The emerging role of liquid biopsy in diagnosis, prognosis and treatment monitoring of pancreatic cancer

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Circulating tumor DNA, circulating tumor cells and tumor-related exosomes may offer new opportunities to provide insights into the biological and clinical characteristics of a neoplastic disease. They represent alternative routes for diagnostic and prognostic purposes, and for predicting and longitudinally monitoring response to treatment and disease progression. Hence, circulating biomarkers represent promising noninvasive tools in the scenario of pancreatic cancer, where neither molecular nor clinical predictors of treatment benefit have been identified yet. This review aims to provide an overview of the current status of circulating biomarker research in pancreatic cancer, and discusses their potential clinical utility to facilitate clinical decision-making.

In the recent years, encouraging advances in exploring accurate and less invasive techniques to discover targetable driver mutations have dramatically changed the therapeutic perspectives and clinical outcomes of patients affected by solid tumors. Unfortunately, molecular predictors of prognosis and treatment outcome have not yet been identified in pancreatic cancer. Radiological techniques, including computed tomography and MRI, are used for disease staging and to assess the effectiveness of the locoregional and systemic therapies [1]. However, due to the retroperitoneal anatomical position of the pancreas, and since pancreatic tumor tissue is surrounded by a dense fibrotic stroma and tumor margins may be obscured, the evaluation of tumor response to therapy is particularly challenging with imaging procedures [1]. Positron emission tomography (PET) may play a role in the diagnosis and staging of the disease and in detecting metabolic changes caused by radiotherapy and chemotherapy; however, its role in monitoring treatment outcomes has not established yet [2]. The CA 19–9 serum marker is usually used to predict prognosis and to monitor tumor response after treatment [3]. However, the following limitations has been reported for CA 19-9: i) sensitivity and specificity of approximately 79 and 82%, respectively; ii) changes in serum levels unrelated to disease progression; and iii) inaccuracy in the differential diagnosis between early tumors and precancerous lesions [3,4]. Therefore, the identification of new predictive biomarkers in pancreatic cancer patients is urgently needed for clinical decision-making.

Since the biology of pancreatic cancer is characterized by many genetic variants that play a role in neoplastic transformation and disease progression [5], the use of circulating biomarkers, including circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), and extracellular vesicles (EVs, i.e., exosomes) may be useful as diagnostic, prognostic and/or predictive tool in these patients [6–10]. This review aims to provide an overview of the current status of circulating biomarkers research in pancreatic cancer. The potential clinical utility of these biomarkers in terms of prognostic and predictive power is discussed.

Future Medicine

Liquid versus tissue biopsy

Traditionally, cancer-related molecular alterations are investigated using tumor samples from surgical or biopsy specimens. In clinical practice, tumor specimens are often obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA) following disease progression or recurrence, especially in locally advanced or metastatic pancreatic patients who will not undergo surgical resection of their primary tumor [11]. Unfortunately, the pooled sensitivity and specificity of EUS-FNA in diagnosis of pancreatic cancer is 86 and 96%, respectively, as confirmed by several studies [12–14]. EUS-FNA, with a sensitivity of 84% and a specificity of 96%, provided superior accuracy and specificity in the evaluation of malignancy in lymph nodes [13]. Moreover, in 58 patients with negative CT-guided biopsies, EUS-FNA had 90% sensitivity for malignancy, 50% specificity for benign disease and 84% accuracy [14]. On the contrary, a series of data reported lower sensitivities, especially in a cohort of patients with chronic pancreatitis [15,16]. In the group of 75 out of 300 patients with chronic pancreatitis, EUS-FNA had low sensitivity for pancreatic mass lesions (73.9 vs 91.3%, respectively; p = 0.02). Additionally, this technique may be inconclusive in up to 20% of early-stage pancreatic cancer cases or in the presence of small metastases difficult to detect [17,18]. Consequently, EUS-FNA could give rise to false negative results and, then, to repetitive biopsies.

Moreover, there are several barriers that may limit sample acquisition by tissue biopsy including the anatomical position of the pancreas within the retroperitoneal space and the presence of peritoneal masses being hardly accessible. Tissue biopsies therefore represent invasive procedures especially for patients having significant complications, including bleeding and tumor dissemination. Sample preservation in formalin-fixed paraffin-embedded (FFPE), which crosslinks DNA, may also represent a barrier, being sometimes inadequate for cancer molecular sequencing [19]. Furthermore, the amount of tumor cells that can be obtained from each biopsy is largely variable and depends on tumor cellularity and sample size. With regards for pancreatic cancer, EUS-FNA only has a sensitivity of 75–94% and a specificity of 78–95% [20]. Indeed, biopsy specimen resulting after a EUS-FNA is often characterized by a higher amount of stromal cells than tumor cells [21–23]. Consequently, mutant DNA from pancreatic cancer tumor cells can be over-shadowed by the large excess of wild type DNA derived from normal stromal cells [21]. Thus, false results are common and repeated biopsies are frequently required. Finally, tumor heterogeneity is an additional limitation, and analyses carried out on small samples do not reflect tumor complexity (especially in presence of metastasis) and cannot be applied to the assessment of drug sensitivity, which may change during treatment due to tumor evolution and clonal selection [24,25].

To overcome the above-mentioned limitations, less invasive tools able to capture tumor heterogeneity, detect residual disease or recurrences, allowing for real-time analyses of acquired molecular changes are urgently needed. Recently, liquid biopsy has emerged for pancreatic cancer with the promise to address these unmet clinical needs. Such an approach is characterized by the following advantages: it is a source of fresh nucleic acids; sampling involves a minimally invasive procedure, thus reducing any complications from tissue biopsies; it can be repeated many time allowing for the dynamic monitoring of tumor heterogeneity; it is useful for monitoring tumor burden (especially when imaging bring to inconclusive results) and for detecting minimal residual disease after treatment or surgery; and it is useful to evaluate drug response and identify mechanisms of resistance [19].

CA 19–9 and other traditional serum markers

The analysis of CA 19-9 levels in the peripheral blood of pancreatic cancer patients has received formal the US FDA approval as a test for monitoring drug treatment supportive to imaging approaches [3]. Several studies have examined changes in CA 19-9 serum levels before and during chemotherapy [26–32]. Some of them reported increasing CA 19-9 levels in chemotherapy-treated patients, in presence of tumor progression on restaging CT scan [28–31], whereas others have called into question the reliability of CA 19-9 as a marker of drug response and survival [26,27]. Some major limitations of CA 19-9 as biomarker are low sensitivity (79–81%) and specificity (82–90%); according to this, CA 19-9 levels can be found elevated in subjects with normal pancreatic function or in patients with nonmalignant diseases, including pancreatitis, liver cirrhosis, obstructive jaundice [3,4]. The use of CA 19-9 as a universal biomarker is also limited by the fact that approximately 5–10% of the Caucasian population is Lewis–null blood type, not producing CA 19-9 antigen, leading to high false-negative rate (Table 1) [3,33,34]. CEA is the second most common biomarker used for detecting pancreatic cancer. Some studies have shown that a combination of CEA with other serum markers, such as CA 19-9 and CA 125, should increase the accuracy in differentiate pancreatic cancer patients from healthy subjects [35,36]. Results from a recent meta-analysis involving 57 studies demonstrated a mean sensitivity of 78.2% and a mean specificity of 82.8% of CA 19-9 for discriminating pancreatic cancer from benign disease and a sensitivity of 44.2% and a specificity of 84.8% for CEA as a diagnostic serum marker [4]. As

Table 1. Comparison of reported sensitivity and specificity of CA 19-9 and circulating tumor cells, circulating tumor DNA and exosomes, respect to their diagnostic, prognostic and predictive role in pancreatic cancer.

