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Review article

Liquid biopsy in pancreatic cancer: current advances, limitations, and future perspectives

Silvia Carrara[a,b], Michele Bevere[c], Donatella Delle Cave[d], Manuel Gentiluomo[e], Irene Esposito[f], Claudio Luchini[c,g,*]

[a] Department of Biomedical Sciences, Humanitas University, 20090 Pieve Emanuele, Milan, Italy
[b] IRCCS Humanitas Research Hospital, Department of Gastroenterology, 20089 Rozzano, Milan, Italy
[c] ARC-Net Applied Research on Cancer Center, University of Verona, 37134 Verona, Italy
[d] Institute of Genetics and Biophysics 'Adriano Buzzati-Traverso', National Research Council CNR, 80131 Naples, Italy
[e] Department of Biology, University of Pisa, 56126 Pisa, Italy
[f] Institute of Pathology, Heinrich-Heine University and University Hospital of Düsseldorf, 40225 Düsseldorf, Germany
[g] Department of Diagnostics and Public Health, Section of Pathology, University of Verona, 37134 Verona, Italy

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ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic malignancy and is usually diagnosed at advanced stages. The diagnosis of PDAC is established through pancreatic cytology/tissue biopsy, most commonly obtained under endoscopic ultrasound-guidance. Although this approach is generally safe and accurate for the histological diagnosis, maintaining/obtaining material for additional molecular analysis may be challenging. Thus, there is growing interest in minimally invasive approaches capable of providing molecular information without necessarily requiring tissue sampling. Liquid biopsy (LB) represents a significant advancement in this regard, being a minimally invasive tool for biomarker analysis and with the potential to improve patient management at various levels. The most relevant biomarkers that LB can detect are circulating tumor cells, cell-free DNA, circulating miRNA, and extracellular vesicles. Integrating LB into the clinical workflow may improve the diagnosis, prognosis, and monitoring of the disease, even for patients affected by PDAC and with potential implications on early detection approaches. Despite significant progress, no LB assay is yet validated for routine PDAC management; however, growing evidence suggests that multimodal LB integration may soon reshape diagnostic and surveillance strategies. Here we provide a comprehensive overview on liquid biopsy in pancreatic cancer, presenting its current progress, limitations, and future perspectives.

1. Introduction

Cancer is the second major cause of death worldwide, representing an increasingly central public health problem [1]. Two key factors have been identified for improving patients' prognosis and their chances of survival, namely early detection and appropriate therapies [2]. Currently, the gold standard for cancer diagnostics is still represented by tissue biopsy and/or cytology, with pathologists playing a crucial role in this field [3]. Although this approach can provide a definitive diagnosis of cancer, following existing guidelines of tumor taxonomy, tissue biopsy is an invasive test with potentially harmful effects related to the procedure itself and depending on tumor site. Furthermore, in a non-negligible fraction of cases, it may be difficult to collect and cannot be efficiently used for the longitudinal monitoring of disease progression [3,4].

In this challenging scenario, one of the most recent and promising advances in the field is represented by liquid biopsy (LB). LB is a methodology for collecting patients' samples using a minimally-invasive approach. It is focused on acquiring blood or body secretions for the detection and analysis of circulating tumor cells (CTCs), metabolites, and molecular alterations [3–6]. Typical specimens for LB are peripheral/venous blood and urine [3,4,7]. In those samples, different molecular markers can be detected and analyzed, including CTCs, cell-free DNA (cfDNA), circulating-tumor DNA (ctDNA), circulating-free RNA (cfRNA), exosome/cancer-

* Corresponding author at: Department of Diagnostics and Public Health, Section of Pathology, and ARC-Net Research Center, University of Verona, Piazzale Scuro, 10, 37134, Verona, Italy.
E-mail address: claudio.luchini@univr.it (C. Luchini).

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derived extracellular vesicles (EVs), and tumor-educated platelets [3,4]

Compared to tissue biopsy, the most relevant advantages potentially introduced by LB-based approaches include: i) the non-invasive nature, which may be very useful in selected patients (e.g., fragile patients with multi-morbidities), ii) potential feasibility of its adoption for cancer screening, iii) a more applicable and efficient tool for longitudinal and residual disease monitoring [8–13].

This review aims to provide a general overview of the state-of-the-art landscape of LB in the specific field of pancreatic cancer, and specifically on pancreatic ductal adenocarcinoma (PDAC), the most prevalent and lethal subtype of this malignancy. Furthermore, it discusses and highlights current limitations of LB in the field and what is realistically ready in the next future for clinical translation.

2. PDAC: Biology and current challenges

PDAC arises from the exocrine compartment of the pancreas and develops through well-defined precursor lesions. The most common of them are pancreatic intraepithelial neoplasia (PanIN), intraductal papillary mucinous neoplasms (IPMN), and mucinous cystic neoplasms (MCN), which accumulate genetic and epigenetic alterations over time, evolving from low-grade lesions to lesions with high-grade dysplasia and ultimately to invasive cancer [14–18]. PDAC has a complex biology, resulting from both tumor cell intrinsic factors and extrinsic aspects related to its complex tumor microenvironment (Fig. 1).

