



# Diagnostic interest of liquid biopsy in the management of early stage pancreatic ductal adenocarcinoma

Etienne Buscail

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**Docteur de**

**L'université de Bordeaux**

École doctorale Sciences de la Vie et de la Santé

Biologie cellulaire et physiopathologie

**Par Etienne BUSCAIL**

**INTÉRÊT DIAGNOSTIQUE DE LA BIOPSIE LIQUIDE DANS  
LA PRISE EN CHARGE DE L'ADÉNOCARCINOME  
CANALAIRE DU PANCRÉAS À UN STADE PRÉCOCE**

Sous la direction du Pr. Laurence Chiche

Co-encadrement : Pr. Sandrine Dabernat

Soutenue le 14 Juin 2019

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M MERLIO Jean-Philippe	PUPH	Université de Bordeaux	Président
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Mme ALIX-PANABIERES Catherine	MCUPH	Université de Montpellier	Membre invité



« Il n'y a pas d'enthousiasme sans sagesse, ni de sagesse sans générosité »

Paul Eluard



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## 1-Objectifs de thèse

### 1-1 Justification scientifique et description générale

#### *1-1-1 Etat actuel des connaissances sur la pathologie*

Le cancer du pancréas (CP) est l'un des plus létaux avec un taux de survie à 5 ans <5%, tous stades confondus. Selon l'Institut national contre le cancer, il y a eu plus de 11000 cas en 2012 soit une augmentation de plus de 4,7%/an entre 2005 et 2012. Son incidence augmente encore et il pourrait devenir la deuxième cause de décès par cancer d'ici 2025 en Europe et aux Etats Unis[1]. Le pronostic reste mauvais malgré les progrès du diagnostic par imagerie à haute résolution et les traitements avec de nouveaux protocoles de chimiothérapie (gemcitabine, FOLFIRINOX et nab-paclitaxel). En effet, une grande majorité des patients (85%) consultent tardivement pour des tumeurs localement avancées et / ou avec des métastases. Ceci s'explique par l'absence de symptômes spécifiques et de marqueurs précoces pour cette maladie par ailleurs très agressive. Pour ces patients, la survie globale a doublé au cours des 15 dernières années (la survie médiane est passée de 6 à 12 mois), mais la survie à 5 ans reste inférieure à 3%[2,3].

#### *1-1-2 Etat des connaissances sur les traitements/stratégies/procédures de référence et à l'étude.*

A côté du caractère agressif du CP se pose en pratique clinique le problème majeur du temps de latence entre la suspicion du cancer et la mise en place du traitement. En particulier, dans le cadre de traitements néoadjuvants à la chirurgie, une preuve histologique est obligatoire avant d'engager tout traitement, malgré les progrès de l'imagerie médicale. Or, le délai d'obtention d'une réponse non ambiguë peut encore aggraver le retard de la prise en charge. De plus les données de la littérature



confirment que l'allongement du délai de prise en charge diagnostique grève le pronostic[4,5]. L'écho-endoscopie ponction est l'examen de référence pour l'obtention de cette preuve histologique avec une sensibilité variant de 75 à 98 % et une spécificité variant de 71 à 100% [6]. Néanmoins, sa valeur prédictive négative varie selon les séries de 33 à 85%. Ceci rend compte d'un taux de faux négatifs de 50% en moyenne, impliquant des biopsies itératives morbides et des anesthésies répétées[6]. De plus, le rendement de l'écho-endoscopie avec ponction est influencé par l'expérience de l'opérateur et la taille de la tumeur. Par ailleurs, le seul marqueur circulant biologique actuellement utilisé en routine est le dosage du CA 19-9 sanguin, mais il n'est pas recommandé par la HAS pour le diagnostic car peu sensible et peu spécifique respectivement 68 et 70%[7].

## 1-2 Hypothèses de la recherche et résultats attendus

Les tumeurs primaires relarguent dans le sang des fragments de la tumeur primaire comme les cellules tumorales circulantes CTCs, des acides nucléiques ou des EVs, qui témoignent de sa présence, et dont l'identification et la quantification constituent un nouveau champ d'analyse de biomarqueurs circulants appelé biopsie liquide[8], lorsqu'ils sont recherchés dans les fluides biologiques comme le sang.

La détection de CTCs, de ctDNA et d'exosomes GP1C<sup>+</sup> pourrait constituer une biopsie liquide, non invasive, facilement réalisable en amont ou en absence d'examen anatomo-pathologique concluant pour le diagnostic de l'adénocarcinome pancréatique. De nombreuses publications récentes concernant le cancer du sein, le cancer colorectal et le cancer de la prostate ont montré que la recherche de CTCs avec la méthode CellSearch® (approuvée FDA–USA)[9] était un outil très prometteur et très performant pour l'évaluation du pronostic des patients [10,11]. De plus, l'identification de mutations dans les CTCs est utile pour prédire la résistance au

traitement de certaines tumeurs comme cela a été démontré pour le cancer du sein [12]. Ce test pronostique est actuellement implanté en routine dans la pratique clinique et approuvé par la FDA pour le monitoring du cancer du sein, prostate et colon[13]. Des études récentes ont montré que des CTCs sont détectables dans le cancer du pancréas mais lorsque ce travail a débuté, il y avait peu de données sur la sensibilité et la spécificité de la détection de CTCs dans le sang périphérique et portal chez les patients résécables d'emblées [14,15].