Biomarker	Diagnostic		Prognostic		Predictive		Ref.
	Sensitivity (mean)	Specificity (mean)	Sensitivity (mean)	Specificity (mean)	Sensitivity (mean)	Specificity (mean)	
CA 19-9	78%	83%	79%	90%	80%	88%	[3,4,28–31]
CTCs	70%	95%	82%	90%	45%	76%	[8,9,42–45]
ctDNA	47%	87%	85%	88%	90%	92%	[6,46–50]
Exosomes	89%	90%	75%	93%	–	–	[10,51]

CTC: circulating tumor cell; ctDNA: circulating tumor DNA.

prognostic tool, increased levels of CEA were associated with a higher tumor burden and worse prognosis [37,38]. CA 125 is another serum marker studied in connection to pancreatic cancer primarily to circumvent the limitation of CA 19-9. The combined evaluation of both CA 125 and CA 19-9 showed a sensitivity of 87.8%, which was higher than that of CA 19-9 alone (80.8%) [39]. In a study of 212 patients with proven pancreatic cancer, CA 125 had shown its superiority compared with CA 19-9 in predicting the resectability of pancreatic cancer (CA 125: sensitivity 78.68% and specificity 71.05%; CA 19-9: sensitivity 63.24% and specificity 71.05%). Moreover, aberrant high levels of CA 125 seemed also to indicate unresectable pancreatic cancer [40]. Furthermore, a recent study showed that CA 125 may be a promising, noninvasive, predictive biomarker for pancreatic cancer metastasis, with a sensitivity of 72.3% and a specificity of 63.9% [41].

Circulating tumor cells
Methodological issues
The existence of CTCs in the peripheral blood of cancer patients was demonstrated for the first time in 1869 and their presence has also been reported in metastatic disease. CTCs are released into the bloodstream through shedding from the primary tumor, and they can disseminate into the blood vessels invading the tissue stroma [52–54]. Their utility for clinical purposes is suggested by several studies that have clearly shown the extremely rare presence of CTCs in the blood of healthy subjects and patients with nonmalignant disorders, and a higher number in patients with malignant disorders [55]. CTCs can be disseminated to distant sites, with the potential to promote metastasis. This hypothesis is supported by clinical data showing that CTCs may have a significant prognostic impact, being related to decreased progression-free survival (PFS) and overall survival (OS) in patients with colorectal, lung, breast and prostate cancer. In addition, these cells have also been isolated for treatment monitoring purposes [56–60]. The frequency of CTCs in the whole blood is around 1 for 10^7 leukocyte per ml, and their half-life was estimated to be in the range of 1–2.4 h [61]. Nowadays, two techniques have been developed for improving enrichment and detection of CTCs: an antibody-based enrichment method that use antibodies directed against cell surface markers and biological/physical assays that allow isolating CTCs on the basis of bioelectric characteristics or cells size [61]. Furthermore, CTCs detection can also be achieved by genomic, transcriptomic and proteomic approaches; among them, the FDA-approved CellSearch® technique is the most widely used CTC platform [61]. However, CTCs detection and isolation are factual challenge for cancer monitoring and a number of technical concerns need to be solved before translating this approach into clinical practice (Figure 1 & Table 2) [62–65].

Diagnostic role of CTCs
Several findings reported that CTC analysis has sufficient and high sensitivity (70%) to detected stage I and II pancreatic cancer [42–44,66]. Additionally, CTCs are able to identify with a sensitivity of 30% patients with pre-adenocarcinoma disease [43,67]. Kulemann and colleagues using a filtration-based method and *KRAS* mutational analysis detected CTCs in eight out of ten patients (80%) with early stage, while no CTCs were found in nine healthy controls [42]. Interestingly, diagnostic accuracy improved in the study conducted by Xu and colleagues who found CTCs in 6/8 patients with benign pancreatic disease (75%) and in 8/11 patients with early-stage pancreatic cancer (73%) (Table 1) [43]. Therefore, even if studies with a higher number of patients are needed, CTCs analysis may be useful for early-stage pancreatic cancer diagnosis.
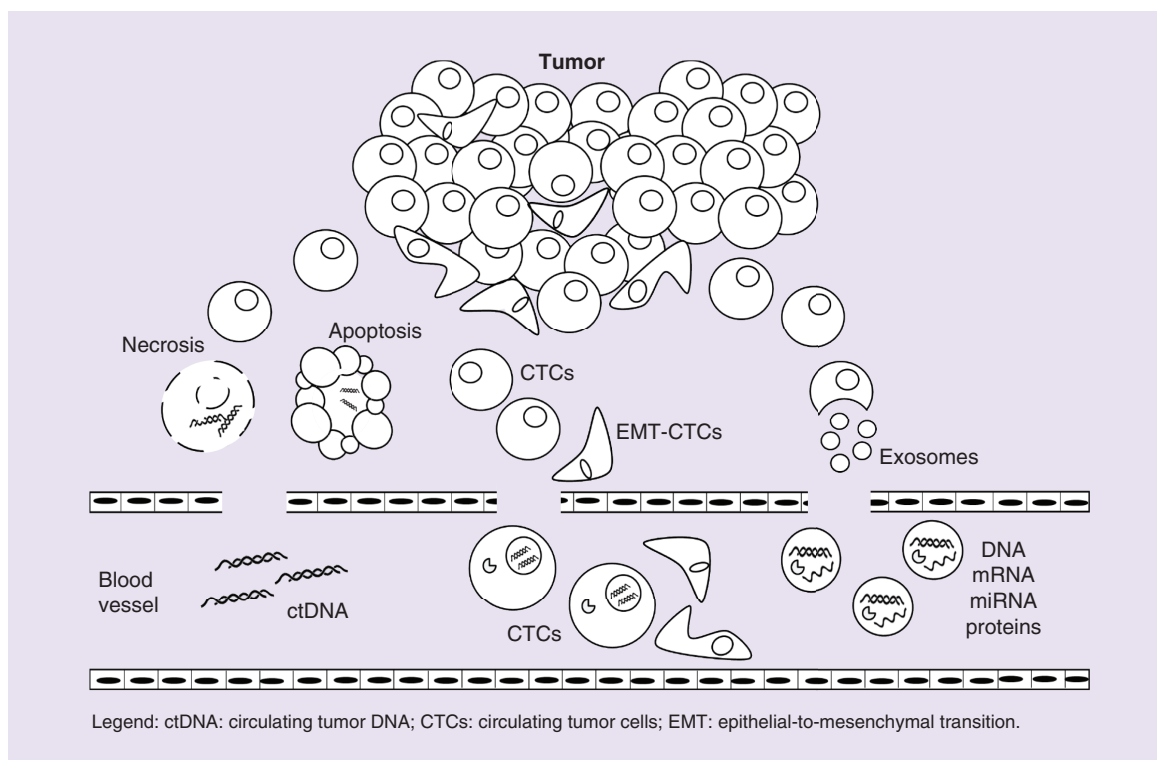
Legend: ctDNA: circulating tumor DNA; CTCs: circulating tumor cells; EMT: epithelial-to-mesenchymal transition.