A relatively small set of recurrent driver mutations characterizes PDAC cells. They involve the proto-oncogene *KRAS*, which is affected by activating mutations in >90% of cases of PDAC and initiates tumorigenesis by driving MAPK- and PI3K-signaling [19–22]. The tumor-suppressors *TP53, CDKN2A*, and *SMAD4*, which control genome stability, cell cycle, and TGF-β signaling, are frequently inactivated [23]. Together, these "core four" genes shape PDAC's hallmark resistance to apoptosis and growth control. Additional alterations, including those in chromatin remodelers (*ARID1A, KDM6A*), DNA damage repair genes (MMR genes, *BRCA1/2, ATM, PALB2*), and specific gene rearrangements/fusions, define molecular subgroups with therapeutic relevance [24–27]. However, actionable alterations are typically found in the small fraction of PDAC that are *KRAS* wild-type [28,29]. Comprehensive profiling has defined distinct PDAC subtypes: classical and basal-like. These differ in transcriptional programs, prognosis, and therapeutic vulnerabilities, with basal-like tumors being usually chemoresistant and bearing a worse prognosis [30]. "Hybrid" tumors with mixed features are frequent, reflecting intratumoral heterogeneity and dynamic plasticity during tumor evolution and under treatment pressure. These tumors show an intermediate/hybrid molecular and clinical (prognostic) profile [30].

PDAC cells exhibit profound metabolic plasticity. They rely on enhanced glycolysis, glutamine metabolism, and autophagy to survive in hypoxic and nutrient-poor conditions [31,32]. PDAC is notorious for its desmoplastic stroma, composed of cancer-associated fibroblasts, extracellular matrix, immune cells, nerves, and endothelial cells. This microenvironment not only acts as a physical barrier to drug delivery but also actively supports tumor growth, immune evasion, and metabolic reprogramming. Crosstalk between tumor cells and stroma through MAPK, TGF-β, Hedgehog, and inflammatory pathways drives progression and therapy resistance [33–35]. In addition, PDAC is an immunologically "cold" tumor. It suppresses anti-tumor immunity via recruitment of regulatory T cells, myeloid-derived suppressor cells, and tumor-associated macrophages, and through the upregulation of immune checkpoints, such as PD-L1. The lack of effector T-cell infiltration explains the limited success of immunotherapies to date [35]. Early dissemination of cancer cells, even before overt tumor formation, contributes to PDAC's high metastatic potential. Subclonal diversification and selection in response to hypoxia, immune pressure, and therapy drive disease progression. Metastatic niches (liver, peritoneum, lung) are shaped by exosomes, inflammatory signaling, and metabolic adaptation [36].

Intensive scientific efforts have been and are currently being made to decipher the molecular basis of the above-mentioned factors and their complex interactions, which are responsible for the clinical aggressiveness of PDAC. While the main goal remains the identification of vulnerabilities to be exploited for therapeutic purposes, the expanding knowledge of the molecular biology of PDAC has also paved the way for the development of LB-based approaches to tackle all phases of this deadly disease, from early diagnosis to disease monitoring and assessment of therapy response (Table 1).

3. Circulating tumor cells

Interest is growing in minimally invasive approaches to PDAC diagnosis capable of providing molecular information without necessarily requiring tissue sampling. In this context, a critical role is played by CTCs. They are released into the bloodstream from primary or metastatic lesions and offer a valuable, minimally invasive window for investigating PDAC features and its biology (Fig. 2) [37]. Their presence enables real-time monitoring of tumor evolution and therapeutic response, capturing the genotypic and phenotypic heterogeneity that drives disease progression [38]. As intact entities, CTCs may also provide a unique opportunity for research purposes, enabling advanced single-cell multi-omics analyses for an integrative characterization of tumor complexity at the molecular level. Furthermore, recent technological advancements have facilitated the successful *in vitro* and *in vivo* culture and expansion of CTCs, thereby supporting patient-specific investigations of tumor evolution, metastatic potential, and therapeutic susceptibility [39]. Collectively, these developments represent a critical step toward the implementation of precision oncology and the realization of personalized medicine.

Several meta-analytic studies support the prognostic relevance of CTCs in PDAC. Combined analyses across multiple studies report that the presence of CTCs is consistently associated with worse overall survival (OS) and progression-free survival (PFS) compared with CTC-negative status [40]. Of note, CTCs have also been found in the blood of patients with pre-malignant pancreatic lesions, making them a suitable tool for the early detection of neoplastic transformation [41]. Despite such encouraging data, it is important to acknowledge that some biological and technical challenges limit their clinical applicability. While CTCs have been extensively investigated across several malignancies, their biological significance and clinical relevance in PDAC at different disease stages remain indeed incompletely elucidated [42]. In addition, CTCs exhibit significant phenotypic plasticity in PDAC, primarily driven by several biological mechanisms such as epithelial-to-mesenchymal transition (EMT), leading to an extensive reprogramming of gene and protein expression. This biological plasticity influences their release into the blood and challenges the ability of existing detection methods to identify and isolate CTCs. Moreover, methodological heterogeneity across enrichment and detection platforms limits comparability between studies and consensus on clinical levels [8,43]. Currently, significant interest has been generated regarding a platform for CTCs detection, which has recently received Federal Drug Administration (FDA) approval. It is the antibody-based CellSearch system, which can isolate EpCAM- and cytokeratin (CK)-positive epithelial tumor cells, while simultaneously confirming CD45-negativity to exclude leukocytes from the analysis [44–46]. The combination of diagnostic leukapheresis (DLA) with the CellSearch platform increases the adequate blood

