verify the diagnostic and prognostic value of liquid biopsies in PC before their routine clinical implementation can be recommended.

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