Figure 1.   Schematic representation of circulating biomarkers. Using several mechanisms, tumor cells release different circulating biomarker that freely swim into the bloodstream.
ctDNA: Circulating tumor DNA; CTC: Circulating tumor cell; EMT: Epithelial-to-mesenchymal transition.

Table 2. Comparison between circulating tumor DNA, circulating tumor cells, exosomes and their mechanism of release, main methods of isolation and analysis, test capability and clinical applications.

	ctDNA	CTCs	Exosomes
Mechanisms of release	• Released by tumor necrosis or apoptosis, lysis of circulating tumor cells or micrometastases, active cellular processes	• Released into bloodstream by an active mechanism involving the EMT or through the shedding from the primary tumor	• Fusion of the multivesicular endosome with the plasma membrane
Isolation and analysis methods	• Isolation: ✓ commercial kits; ✓ individual laboratory protocols; ✓ company inhouse procedures. • Analysis methods: ✓ Sanger; ✓ Pyrosequencing; ✓ PCR (Real-Time PCR, PNA clamping, ddPCR, BEAMing, NGS); ✓ Mass spectrometry	• Isolation and enrichment: ✓ density gradient centrifugation; ✓ flow cells; ✓ immunomagnetic beads; ✓ membrane filtration. • Analysis methods: ✓ PCR (RT-qPCR); ✓ immunological approach (CellSearch, Immunocytochemistry, FAST)	• Isolation: ✓ ultracentrifugation; ✓ filtration; ✓ polymer-based precipitation; ✓ chromatography; ✓ immunological separation. • Analysis methods: ✓ Western blot; ✓ PCR (Real-Time PCR, RT-PCR, ddPCR, NGS)
Test capability	• Epigenetic modifications (methylations) • Mutations (point mutations, amplifications, deletions, translocations).	• Epigenetic modifications (methylations); • Mutations (point mutations, amplifications, deletions, translocations); • RNA expression and fusion transcripts; • Protein expression; • *In vitro/in vivo* culture	• Epigenetic modifications (methylations); • Mutations (point mutations, amplifications, deletions, translocations); • RNA expression and fusion transcripts; • Protein expression
Applications	Diagnosis, prognosis and prediction of response to treatment. Monitoring of the disease.		

ctDNA: Circulating tumor DNA; CTC: Circulating tumor cell; EMT: Epithelial-to-mesenchymal transition; PNA: Peptide nucleic acid; ddPCR: Droplet digital PCR; NGS: Next-generation sequencing; RT-PCR: Reverse transcription PCR; RT-qPCR: Quantitative reverse transcription PCR.

Prognostic and predictive role of CTCs

Evidence supports the feasibility of using CTCs to analyze gene expression or mutation both in operable and inoperable pancreatic cancer patients. However, pancreatic cancer is characterized by a low CTCs detection rate (from 5 to 100%), as compared with other solid tumors [8,9,42,45,55,63–66,68–75]. This large variability in results may depend on the detection method; for example, a significant loss of tumor cells has been reported during density gradient purification, which includes several washing steps [76]. Furthermore, magnetic bead enrichment (CellSearch) tends to cause cell destruction and requires a certain level of expression of tumor specific antigens (i.e., EpCAM) to maintain cells in the magnetic support. This may account for the low CTCs detection rate frequently observed in pancreatic cancer patients, most probably due to the high heterogeneous expression of EpCAM [77]. Another obstacle for CTCs isolation using the antigen-dependent approach is detection during the disease progression by epithelial-to-mesenchymal trasnsition process, where EpCAM are down-regulated in pancreatic cancer [78]. Several other mechanisms involved in the down-regulation of EpCAM have also been described, including internalization and proteolysis [79].

There are much evidences that CTCs are identified in patients with pancreatic cancer and not in healthy subjects. Funaki and colleagues demonstrated the presence of CEA mRNA in the peripheral blood of patients with metastatic pancreatic cancer and of those affected by pancreatitis (as control) by using RT-PCR. Three out of nine metastatic patients (33%) were CEA mRNA positive, while all of controls were negative [68]. Following this study, other research groups attempted to monitor CEA mRNA expression as a method for detecting CTCs in pancreatic cancer patients [65,80]. Using nested RT-PCR, Chausovsky and colleagues reported that 22 (79%) out of 28 metastatic pancreatic cancer patients were positive for CK-20 mRNA in peripheral blood, indicating that CK-20 could be a potential biomarker for CTCs detection in pancreatic cancer [70]. Later, similar findings were shown by other researchers [71,76,81]. After immunomagnetic enrichment, Zhou and colleagues used a multi-marker approach as an indicator for CTCs by conventional RT-PCR. Interestingly, by combining mRNA expression analyses of CK20, CEA, C-MET and the human telomerase reverse transcriptase, they were able to distinguish pancreatic cancer patients (n = 25) from benign controls (n = 15) with high specificity and sensitivity (CK20: sensitivity 84% and specificity 93%; CEA: sensitivity 80% and specificity 100%; C-MET: sensitivity 80% and specificity 100%; human telomerase reverse transcriptase: sensitivity 100% and specificity 100%). Moreover, mRNA expression levels of CK-20, CEA and C-MET were statistically higher during later stages (stages III and IV) than earlier stages (stages I and II), suggesting that assessment of CTCs may be useful for monitoring disease progression [66].

The following findings suggest that CTC-derived mRNA can be a reliable biomarkers for the early detection of relapse in pancreatic cancer patients: in particular, the mRNA expression of several biomarkers has been evaluated. Mataki and colleagues used RT-PCR to evaluate CEA mRNA expression as molecular biomarker of CTCs in the peripheral blood of 53 pancreatic cancer patients undergoing curative surgery. During the follow-up period, 16 out of 53 patients had a relapse and the time to the first increase of CEA mRNA expression levels in the blood of 8 relapsing patients was shorter than the period required for detection by imaging (303 ± 169 days vs 375 ± 268 days) [65]. Similar results were obtained using a multi-marker mRNA panel consisting of CK-19, MUC1, EPCAM, CEA, CAM5 and BIRC5 for CTCs detection [72]. This study demonstrated that 16 CTCs-positive patients had significantly shorter PFS, compared with CTCs-negative patients (66 days vs 138 days, respectively) [72].