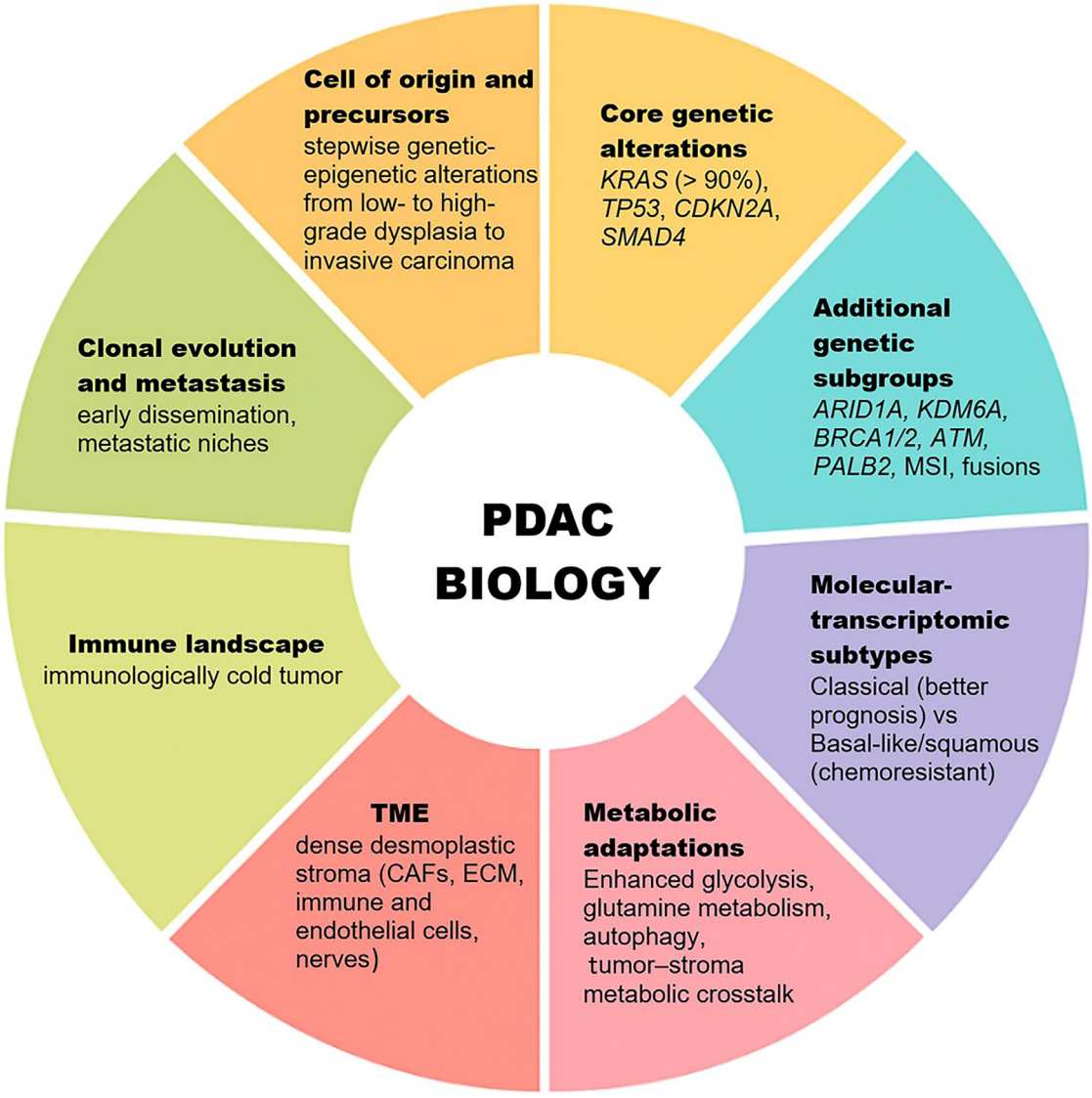

Fig. 1. Key Biological Features of Pancreatic Ductal Adenocarcinoma (PDAC). PanIN: pancreatic intraepithelial neoplasia; IPMN: intraductal papillary mucinous neoplasm; MCN: mucinous cystic neoplasm; MDSCs: myeloid-derived suppressor cells; TAM: tumor-associated macrophages.

Table 1
Summary of advantages and limitations of the different biomarkers identified in liquid biopsy in the field of pancreatic cancer, also considering future perspectives and room for improvement.

Biomarkers	Advantages	Disadvantages	Future perspectives and room for improvement
CTCs	Complete cellular information and functional studies (monitoring of the disease). Prognostic relevance.	Difficult to isolate; loss of cell-membrane molecules after epithelial-to-mesenchymal transition may cause false-negative results.	Development of isolation methods independent of epithelial markers (EpCAM-free platforms) to overcome EMT-related false negatives.
cfDNA/ ctDNA	Useful for detecting single nucleotide variants (e.g., *KRAS*) and for disease monitoring; reflect tumor heterogeneity.	Low levels above all in early-stage of PDAC.	Implementation of ultra-sensitive technologies (e.g., digital droplet PCR) to allow reliable detection in early-stage PDAC.
c-miRNAs	Stable; low-cost analysis.	No standardized workflows, low specificity (possible high levels in non-tumor conditions).	Establishment of standardized extraction and normalization workflows to reduce intra/inter-study variability
EVs	Usually abundant; stable; useful for various multi-omic approaches; high diagnostic potential.	Isolation and characterization remain technically challenging; high inter-laboratory variability and low standardization.	Optimization of scalable and reproducible isolation methods suitable for clinical routine; definition of consensus markers for EV characterization and quality control

Abbreviations: PDAC: pancreatic ductal adenocarcinoma; CTCs: Circulating tumor cells; cfDNA: cell-free DNA; ctDNA: circulating tumor DNA; c-miRNAs: circulating micro-RNA; EVs: extracellular vesicles.