Moreover, several lines of evidence highlighted the clinical relevance of CTCs as a negative prognostic factor able to predict disease recurrence in pancreatic cancer patients [8,64,65,72,82,83]. Kurihara and colleagues showed significantly shorter OS in 11 (42%) of the 26 pancreatic cancer patients (stage II–IV) who were CTCs-positive (110.5 days vs and 375.8 days) [64]. More recently, a study showed that CTC-positive patients had significantly poor OS compared with CTC-negative patients (88 days vs 393 days, respectively) [8]. A meta-analysis including a total of 623 pancreatic cancer patients (268 of which were CTCs positive), confirmed the negative prognostic value of CTCs, associating the presence of CTCs with poor correlation between survival and CTCs positivity [83]. Z'graggen and colleagues showed that the CTC prevalence was higher among pancreatic cancer patients with unresectable disease compared with those having a localized, resectable cancer (33 vs 9%); however, the correlation between CTCs detection and disease progression was not statistically significant [45]. Furthermore, in a relatively small study, the presence of CTCs did not affect median survival in patients with locally advanced/metastatic pancreatic cancer (16 vs 10 months in CTC-positive vs negative patients, respectively) but, patients with a *KRAS* mutation (12/21) in CTCs had a better median survival than those having wild type *KRAS* (19.4 vs 7.4 months) [42].

Few studies have investigated whether changes of CTC levels before and after therapies may be predictive of treatment efficacy, providing a longitudinal monitoring of the disease. In particular, a correlation was observed between the reduction in the number of CTCs and the decrease in tumor size and/or tumor response [9,84,85]. Sheng and colleagues founded CTCs in 17 (94%) out of 18 pancreatic cancer patients, with an average number of 3 CTCs per ml peripheral blood. Subsequently, they also found a correlation between CTC number and tumor size in three metastatic pancreatic cancer patients undergoing chemotherapy. The results showed a reduction in CTCs number during treatment, together with the decrease in tumor size, as confirmed by CT-scan [85]. More recently, a mean CTC number of 4.9 in 7.5 ml blood was found in 32.3% of unresectable pancreatic cancer patients who had significantly lower OS than those without CTCs (203 vs 399 days). In addition, 40 patients were monitored during chemotherapy or chemo-radiotherapy regimes to determine whether quantitative variations in CTCs could predict tumor response. At 3 months after the start of treatment, the CTC positivity rate was 45.4% for patients with progressive disease and 24.1% for those with stable disease or partial response. Furthermore, CTC-positive patients had lower OS than those without CTCs (354 vs 487 days) (Tables 1 & 3) [9].

In summary, the clinical relevance of CTC characterization in pancreatic cancer patients is still controversial. Indeed, although the majority of data support the prognostic value of CTCs in pancreatic cancer patients, others have not demonstrated a significant correlation between CTCs and survival. Negative results could be due to limitations in the study design (e.g., small sample size resulting in low statistical power) as well as to different methodological approaches used to detect CTCs. To deeply understand whether CTCs can be reliable markers in predicting treatment success or failure faster and better than conventional approaches, comprehensive well-designed studies are needed. For these purposes, rigorous evaluations of larger, prospective clinical trials are also warranted. Even more important would be the identification of a CTC threshold able to early differentiate metastatic from non metastatic pancreatic cancer.

Circulating-free tumor DNA
Methodological issue
In 1948, circulating-free DNA was discovered in the blood of healthy individuals [86]; since then, several groups have shown the presence of DNA with neoplastic characteristics in the circulation [87]. Although the exact mechanism determining the release of ctDNA in blood is unclear, several hypotheses have been postulated including DNA release by tumor necrosis or apoptosis and lysis of CTCs or micrometastases [88]. Alternatively, all living cells could actively release DNA into the blood [89]. Usually, ctDNA is detectable as small fragments with the length of 170–180 base pairs [88]. The concentration of ctDNA in biological fluids is very low and varies considerably among different subjects, ranging from 1 to 100 ng/ml, also depending on type and dimension of the tumor burden [90]. Consequently, even if sensitivity and specificity for ctDNA detection are generally low, tumor DNA can be characterized by detecting somatic mutations localized in the genome of cancer cells only [91]. High analytical sensitivity and specificity are therefore required for both ctDNA extraction and detection. In this regard, several technologies are available to analyze ctDNA mutations, including real-time PCR, automatic sequencing, mass spectrometry genotyping, next-generation sequencing (NGS), digital PCR platforms (such as digital droplet PCR, ddPCR). However, the sensitivity of these methods varies from 15 to 0.01% [92–95] and the lack of protocol standardization still remains the major challenge. Finally, the cost-effectiveness and the reliability of ctDNA methods in clinical practice also need to be considered (Figure 1 & Table 2).

Diagnostic role of ctDNA
CtDNA analysis seems to be of little use in the diagnosis of pancreatic cancer, especially due to the low abundance of ctDNA detectable in early stages. A study showed that four out of four patients with chronic pancreatitis and ctDNA-*KRAS* mutations were diagnosed with pancreatic cancer during follow-up [96]. Maire and colleagues reported that the sensitivity and specificity of ctDNA *KRAS* mutations for the diagnosis of pancreatic cancer were 47 and 87%, respectively. Nevertheless, the combination of ctDNA *KRAS* mutations and CA19-9 had a sensitivity and specificity of 98 and 77%, respectively [46]. Additionally, CA 19-9 was superior to ctDNA for detection of pancreatic cancer in low burden disease (Table 1) [97].

Prognostic and predictive role of ctDNA
Shapiro and colleagues first reported higher ctDNA plasma levels in patients with pancreatic cancer than healthy subjects [98]. Since then, many investigations have explored tumor-derived genetic alterations in plasma of pancreatic

Table 3. Principal studies reporting the detection of circulating tumor cells in pancreatic cancer.