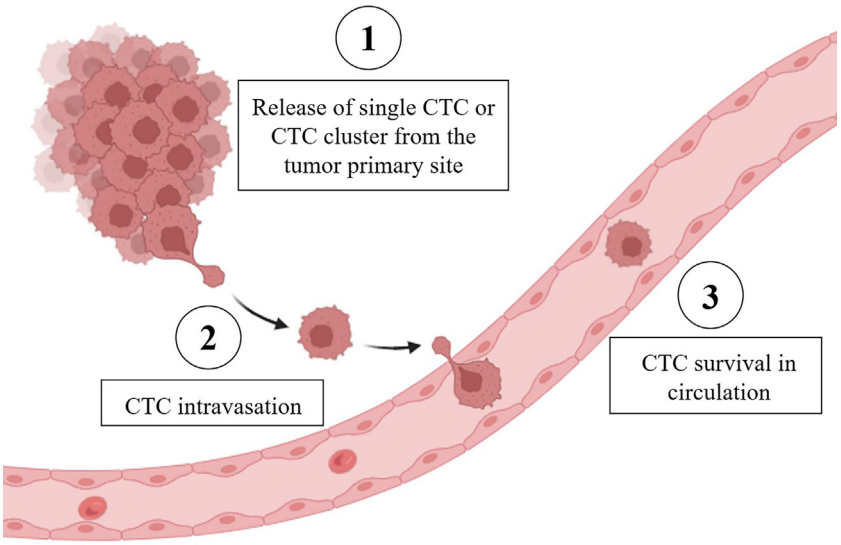

Fig. 2. Summarizing representation of circulating tumor cells (CTC) and their modality of entering blood circulation.

volume that can be analyzed [47], enhancing CTCs detection in both non-metastatic and metastatic PDAC. This approach also enables downstream molecular analysis.

Other significant studies and research efforts have been published in the literature and are summarized in Supplementary Table 1. Along this line, using a combined approach involving magnetic–activated cell separation (MACS) based on anti–CK7/8 and anti–EpCAM microbeads, followed by anti–CD45/DAPI staining, Effenberg et al. showed that CTCs status can reliably discriminate between patients with longer (absent or lower levels of CTCs) versus shorter (higher CTCs levels) PFS and OS, independently of other well-established prognostic moderators [48]. Interestingly, Cheng et al. developed a molecular approach that combines the detection of folate receptor–positive (FR^+) CTCs via ligand-targeted PCR (LT-PCR) with the status of conventional biomarkers such as CA19-9 [49]. This strategy improved diagnostic accuracy and enabled early–stage detection, representing a promising multimodal approach for analyzing early-stage PDAC. Later on, in a study by Tang et al., a VAR2-based platform targeting oncofetal chondroitin sulfate (ofCS), a surface biomarker overexpressed in PDAC and its CTCs [39], was established. CTCs isolated using this method were expanded to long-term organoid cultures enriched in $CXCR4^+$ cancer stem cells (CSCs), capable of tumor initiation from a single cell. Drug screening of these $CXCR4^+$ CTC-derived organoids identified stearoyl-CoA desaturase (SCD1) as a promising therapeutic target.

In the context of microfluidics, it is worth noting that some systems can capture CTCs through interactions with EpCAM-coated microposts, while others utilize CD133-coated surface-based systems, all under precisely controlled laminar flow conditions [50,51]. Ankeny et al. employed the NanoVelcro CTC chip, which combines anti-EpCAM-coated nanosubstrates with microfluidic technology to enable single-CTC capture and molecular characterization [52]. PDAC CTCs were identified using multi-color immunocytochemistry and validated through the detection of a *KRAS* codon 12 mutation. Assessment of CTCs in 100 patients demonstrated a correlation with tumor stage.

Although CTCs show an evident potential in prognostic stratification, technical variability and the lack of directly associated therapeutic strategies currently limit their clinical application. Standardized detection methods, prospective trials, and integration with other LB biomarkers (such as ctDNA, miRNAs, and extracellular vesicles) are essential for establishing CTCs as a valuable tool in personalized cancer management. Currently, the principal clinical value of CTCs remains essentially prognostic, with a higher number of CTCs in LB being strictly associated with worse clinical outcomes, and a lower CTC level being associated with prolonged survival indices.

4. Cell-free DNA

cfDNA is composed of DNA fragments of 150–180 base pairs found in the bloodstream with a half-life of about an hour [53]. cfDNA is derived mainly from apoptotic, necrotic, and secreted fragments originating from healthy and cancer cells, whereas circulating tumor DNA (ctDNA) is released from neoplastic cells [54–56]. Regarding PDAC, the first study to detect *KRAS* mutations using PCR in cfDNA was published in 1994 [57]. Following this study, additional work confirmed these initial findings and further extended the use of cfDNA/ctDNA detection via PCR, droplet digital PCR (ddPCR), and next-generation sequencing (NGS) across both early and late stages of cancer, albeit with variable sensitivity [58–75]. The potential significance of cfDNA has also been explored in the setting of PDAC precursor lesions, and above, all in cases of IPMN. Indeed, *KRAS* and *GNAS* mutations have been detected in cfDNA from patients with IPMN, with reported detection rates ranging from 31% to 88% and 41% to 79%, respectively [76–81]. Such results lay the foundation for employing cfDNA monitoring as a potential method to track high-grade dysplasia precursor lesions and cancer transformation over time.

Positive detection of *KRAS/TP53* mutations in cfDNA/ctDNA has been correlated with poor prognosis and might predict clinical relapse [82–85,79–82]. Moreover, the positive detection of *KRAS* in cfDNA pre- and post-chemotherapy (e.g., gemcitabine or FOLFIRINOX) may be able to identify early relapses and tumor progression [86]. Changes in cfDNA/ctDNA levels are also correlated with radiological data and CA19-9 levels, indicating the potential integrative use of cfDNA in the follow-up [86–93]. Similar to that observed for CTCs, patients negative for *KRAS* cfDNA after surgery showed significantly better prognosis/curative resection compared to those with positive detection [94–97]. Along this line, an interesting investigation showed that *KRAS* or *TP53* variant allele frequency (VAF) after 2 months of treatment (gemcitabine/nab-paclitaxel/xeloda in combination with cisplatin and irinotecan) may predict PFS and OS better than CA19-9 or CEA alone [98].