Number of patients	Characteristics	CTCs count	Methods	CTCs status (number)		CTCs markers	Progression-free survival (months)			Overall survival (months)			Role of CTCs	Ref.
	Tumor			Positive	Negative		Positive	Negative	p-value	Positive	Negative	p-value		
67	Resectable	10/5 ml of blood	RT-PCR	32	35	CEA	–	–	–	17	36	n.s.	Prognostic	[80]
26	Locally advanced/metastatic	≥1/7.5 ml of blood	CellSearch	11	15	CK8-18-19 CA19-9 CEA	–	–	–	3.7	12.4	≤0.001	Prognostic	[64]
34	Locally advanced/metastatic	–	Immuno-magnetic beads	16	18	KRT19 MUC1 EpCAM CEA CAM5 BIRC5	2.2	4.6	0.01	–	–	–	Prognostic	[72]
35	Localized and locally advanced/metastatic	≥1/7.5 ml of blood	CellSearch	7	28	EpCAM CD45 CK	–	–	–	2.9	13.1	0.0108	Prognostic	[8]
53	Localized and locally advanced/metastatic	≥1/7.5 ml of blood	ISET	21	32	CD45 EpCAM CK4-5-6-8-10-13-18 Vimentin E-Cadherin	3.1	4.7	0.13	4.2	5.5	0.26	Prognostic	[82]
105	Localized and locally advanced/metastatic	–	CellSearch	27	78	AE1/AE3 CK7-19-20 BER-EP4 CA 19-9	–	–	15.6	17.4	0.95–1	–	Prognostic	[45]
21	Localized and locally advanced/metastatic	Clusters of 3-50 cells or as single cells	ScreenCell filtration	18	3	CK ZEB1	–	–	–	16.0	10.0	n.s.	Prognostic	[42]
41	Advanced/metastatic	≥1/7.5 ml of blood	Immunofluorescent staining	33	8	CK8-18 CA19-9	–	–	–	–	–	–	Predictive	[84]
18	Metastatic	≥1/7.5 ml of blood	GEM chip	17	1	EpCAM DAPI cytokeratin CD45	–	–	–	–	–	–	Predictive	[85]
56	Advanced	>0/7.5 ml of blood	CellSearch	21	35	EpCAM CK8-18-19 CD45	–	–	–	6.7	13.1	0.0051	Prognostic, Predictive	[9]

ISET: Isolation by size of epithelial tumor cells; n.i.: Not investigated; n.s.: Not significant; RT-PCR: Reverse transcriptase-PCR.

cancer patients. Interestingly, few studies can be found regarding the characterization of microsatellite instability, allelic imbalance or methylation [99–102]. Conversely, much attention has been focused on ctDNA *KRAS* mutations detection, since this gene is highly mutated in pancreatic cancer. Therefore, several reports investigated the correlation between mutations in ctDNA, especially in the *KRAS* gene, and matched tumor biopsy, with a concordance ranging from 25 to 70% (Table 4) [96,103]. In 1998, *KRAS* mutation in ctDNA was detected in plasma of nine (60%) out of 15 pancreatic cancer patients; these patients were also found to have the mutation in tumor tissue [104]. Uemura and colleagues found *KRAS* mutations in 26 out of 28 (93%) pancreatic cancer tissues, and nine of them exhibited the same mutation in matched plasma DNA [105]. Moreover, in a recent study, the concordance rate of 77.3% between *KRAS* mutations in tissue and ctDNA was reached [47]. In a study aimed at investigating the feasibility of ctDNA in 26 pancreatic cancer patients, the authors identified more than 90% of the mutations (e.g., *KRAS*, *TP53* and *SMAD4*) found in matched tumor biopsies, using the NGS technology [106]. In contrast to this, Marchese and colleagues found *KRAS* mutations in 70% of tumor tissue samples, whereas no ctDNA *KRAS* mutation was found in matched plasma samples [107]. Similar findings were reported in a recent study, in which the overall concordance between plasma and matched tumor tissues assessed by NGS was 25% [103].

Moreover, several studies have been designed to test the clinical application of ctDNA detection by enrolling patients with either surgically resectable or locally advanced/metastatic pancreatic cancer [6,8,47,48,102,108,109]. In particular, the presence of plasma ctDNA *KRAS* mutations before or after initiation of chemotherapy was almost always reported to be correlated with primary tumor analyses, the presence of metastases, and a significantly shorter OS [6,8,47,48,102,103,108–110]. In agreement with these data, although *KRAS* mutations were found in only 33% of metastatic patients prior to treatment [108], Chen and colleagues reported a median survival of 3.9 months for patients with *KRAS* mutated ctDNA versus 10.2 months for the ctDNA negative patients (p < 0.001) [6]. More recently, a study performed with a high sensitivity ddPCR method, demonstrated that patient with *KRAS* mutations in plasma ctDNA had a worse prognosis when compared with those without (176 vs 489 days) [47]. Similar studies have been carried out by other researchers who focused their attention on patients with resectable tumors [8,49,102,104,109]. Sausen and colleagues reported that ctDNA detection after surgery can predict disease relapse and poor outcome. Of note, recurrence was detected 6.5 months earlier by ctDNA than by standard imaging [102]. Recently, *KRAS* mutations in ctDNA were detected in preoperative (24.4%) and postoperative (44.4%) sera of patients with resectable pancreatic cancer. Patients with *KRAS* mutations in postoperative ctDNA had significantly shorter disease-free survival and OS than those with wild type *KRAS*. Moreover, multivariate analysis demonstrated that the presence of *KRAS* mutations in postoperative patients as well as the switch from wild type *KRAS* in preoperative to mutant *KRAS* in postoperative ctDNA, were independent prognostic factors for poor disease-free survival and OS, respectively [49]. Considering the specific type of mutation, the presence of *KRAS* p.G12V was found to be associated with a significantly shorter survival, compared with *KRAS* p.G12D, p.G12R or the wild type [47].

Several lines of evidence demonstrate that ctDNA analysis may predict response or resistance to targeted drugs and chemotherapeutics across different tumor types (e.g., colorectal, lung and breast cancer) [111]. Although few analyses have been carried out in pancreatic cancer patients, data reported later showed that longitudinal monitoring of ctDNA can predict response to chemotherapy and disease progression, simultaneously or even earlier (5 months before) than conventional monitoring approaches (i.e., radiological imaging and CA 19-9) [48,50]. Recently published data demonstrated that changes in *KRAS* mutation copy numbers in ctDNA was related to radiological data at follow-up and CA19-9 levels, suggesting that mutant *KRAS* can be an efficient and early ctDNA marker of response to treatment in pancreatic cancer [48,50]. Moreover, the paper published by Del Re and colleagues showed a statistically significant difference in PFS and OS in patients with increase versus stability/reduction of ctDNA in samples collected at day 15 compared with the pretreatment samples (median PFS 2.5 vs 7.5 months; p = 0.03; OS 6.5 vs 11.5 months; p = 0.009). Moreover, the authors found that variations in *KRAS* mutant ctDNA levels were better correlated to tumor dynamics than changes in CA 19-9 levels (Table 5) [50].

In summary, ctDNA may be a useful tool to detect genetic alterations in pancreatic cancer patients. As reviewed, ctDNA is of critical interest to address the issue of monitoring treatment outcome and disease progression in pancreatic cancer compared with tissue biopsy, and the analysis of frequently mutated genes, such as *KRAS* mutations in ctDNA, could represent a novel option in this clinical setting, if validated by future larger prospective studies. Nevertheless, because of the limited sizes of the small cohort and the use of different technological approaches for the detection of mutations in ctDNA, current evidence supporting ctDNA use in the clinic are still quite mixed, and future studies are warranted.