However, the simple detection of *KRAS* mutation in cfDNA does not reveal the tissue of tumor origin, since *KRAS* is a driver gene of

several tumor types. Additionally, potentially oncogenic mutations found in cfDNA may arise from non-cancerous conditions, such as clonal hematopoiesis, and from leukocytes, primarily neutrophils [99–101]. Additionally, cfDNA has a >75% detection rate in patients with advanced PDAC, but a lower detection rate (48%) in localized PDAC [102]. This finding further confirms the importance of tumor burden in influencing the quantity of CTCs and cfDNA, with early-stage tumors potentially generating negative results at LB.

To address these limitations, incorporating epigenetic signatures in cfDNA/ctDNA provides an additional layer of information that can support the identification of a specific tumor origin [103,104]. Moreover, methylation methods, such as DNA methylation or 5-hydroxymethylation (5hmC) profiling, have the potential to capture all signals in the plasma, including those deriving from immune cells (a major contributor of cfDNA), ultimately improving the detection rate for early-stage cancers [103–105]. One important study in the field of PDAC epigenetics identified differential 5hmC enrichment and occupancy over chromatin states in cfDNA from PDAC tissue (N=64) compared with non-cancer cfDNA samples (N=243) [106]. Among the genes with higher 5hmC density in PDAC, the authors found those related to pancreas development (*GATA4, GATA6, PROX1*, and *MEIS2*) and/or implicated in cancer (*YAP1, TEAD1, PROX1,* and *IGF1*) [106]. Remarkably, tissue-derived 5hmC features can be used to classify PDAC cfDNA versus non-cancer cfDNA (area under the curve, AUC = 0.88) [106]. Moreover, a seminal study in this specific field performed cfDNA 5hmC profiling from patients with PDAC (N=287) and healthy controls (N=216), identifying five hypermethylated genes (*KCNA3, PRRX, CCNA1, TRIM58*, and *NR2F1-AS1*) that were specifically enriched in PDAC patients [107]. The sensitivity and the specificity for PDAC detection were 88.7% and 96.8%, respectively [107]. The combination of 5hmC profiling and a machine learning algorithm was also able to discriminate early-stage PDAC with a sensitivity of 68.3% (95% confidence interval [CI], 51.9%-81.9%) and an overall specificity of 96.9% (95% CI, 96.1%-97.7%) [108].

An increasing number of studies focused on cfDNA/ctDNA investigated its role in combination with protein and methylation biomarkers. Along this line, a recent study showed that the integration of protein and methylation markers with cfDNA-based data in early-stage PDAC (N=40) and matched healthy controls (N=40) increased diagnostic sensitivity compared to CA19-9 alone [109]. The most relevant study in this field is certainly represented by the CancerSEEK test, which integrated, also with supervised machine learning, data from genomic profiling of 16 cancer-driver genes (i.e., *KRAS, TP53, CDKN2A, GNAS, HRAS, NRAS, CTNNB1, PIK3CA, FBXW7, APC, EGFR, BRAF, PTEN, FGFR2, AKT1,* and*PPP2R1A*) and blood levels of 8 cancer-related proteins (CA-125, CA19-9, CEA, HGF, Myeloperoxidase, OPN, Prolactin, TIMP-1) for identifying the tumor origin of eight different clinically-evident solid tumors including PDAC (93 patients with PDAC) [110]. The sensitivity of PDAC detection was high (>70%) thanks to this integrated approach [110]. Another study employed a highly sensitive bioelectronic approach for the multiparametric (cfDNA/protein)-based detection of high-grade PDAC precursors with very promising results using cyst fluids and plasma [111,112].

A further level of insight has been achieved by analyzing the fragmentation patterns of cfDNA (so-called fragmentomic analysis) alongside low-coverage whole-genome sequencing. This approach enables the characterization of an increasing number of transcriptional and epigenetic alterations, together with somatic variations [113]. The first study to use this new methodology, coupled with stacked machine learning, demonstrated a striking ability to detect PDAC with very high values of both sensitivity (97.8%) and specificity (95.0%) [114]. Later on, another study using cfDNA fragmentomics replicated these high values of diagnostic sensitivity and specificity [115]. One of the most significant studies in the field applied cfDNA fragmentomics on a large sample size, composed of 422 patients with PDAC and 498 healthy controls [115]. The authors, thanks to data integration facilitated by artificial intelligence-based systems, developed two different models with related scores: one diagnostic and one prognostic [115]. The diagnostic score integrated with CA19-9 levels distinguished PDAC from healthy controls with very high values. Moreover, the prognostic score showed an excellent capacity in stratifying patients' survival [115].

5. Circulating miRNAs

MicroRNAs (miRNAs) are small non-coding RNA molecules, 19–25 nucleotides long, playing a crucial role as post-transcriptional regulators of gene expression, with well-documented functions in maintaining cellular metabolism [116]. They also have implications in homeostasis alterations, cell dysregulation, and cancer, including PDAC. Their widespread dysregulation is recognised as a key hallmark of PDAC pathogenesis, affecting fundamental cellular processes such as proliferation, invasion, epithelial-mesenchymal transition (EMT), and resistance to therapy [117]. Circulating miRNAs (c-miRNAs) are stably detectable in various biofluids, such as serum, plasma, and bile, and their stability in those fluids is crucial for their utility as LB markers. It is primarily conferred by their association with carrier proteins or, more significantly, by their encapsulation within EVs, such as exosomes [118]. This protection shields them from plasma RNase degradation, making c-miRNAs minimally invasive candidates that meet the critical need for dependable screening and early detection in PDAC. Additionally, a deeper understanding of the functional roles of deregulated miRNAs may suggest their use as biomarkers [119]. miRNAs act as either oncomiRs (promoting cancer) or tumor suppressors (inhibiting cancer) [119]. The highly upregulated oncomiR hsa-miR-21-5p represents a significant first example [120]. Its elevated expression is directly linked to enhanced cancer cell proliferation and infiltrative capacities [17,121,122]. Mechanistically, miR-21-5p promotes these oncogenic features by targeting and controlling tumor suppressor genes, such as Phosphatase and Tensin Homolog (*PTEN)* and Sprouty Homeolog 2*(Spry2)* [17,121,122]. By downregulating PTEN, miR-21-5p activates the central Phosphoinositide 3-Kinase/Protein Kinase B (PI3K/AKT) signalling pathway, a key driver of cell survival and growth in several cancer types, including PDAC [123]. Simultaneously, Spry2 suppression potentiates the Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase (MAPK/ERK) pathway, further driving uncontrolled proliferation and drug resistance [117]. Additionally, the decrease in multiple tumor-suppressing miRNAs may exert a crucial function in the development of PDAC. For example, miR-30b-5p and miR-320b are consistently decreased in PDAC compared to healthy controls and patients with chronic pancreatitis (CP) [124]. This loss of suppression contributes to the progression of the disease. Other key suppressors, such as those in the miR-200 family, are frequently silenced, contributing to the initiation of EMT, a process essential for tumor progression and metastasis [117].

The limited performance of the protein markers, such as CA 19-9 in early-staged PDAC, along with the aforementioned circulating miRNAs features, makes them a strategic molecule's subtype for developing different potentially effective strategies for improving the management of patients affected by PDAC. Currently, single-marker assays show substantial limitations. Indeed, the highly complex nature of PDAC is also reflected in the heterogeneity of potentially useful markers, highlighting that a single indicator is insufficient. Instead, to achieve a more comprehensive understanding of the disease, also with potential clinical implications, there is a need for the adoption of robust multi-miRNA signatures (panels). Such a multi-marker strategy mirrors

current genetic epidemiology-based approaches in PDAC, where reliance on integrated multifactorial risk scores is preferred over single-variant associations, highlighting the complex, polygenic, and multi-molecular nature of pancreatic carcinogenesis [125,126]. Notably, a consensus signature derived from a comprehensive, robust rank aggregation analysis identified four up-regulated (hsa-miR-21-5p, hsa-miR-31-5p, hsa-miR-210-3p, and hsa-miR-155-5p) and three down-regulated (hsa-miR-217, hsa-miR-148a-3p, and hsa-miR-375) miRNAs, whose combined expression patterns offered a high diagnostic accuracy [120]. In addition to this diagnostic potential, another significant area of application for c-miRNAs may be represented by the field of screening/early diagnostics. Along this line, a prospective cohort study found that specific circulating miRNA signatures were associated with PDAC risk within five years of blood collection, indicating that they can identify early molecular alterations [127]. This capability may be crucial for targeted screening of high-risk populations, such as those with hereditary syndromes, familial pancreatic cancer, and precursor lesions.

Interestingly, c-miRNA may also show a prognostic and predictive significance. Indeed, a high expression of onco-miRNAs, particularly miR-21, was consistently associated with poor prognosis, predicting shorter OS and PFS [119]. At the same time, elevated expression of miR-21 was frequently associated with clinical chemoresistance, particularly to gemcitabine and 5-fluorouracil [128]. Furthermore, post-treatment reduction of PDAC-related c-miRNAs, including miR-18a, miR-196a, and miR-221, following radical surgical resection, indicated a positive treatment response [118]. Conversely, their re-elevation may indicate an impending relapse, even prior to radiological evidence, thus presenting a critical window for early treatment intervention [119].

6. Extracellular vesicles

Extracellular vesicles (EV) are an umbrella terminology that encompasses all heterogeneous cell-derived membranous structures released into the extracellular space [129,130]. Based on their distinct features, such as size, release mechanism, and composition, EVs have been classified into exosomes, microvesicles, and apoptotic bodies [129,130]. In particular, exosomes represent the smallest EVs. Their diameter is 150 nm, and they are released into the extracellular space after the fusion of multivesicular bodies with the plasma membrane [129,130]. At the same time, microvesicles and apoptotic bodies are larger than exosomes, with diameters greater than 200nm; they are released directly from the plasma membranes of living or dying cells, respectively [130–132]. Exosomes, the most studied type of EVs, can carry and deliver various types of load molecules, including lipids, lipoproteins, proteins, and nucleic acids. Thanks to their structure and function, such molecules are not only maintained in their status and conserved from degradation but can ultimately be transferred between cells [130–132]. Consequently, exosomes play a crucial role in facilitating and supporting cellular communications and homeostasis thanks to their capacity to transfer or eliminate specific products in a selective manner (Fig. 3) [130–132].

Notably, it has been demonstrated that EVs also exert specific functions in cancer biology. In several tumors, indeed, an increased rate of release of EVs, and in particular of exosomes, is observed; furthermore, those derived from cancer cells are able to modulate tumorigenic signaling and the tumor microenvironment [133–136]. Indeed, exosomes can be transferred to other cells, first stimulating their growth, survival, and migration, and then supporting various oncogenic mechanisms, such as angiogenesis, modulation of the immune system, and pre-metastatic niche formation [130]. For all these aspects, circulating tumor-derived exosomes can be seen as a valuable cancer biomarker [129]. Intriguingly, although they

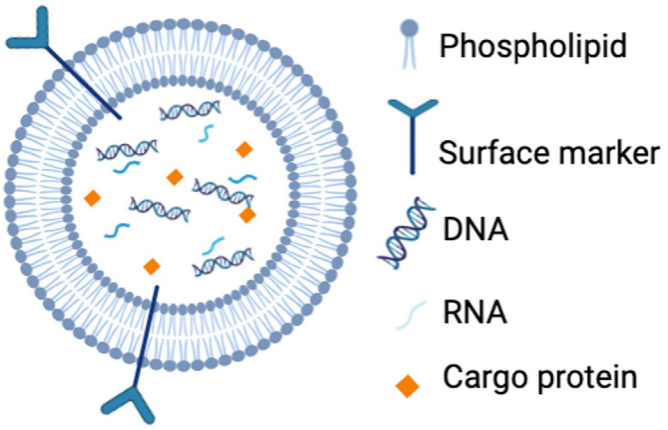

Fig. 3. Schematic figure showing the structure of some of the most extensively studied extracellular vesicles.

can carry several types of molecules, one of the key factors within exosomes is represented by exosomal miRNAs, given their central role in regulating gene expression [137–141]. Therefore, exosomes can contact either neighbor or distant cells and, once inside the receptor cell, exosomal miRNAs can play their roles modulating the expression of target genes [142].