Table 4. Principal studies reporting the correlation between mutations in circulating tumor DNA and matched tumor biopsy.

Characteristics			KRAS mutations				
Number of patients	Tumor	Localization	Tissue (number)	Plasma (ctDNA) (number)	Concordance Tissue/plasma	Perspective/conclusion	Ref.
21	Pancreatic adenocarcinoma	Codon 12	15/21 (71.0%)	9/15 (60.0%)	✓ (60.0%)	Evaluation of the clinical usefulness of detection of such mutations on tumor staging and efficacy of treatment	[104]
21	Pancreatic cancer	Codon 12	7/10 (70.0%)	17/21 (81.0%)	✓ (100.0%)	Detection of mutant plasma DNA for conventional cancer diagnosis	[96]
28	Pancreatic cancer	Codon 12	26/28 (93.0%)	9/26 (35.0%)	✓ (35.0%)	Genetic alterations detected in plasma could potentially applicable for cancer screening and the monitoring of the disease	[105]
30	Pancreatic cancer	Codon 12	28/40 (70.0%)	0/40 (0.0%)	✗ No concordance	KRAS mutations in ctDNA is an unsatisfactory method for the detection in patients with pancreatic cancer and in patients with high risk of progression disease	[107]
75	Pancreatic cancer	p.G12V, p.G12D and p.G12R in codon 12	28/75 (37.3%) p.G12V 22/75 (29.3%) p.G12D 6/75 (8.0%) p.G12R	26/75 (34.6%) p.G12V 29/75 (38.6%) p.G12D 4/75 (5.3%) p.G12R	✓ (77.3%)	ctDNA in serum has been considered a useful candidate for noninvasive cancer diagnosis and also for predicting survival	[47]
26	Pancreatic and biliary carcinomas	Codon 12	11/26 (42.0%)	14/26 (54.0%)	✓ (65.0%)	The detection of tumor-derived mutations with ctDNA-based NGS assays provides a useful measure of disease progression or response to therapy unlike tissue-based NGS	[106]
34	Pancreatic ductal adenocarcinoma	Not reported	20/23 (87.0%)	10/34 (29.0%)	✓ (39.0%)	ctDNA-based NGS assays are not yet an adequate substitute to tissue biopsies, both as prognostic tools and to monitor the disease progression	[103]

ctDNA: Circulating tumor DNA; NGS: Next-generation sequencing.

Table 5. Principal studies reporting the detection of circulating tumor DNA in pancreatic cancer.

Number of patients	Characteristics Tumor	Method	ctDNA Positive (number)	ctDNA Negative (number)	Gene	Progression-free survival (months) Positive	Negative	p-value	Overall survival (months) Positive	Negative	p-value	Role of ctDNA	Ref.
44	Locally advanced/metastatic	RFLP	12	32	KRAS	2.0	6.0	≤0.005	–	–	–	Prognostic	[108]
91	Locally advanced/metastatic	PCR	30	61	KRAS	3.9	10.2	≤0.001	–	–	–	Prognostic	[6]
14	Locally advanced/metastatic	PNA-clamp PCR	10	4	KRAS	2.0	NR	0.064	4.0	9.0	0.066	Prognostic, Predictive	[48]
75	Metastatic	ddPCR	47	28	KRAS	9.1	13.6	0.02	–	–	–	Prognostic	[47]
31	Locally advanced/metastatic	ddPCR	8	23	KRAS	–	–	–	2.0	25.7	0.001	Prognostic	[8]
105	Resectable	ddPCR	86	19	KRAS	–	–	–	18.4	31.0	0.18	Prognostic	[109]
34	Locally advanced/metastatic	NGS	24	10	KRAS TP53 SMAD4 CDKN2A	–	–	–	7.5	NR	0.045	Prognostic	[103]
104	Locally advanced/metastatic	NGS / ddPCR	50	54	KRAS TP53 SMAD4 NRAS PIK3CA STK11	–	–	–	6.5	19	≤0.001	Prognostic	[110]
27	Locally advanced/metastatic	ddPCR	19	8	KRAS	2.5	7.5	0.03	6.5	11.5	0.009	Predictive	[50]
45	Resectable (post-operative)	PNA-clamp PCR	31	14	KRAS	10†	25†	0.014†	20	40	0.044	Prognostic	[49]

†Defined as disease-free survival.

–: Not available; ctDNA: circulating – tumor DNA; n.i.: not investigated; NR: Not reached; PNA-clamp PCR: peptide nucleic acid-mediated PCR clamping; ddPCR: Droplet digital PCR; NGS: Next generation sequencing; KRAS: Kirsten rat sarcoma viral oncogene homolog; TP53: Tumor protein 53; SMAD4: Small mother against decapentaplegic; NRAS: Neuroblastoma RAS viral oncogene homolog; PIK3CA: Phosphatidylinositol-4,5-bisphosphate 3-kinase; STK11: Serine/threonine kinase 11.

Exosomes as a new source for ctDNA and RNA

The highly fragmented nature of nucleic acids in circulation, and the unstable property of circulating RNA transcripts, may influence their detection and measurements, especially for routine use in clinical practice [112,113]. Recently, RNA (i.e., mRNA, miRNAs and noncoding RNA) and proteins have been characterized and obtained from extracellular vesicles, named 'exosomes' [114,115]. Exosomes are 40–120 nm lipid bilayer membrane-bound vesicles derived from the endocytic pathway following the inward budding of multivesicular endosome fusion with the plasma membrane [116]. Following an exocytotic mechanism, exosomes are released in circulation from a wide range of cells, and several pieces of evidence suggest that tumor cells produce and secrete exosomes in increased amounts, compared with normal counterparts [117]. Five methods to isolate exosomes from biological fluids (i.e., blood, plasma, urine and cerebrospinal fluid) are available, including ultracentrifugation, filtration, polymer-based precipitation, chromatography and immunological separation [118]. To date, ultracentrifugation is currently considered as the gold standard of exosome isolation. However, although encouraging results have been obtained in the last few years, existing exosome isolation techniques present challenges to researchers in this field. For example, it has been proven that it can be difficult to rapidly and efficiently isolate exosomes mostly because of the complexity of biological samples, contamination from other extracellular vesicles, as well as exosome heterogeneity itself (Figure 1 & Table 2) [118]. Therefore, research efforts should be focused to establish a standardized technique for isolating high, relatively pure concentrations of exosomes.