Specifically, regarding PDAC, most studies of EVs have focused on exosomal miRNAs and the protein Glypican-1 (GPC1), a cell surface proteoglycan that is enriched in cancer cell-derived exosomes [130,143,144]. On the one hand, exosomal miRNAs appear to have diagnostic potential, given the distinct expression profiles observed in different pancreatic conditions, including malignant tumors, chronic pancreatitis, and healthy individuals [145–147]. Moreover, they showed tumorigenic activities by promoting PDAC cell proliferation, migration, and cytokine release [145]. On the other hand, circulating exosomal GPC1 can also differentiate between tumors and healthy subjects [148]. Moreover, *KRAS* mutant DNA has been detected in circulating exosomes, and higher exosomal *KRAS* mutant allelic fraction has been associated with lower OS and PFS in patients affected by metastatic PDAC [130,147].

7. Liquid biopsy and clinical trials

The possibility of translating LB techniques into clinical practice for PDAC depends first of all on rigorous preanalytical standardization (sample collection, processing, storage, and biomarker choice) and prospective trials that can validate their diagnostic, prognostic, and predictive values. To date, clinical trials in PDAC have remained limited; however, a growing number of initiatives are underway to bridge the gap between promising biomarkers and the implementation of standard-of-care treatments. Multiple obstacles hinder the success of LB trials in PDAC. First, the low abundance of tumor-derived analytes in early-stage disease limits the sensitivity of assays, particularly for cfDNA and CTCs. Therefore, many trials tend to enroll patients with advanced or metastatic disease, limiting conclusions about early diagnostic potential [149]. Second, heterogeneity in assay platforms (ddPCR, targeted NGS, methylation panels, size-selection fragmentomics, epigenetic classifiers) and lack of standardization make cross-study comparison very difficult [149]. Moreover, trial design must carefully choose control arms (e.g., chronic pancreatitis, non-invasive cancer precursors) to assess specificity in a clinically relevant spectrum of pancreatic diseases. Some ongoing trials (such as PANLIPSY) explicitly include benign lesions as comparator arms [150]. Finally, a long follow-up is needed to correlate biomarker dynamics with clinically meaningful endpoints (recurrence-free survival, overall sur-

Table 2
Potential future clinical applications of liquid biopsy in pancreatic cancer.

Clinical scenario	Potential role of liquid biopsy	Most relevant biomarkers	Current level of evidence	Main challenges
Early detection in high-risk subjects	Screening/risk stratification	cfDNA methylation, fragmentomics, miRNA panels, EVs	Emerging	Low tumor burden, false positives
Differential diagnosis (PDAC vs CP/IPMN)	Non-invasive discrimination	c-miRNAs, EV-associated markers, ctDNA	Emerging	Overlapping molecular patterns
Prognostic stratification	OS and PFS prediction	CTCs, ctDNA VAF, c-miRNAs	Moderate	Standardization
MRD	Detection of Early relapse	ctDNA, fragmentomics	Emerging	Sensitivity
Follow-up	Assessment of early response	ctDNA kinetics, CTC dynamics	Moderate	Timing, cutoffs/thresholds
Resistance mechanisms	Detection of actionable mutations	ctDNA NGS	Moderate	Clonal hematopoiesis
Treatment selection	Molecular profiling	ctDNA	Limited	Low concordance in early stages

Abbreviations: PDAC pancreatic ductal adenocarcinoma; CP: chronic pancreatitis; IPMN: intraductal papillary mucinous neoplasm; MRD: minimal residual disease; OS: overall survival; PFS: progression-free survival; CTCs: Circulating tumor cells; cfDNA: cell-free DNA; ctDNA: circulating tumor DNA; c-miRNAs: circulating micro-RNA; EVs: extracellular vesicles; VAF: variant allele frequency; NGS: next-generation sequencing.

vival, response to chemotherapy). The high rate of early relapse in PDAC demands that trials could enroll sufficient numbers of patients and include serial/longitudinal sampling time points post-surgery and/or during the administration of systemic therapy.

As briefly introduced, one of the most advanced efforts is the PANLIPSY study, a non-interventional trial designed to achieve early detection of PDAC, based on the integration of multiple circulating biomarkers (CTCs, ctDNA, EVs, circulating cell-free nucleosomes, proteins, and microbiota) managed by AI-based systems. The primary objective is to develop a composite LB signature that can distinguish early-stage PDAC from benign pancreatic lesions [150]. Although still ongoing, PANLIPSY exemplifies the future direction of the field: the integration of comprehensive molecular biomarker profiling with the use of artificial intelligence–based predictive systems to enhance PDAC early detection and risk stratification of healthy subjects and patients. It is also worth mentioning that the Mayo Clinic group proposed a prospective multicenter study, in which a panel combining methylated DNA markers in pancreatic juice and the levels of CA 19-9 in plasma was adopted [151]. This hybrid fluid-based approach may discriminate PDAC from healthy patients with high accuracy and demonstrate how sampling from pancreatic juice and blood can be successfully integrated.