Diagnostic role of exosomes

Due to the ability to carry cargo with disease-specific nucleic acids and proteins, exosomes seem to be promising as screening and diagnostic tools in pancreatic cancer. Melo and colleagues showed that circulating exosomes positive for glypican-1 distinguished pancreatic cancer patients from healthy controls and individuals with benign diseases [51]. Exosomal *KRAS* mutations have been shown to be superior to ctDNA *KRAS* mutations in diagnosing pancreatic cancer patients at different stages. However, mutant *KRAS* in exosomes was also detected in healthy controls [10]. A recent study showed that exosomal miRNA levels analysis is superior to exosomal GPC1 or serum CA 19-9 levels in diagnosing pancreatic cancer patients and differentiating neoplasia from chronic disease (Table 1) [119]. Moreover, a recent large study identified a metabolic tumor biomarker signature comprising nine metabolites and CA 19-9 able to discriminate pancreatic cancer from chronic pancreatitis, with a higher diagnostic accuracy than CA 19-9 alone [120].

Functional significance of exosome-derived DNA and RNA and their role in prognosis and treatment monitoring

Emerging evidence suggests that cancer-derived exosomes participate in multiple steps of cancer development and their impact on the pathogenesis of pancreatic cancer is well recognized. In fact, they play a critical role in cell-to-cell communication between the tumor and neighboring tissues and systemic microenvironment, facilitating promotion of tumor invasiveness, angiogenesis, inhibition of apoptosis, development of a premetastatic niche, induction of immune tolerance and treatment resistance [121–125]. Therefore, also considering that exosomes are stable under different conditions and can protect their biological cargo against degradation or denaturation in the extracellular space, they are evaluated as new promising prognostic and predictive biomarkers in cancer (Figure 1 & Table 2).

Recent publications have shown that circulating exosomes, extracted not only from pancreatic cancer cells but also from plasma/serum of patients, contain a repertoire of molecular cargo, including DNA, mRNA, miRNAs and proteins. Of note, the last years have been characterized by the growing interest in miRNAs, in other words, small noncoding RNA able to target multiple mRNAs, causing translational repression and/or mRNAs degradation [126]. Several studies reported their involvement in proliferation, migration, invasion, metastasis and chemoresistance of several tumor types, including pancreatic cancer [127]. Interestingly, Lee and colleagues identified a miRNA expression signature associated with pancreatic cancer highlighting the presence of 100 out of 201 miRNA precursors with aberrant expression [128]. Deregulation of miRNAs' functions indicates that they can act both as oncogenes and tumor suppressors [129]. Data from several investigations reported miRNA-21, miR-17-5p, miRNA-31, miRNA-210, miRNA-221, miRNA-224 and miRNA-486, as the most highly upregulated miRNAs in pancreatic cancer [130–134]. On the other hand, the most down-regulated miRNAs are miRNA-96, miRNA-148a, miRNA-216 and miRNA-217 [132,135]. The upregulation and the down-regulation of these oncogenic and tumor suppressor miRNAs appear to be involved at several steps along pancreatic tumorigenesis, above all in metastasis

promotion and chemoresistance, especially through their direct interaction with specific target genes implicated in angiogenesis, cell cycle, apoptosis and metastasis signaling [128,136–142].

Although most studies have shown the potential utility of miRNAs as diagnostic biomarkers useful to differentiate normal from pancreatic cancer tissues [143,144], an increasing interest in these miRNAs is attributable to their role as predictive markers, in relation to prognosis and/or response to pharmacological treatment. Until recent years, the expression of miRNAs profiling has been conducted using samples from tumor tissues; however, in the last years, circulating miRNAs have been largely studied in blood samples of pancreatic cancer patients [145]. One of the most studied is the miRNA-21, which acts as a key factor mediating for growth, development and progression of tumors, including pancreatic cancer, being also associated with poor prognosis of pancreatic cancer when analyzed in tissue [130,146–149]. Similar results were obtained using blood as source of circulating miRNAs [149,150]. Wang and colleagues evaluated miRNA-21 expression in 65 resectable pancreatic cancer patients, highlighting a significantly shorter median survival in patients with a high miRNA-21 expression level than in patients with a low miRNA-21 expression level (12.0 vs 32.0 months; p = 0.003) [150]. Since the sensitivity and the specificity of miRNA detection in blood could be decreased by the presence of ribonucleases, exosomes can represent a protective and enriched envelope for miRNA compared with intracellular and cell-free blood [151]. To date, most literature reports a differential exosomal-miRNA profile by comparing miRNAs expression in pancreatic cancer patients and healthy controls, highlighting the potential role of exosomal-miRNAs as a diagnostic biomarker for pancreatic cancer [131,152]. Unfortunately, there is a lack in the investigation of exosomal-miRNAs as biomarkers useful to evaluate the clinical outcome and to determine the early success of resection or response to any therapy.

Studying circulating exosomes may also aid in the identification of characteristic DNA alterations, mostly in *KRAS* and *TP53* genes [10,153,154]. Recently, San Lucas and colleagues showed a comprehensive profiling of exosomal DNA and RNA by whole genome, exome and transcriptome sequencing from plasma of 2 advanced and 1 resectable pancreatic cancer patient, using an NGS platform. Interestingly, they revealed a wide number of pancreatic cancer biomarkers, such as point mutations, insertions and deletions, copy number profiles, and gene fusions, which could be found within DNA and RNA cargo of shedded exosomes [154]. Kahlert and colleagues provided evidence that exosomes isolated from serum of resectable pancreatic cancer patients contain large fragments (>10 kb) of double-stranded genomic DNA with *KRAS* and *TP53* mutations [153]. Noteworthy, recently, investigations have introduced tumoral exosomes as a potential tool for predicting the outcomes of patients. Allenson and colleagues have recently shown data from a large study comparing *KRAS* mutations by the ddPCR system in exosomal DNA and in ctDNA in pancreatic cancer patients at all stages. The detection rate of mutant *KRAS* from exosomal DNA appeared to be greater than ctDNA across all stages, with a sensitivity and specificity of 75.4 and 92.6%, respectively. The study was conducted including two cohorts: the discovery cohort, with 88 pancreatic cancer patients and 54 healthy controls, and the validation cohort, with 39 localized pancreatic cancer patients and 82 healthy controls. In the discovery cohort, exosomal DNA *KRAS* mutations were detected in 66.7, 5, 80 and 85% of localized, localized postsurgical, locally advanced and metastatic pancreatic cancer patients, respectively, and in 7.4% of controls. Conversely, *KRAS* mutations in ctDNA were identified in 45.5, 0, 30.8, 57.9 and 14.8% of these patients, respectively. In the validation cohort, exosomal DNA *KRAS* mutation were detected in 43.6% (17/39) of patients with localized disease and in 20.7% (17/82) of healthy controls. The authors also observed that a higher mutant allele frequency (MAF) of *KRAS* in exosomal DNA was associated with a poor prognosis in patients with localized disease (MAF <1%: 441 days vs MAF >1%: 127 days; p = 0.031) [10]. A further encouraging result is the observation of a drastic reduction in *KRAS* mutations rate in the cohort of pancreatic cancer with blood sampled after resection compared with localized pre-resected patients (5 vs 66.7%). Consequently, plasma *KRAS* detection may be an effective tool to predict early the success of surgery. Finally, as previously reported, a drastical minority of healthy donors showed mutant *KRAS* in exosomal DNA [10,155].

In summary, although to date only few studies have been reported using tumor-related exosomes in pancreatic cancer, results suggest a new source of circulating nucleic acids, in addition to ctDNA or CTCs. Exosomes encapsulation protects DNA and RNA species from degradation in circulation, offering an advantage for PCR based detection of genetic alterations. However, the identification of mutations in healthy subjects suggests that circulating exosomal nucleic acid analyses could allow potential assessment of cancer risk but the detection of pancreatic tumors cannot be assumed. Finally, their prognostic value has yet to be more investigated, as well as their contribution in determining the early success of resection or response to any therapy.

Future perspective and conclusion

Recently, the explosion of research in the field of circulating biomarkers has also affected pancreatic cancer. Indeed, several tumor components as CTCs, ctDNA and tumor-related exosomes released by tumor into the circulation can be detected in pancreatic cancer patients, providing an alternative route for screening and diagnosis, as well as for selecting the best treatment regimen, longitudinally monitoring the response of tumor to therapy and evaluating the clinical outcome [10,60,61,90,92]. Circulating biomarkers able to identify the amount of residual tumor could be helpful in monitoring specific clinical situations, such as response to medical treatment in locally advanced disease, in order to plan subsequent local treatment. Another application of circulating biomarkers could be the implementation of prognostic markers in resected pancreatic cancer with the purpose of optimizing adjuvant treatment as under investigation in other tumor types (NCT03637686).

However, there is no clear consensus on which circulating biomarker could be used and, therefore, may be translated into clinical practice. Most likely, the main reasons for diverging data reside in the small sample size of the majority of the studies, in the enrollment of heterogeneous population, as well as in the use of different technological approaches that are generally employed to investigate circulating biomarkers.

In conclusion, we are convinced that the in-depth study of circulating biomarkers is the most appropriate path that researchers must travel, in order to preclude the need for direct tumor sampling, complement conventional monitoring approaches and, therefore, achieve a more effective management of pancreatic cancer patients.

Executive summary

- In the scenario of pancreatic cancer, imaging procedures are used for disease staging and to assess the effectiveness of the locoregional and systemic therapies; while the CA 19-9 and other serum markers are usually used to predict prognosis and monitor the tumor response to therapies.
- Since they have several limitations, circulating tumor biomarkers represent promising noninvasive tools for predicting and longitudinally monitoring response to treatment and disease progression.

Liquid versus tissue biopsy
- In clinical practice, cancer-related molecular alterations are investigated using tumor samples from surgical or biopsy specimens.
- In the specific context of pancreatic cancer, tissue biopsy creates several barriers, in terms of sample acquisition (e.g., due to the anatomical retroperitoneal position of the pancreas), as well as for the characterization of the entire tumor heterogeneity (e.g., a higher amount of stromal cells than tumor cells).
- Liquid biopsy use has also emerged for pancreatic cancer, becoming the tool to identify circulating biomarkers in biofluids (e.g, circulating tumor cell [CTCs], circulating tumor DNA [ctDNA], exosomes).

CA 19-9 and other traditional serum markers
- CA 19-9 and other traditional serum markers have several limitations, including: sensitivity and specificity; changes in serum levels unrelated to disease progression may occur; inadequacy in the differential diagnosis of early tumors and precancerous lesions.

Circulating tumor cells

Methodological issues
- Sophisticated tools have been developed for efficient enrichment (e.g., density gradient centrifugation, immunomagnetic beads) and detection of CTCs (e.g., genomic, transcriptomic and proteomic approaches), respectively.
- Several issues in CTCs detection and isolation, leading to extremely varying results, should be resolved for their clinical applications.

Diagnostic role of CTCs
- Several findings reported that CTCs analysis has sufficient and high sensitivity (70%) to detected stage I and II pancreatic cancer. Moreover, CTCs are able to identify with a sensitivity of 30% patients with pre-adenocarcinoma disease.
- Even if studies with a higher number of patients are needed, CTCs analysis may be useful for early-stage pancreatic cancer diagnosis.

Prognostic and predictive role of CTCs
- Several lines of evidence support CTCs detection as a useful tool for prognosis in pancreatic cancer patients, while other findings have not demonstrated a significant correlation between CTCs presence and survival.
- Rigorous evaluation of larger, prospective clinical trials is warranted to better understand the clinical implications of CTCs.

Circulating-free tumor DNA

Methodological issues
- The concentration of ctDNA in biological fluids is very low and varies considerably among different subjects, depending also on type and dimension of the tumor burden.
- High-analytical sensitivity and specificity are required for both ctDNA extraction (e.g., commercial kits and company in-house procedures) and detection (e.g., automatic sequencing and digital PCR).
- The lack of standardization of techniques remains the major challenge in the ctDNA field.

Diagnostic role of ctDNA
- ctDNA analysis seems to be of little use in the diagnosis of pancreatic cancer, especially due to the low abundance of ctDNA detectable in early stages.
- Nevertheless, the combination of ctDNA KRAS mutations and CA 19-9 had a sensitivity and specificity of 98 and 77%, respectively. Additionally, CA 19-9 was superior to ctDNA for detection of pancreatic cancer in low burden disease.

Prognostic and predictive role of ctDNA
- ctDNA could be a useful tool to detect genetic alterations in pancreatic cancer patients.
- Several studies show how ctDNA is a valuable biomarker either as predictor of disease recurrence and prognosis and as predictor of response or resistance to treatment.

Exosomes as a new source for circulating tumor DNA and RNA
- Existing exosome isolation techniques present challenges to researchers (e.g., contaminations from other extracellular vesicles).
- The development of standardized technique for isolating high, relatively pure concentrations of exosomes should be considered, especially for a routine use in clinical practice.

Diagnostic role of exosomes
- Due to the ability to carry cargo with disease-specific nucleic acids and proteins, exosomes seem to be promising as screening and diagnostic tools in pancreatic cancer.

Functional significance of exosome-derived DNA and RNA and their role in prognosis and treatment monitoring
- Circulating exosomes, extracted not only from pancreatic cancer cells but also from plasma/serum of patients, contain a repertoire of molecular cargo, including DNA, mRNA, miRNAs and proteins.
- Future studies should evaluate their prognostic value, as well as their contribution in determining the early success of resection or response to any therapy.

Future perspective and conclusion
- Several tumor components as CTCs, ctDNA and tumor-related exosomes released by a tumor into the circulation can be detected in pancreatic cancer patients, providing an alternative route for selecting the best treatment regimen, longitudinally monitoring the response of tumor to therapy and evaluating the clinical outcome.
- A deeper knowledge of which circulating biomarker could be used and its prognostic and/or predictive role may significantly contribute to evidence-based decision making in the routine clinical practice.

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