To accelerate clinical translation, future trials in PDAC should consider multimodal biomarker panels and integration between imaging and conventional biomarkers: LB signals should therefore be correlated with imaging features (CT, MRI, EUS, PET), CA 19-9 kinetics, and histopathology to validate their clinical impact. Trials in high-risk populations (e.g., germline mutation carriers, familial PDAC risk, new-onset diabetes) should also be proposed to enhance early-detection strategies [152]. Rather than solely biomarker accuracy, trials should measure whether LB could improve clinical decision-making, also including early detection of recurrences before imaging, guiding therapy modifications, and/or sparing ineffective treatments.

8. Limitations, different substrates, future perspectives, and conclusions

As highlighted in this overview on LB in PDAC, there are different and potentially significant biomarkers that can be detected and analyzed with an LB-based assay. In addition to those presented in this overview, there are also other interesting areas related to this field, including LB-based approaches on bile or urine samples and other more specific analytical substrates that merit consideration. First, it is important to acknowledge that bile fluid represents a reliable source of cfDNA for molecular analyses in patients affected by PDAC. In a recent study on 80 patients, Driescher et al. demonstrated a high concordance rate between the mutational profiles of bile-derived cfDNA and matched tissue samples, therefore suggesting a potential role of this strategy in disease monitoring and possible clinical adoption when tissue sampling is not feasible [153]. The suitability of bile as a reliable substrate for cfDNA-based molecular analyses has been further confirmed by subsequent investigations, which highlighted diagnostic and prognostic performances comparable to those reported for plasma-based analyses [154,155]. Notably, promising results have also been reported for urine samples. A recent study demonstrated the feasibility of detecting *KRAS* mutations by analyzing urine-derived cfDNA [156], with detection rates comparable to those observed in plasma samples. A specific advantage of urine-cfDNA analysis is that samples can be collected even at home, both repeatedly and in larger volumes [157]. However, it should be highlighted that the detection rate of *KRAS* mutations in urine may be influenced by renal function [156]. Lastly, with regard to alternative substrates for liquid biopsy in patients with PDAC, a small but growing body of evidence has focused on blood sampling from the portal vein. As also discussed in this review, LB-based approaches based on peripheral blood can be largely influenced by tumor burden and may yield limited sensitivity in non-metastatic disease. In this challenging scenario, portal venous blood sampling may provide higher concentrations of tumor-derived biomarkers, potentially improving the performance of LB–based analyses [158–160].

A key point that should be mentioned in an overview on LB in general and in PDAC in particular is certainly about different levels of limitations, including potential issues in pre-analytical and analytical phases, and the lack of standardization of the different methodologies in the field. Indeed, despite the relevant clinical promise, the transition of LB biomarkers from research to routine clinical practice is severely hampered by a lack of standardization across the research workflow. Addressing these analytical and methodological challenges is of paramount importance for their translation into the clinic. Of note, the pre-analytical phase also introduces notable variability. This phase includes the different modalities of sample collection and storage as general issues and the risk of hemolysis as a specific issue. Different protocols for blood sampling, centrifugation speed, and storage conditions (such as temperature and the number of freeze-thaw cycles) can sig-

nificantly impact the quantity and quality of isolated LB biomarkers. Hemolysis is significant confounding factor, as red blood cells contain high concentrations of certain miRNAs (e.g., miR-16, miR-451a), which can artificially inflate miRNAs levels, rendering them unreliable as tumor-specific biomarkers. Thus, standardized methods for assessing and filtering out hemolyzed samples are essential [118,140,161–163].

The analytical phase also suffers from a lack of consensus/standardization regarding quantitative methods of analysis and different platforms, as well as modalities for quality/internal controls. Quantification platforms, including NGS, microarray analysis, and quantitative PCR, can yield varying results. While NGS represents the best approach for biomarker discovery, RT-PCR remains the gold standard for clinical purposes due to its high sensitivity [7,164,165]. However, a consensus on this crucial point is still lacking, representing a limitation for the introduction of LB in clinical practice. Another critical challenge in the analytical phase is represented by the controls, particularly quality controls and internal/reference controls for data normalization. One of the most relevant examples in this field regarding circulating miRNA is that endogenous controls used in tissue (e.g., U6 snRNA) are often unstable in plasma. Furthermore, the use of miRNAs such as miR-16, a common normalizer, is compromised by its high expression in red blood cells. Consequently, the optimal normalization strategy remains unresolved, often requiring empirical validation via global mean normalization or the use of exogenous synthetic RNA spikes.

Another important issue that should be highlighted is that all the different LB-based approaches and LB-related biomarkers need large-scale validation before their potential consideration as a tool for clinical practice. The discovered signatures suffer from limited validation and significance, as the majority of studies are retrospective, employ small sample sizes, and are often monocentric in design. Large-scale, prospective, and multicenter validation studies, ideally comparing LB-biomarkers to standard-of-care assays, are required to provide that level of evidence that is mandatory for clinical accreditation.

Concluding, while LB for PDAC is still mainly in the research domain, the emerging findings are increasingly promising, showing potential practical applications at prognostic, diagnostic, and predictive levels, as well as in the fields of early detection and clinical monitoring/follow-up (Table 2). Since all these areas are crucial for improving the survival of patients affected by PDAC and considering that they may be improved by LB-based approaches, the pancreas-community should consider LB as a crucial focus for current and future efforts, including research studies with clinical applications.

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Specific author contributions

Study conception and design: SC, CL; review of the literature: all authors; writing original draft: all authors; writing review/editing and approval of the final version: all authors.

Ethical Statement

Not applicable (literature review)

Availability of data and material

All data are available in the manuscript and in the supplementary material.

Declaration of competing interest

S.C. reports honoraria from Boston Scientific and Olympus (consulting). C.L. reports honoraria from Gilead Sciences (advisory board), Astellas (consulting, steering committee, speaker bureau), Bayer, MSD, and Medica s.r.l. (consulting and speaker bureau). All the other authors report no conflicts of interest.

Supplementary materials

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