L'ADN tumoral circulant (ctDNA) dans le plasma est une cible potentielle non invasive pour le diagnostic du CP. Le ctDNA, qui porte des mutations génétiques spécifiques, a été utilisé pour analyser les altérations de séquences somatiques dans divers cancers grâce au séquençage de nouvelle génération (NGS) ou à la PCR digitale (ddPCR)[16]. Récemment, plusieurs études ont montré la faisabilité et la valeur clinique de l'utilisation du ctDNA pour dépister et détecter d'éventuelles altérations génétiques dans de multiples cancers[17]. Mais peu d'études avaient étudié l'utilité clinique du ctDNA dans le cancer du pancréas en dehors des stades avancé et métastatique[14].

Les exosomes, sécrétés par toutes les cellules saines et cancéreuses, contiennent des biomolécules fonctionnelles (y compris des protéines, des acides nucléiques et des lipides). Ils se distinguent des corps apoptotiques et des microvésicules par leur taille, leur origine et leur composition hétérogène. Les exosomes de 30-150 nm de diamètre sont des vésicules extracellulaires générées par le bourgeonnement de la membrane vers l'intérieur (endocytose), ce qui conduit à la formation de corps multivésiculaires et à leur libération par exocytose. Les exosomes sont des médiateurs importants pour les communications intercellulaires. Ils régulent le

microenvironnement de la cellule émettrice, mais peuvent être capté par des cellules cibles d'organes distants[18]. En cancérologie, il a été démontré que les exosomes peuvent promouvoir le développement du cancer, stimuler la transition épithélio-mésenchymateuse, en particulier pour le cancer du pancréas, stimuler l'angiogénèse, activer les fibroblastes dans le stroma, générer une niche pré-métastatique et inhiber les réponses immunitaires de l'hôte[19, pour revue]. Ces fonctions sont probablement liées au contenu spécifique des exosomes. Ils sont caractérisés par des marqueurs spécifiques de leur nature exosomale, mais aussi du type cellulaire qui les a émis. De façon très intéressante, ils peuvent aussi porter des marques de l'état physiopathologique de la cellule émettrice. Le diagnostic et le suivi du cancer par le biais des exosomes ont été largement encouragés en raison de l'apparente facilité d'isolement et d'identification des exosomes dans les fluides corporels, en particulier le sang périphérique[20]. Dans ce sens, la publication de Melo *et al*[21], a permis d'identifier des exosomes Glypican-1<sup>+</sup> (GPC1<sup>+</sup>) comme marqueurs diagnostiques, prédictifs et pronostiques de l'adénocarcinome canalaire pancréatique. Le GPC1 est un protéoglycane de type héparane sulfate normalement présent à la surface cellulaire. Les héparan sulfates sont des coactivateurs de voies impliquant des facteurs de croissance dépendants la liaison à l'héparine (comme les FGFs). Ils sont surexprimés dans les cancers du pancréas. Ces protéines jouent un rôle dans le contrôle de la division cellulaire et de la régulation de la croissance[21].

Ainsi, la détection de l'ensemble des éléments de biospie liquide (CTCs, ctDNA, exosomes GP1C<sup>+</sup>) dans le sang de patients atteints d'adénocarcinome pancréatique semble être une approche particulièrement prometteuse à ajouter dans l'arsenal pauvre des outils diagnostiques actuellement disponibles.

### 1-3 Objectifs de la recherche

#### *1-3-1 Objectif principal*

L'objectif principal était d'estimer la sensibilité diagnostique des méthodes CellSearch®, Oncoquick® et Rosettesep<sup>TM</sup> pour la détection de CTC, la méthode ddPCR pour la détection d'ADN tumoral circulant (recherche de KRAS muté) et la détection/quantification des GP1C+ dans le sang portal et périphérique, dans le diagnostic de cancer du pancréas, chez les patients avec suspicion de cancer de la tête du pancréas résécable d'emblée.

#### *1-3-2 Objectifs secondaires*

Les objectifs secondaire étaient :

- D'estimer les performances diagnostiques des différentes procédures de détection des CTCs, de ctADN et GP1C+ dans le sang périphérique dans le diagnostic du cancer du pancréas.
- De comparer les performances diagnostiques des différentes procédures entre elles dans le sang portal. Comparer les performances diagnostiques des procédures selon si elles portent sur le sang portal ou le sang périphérique
- De comparer les éléments tumoraux détectés avec les paramètres clinico-biologiques en lien avec et les outils diagnostiques conventionnels utilisé pour le suivi de la maladie (évaluation TDM de la tumeur taille et envahissement locorégional et métastatique, dosage CA 19-9)
- D'évaluer l'association entre l'évolution du patient à 15 mois (décès, récurrence, localisation secondaire) et le nombre de CTC détectées la quantité de ctDNA et d'exosomes GP1C+.

## Présentation du manuscrit

Le manuscrit se présente sous la forme de quatre articles :

-Deux articles de revues :

le premier article étudie la littérature sur les modalités diagnostiques du CP. Une première partie de dernier effectue un rappel des modalités diagnostiques avec les outils de la pratique clinique courante (ie dosage du CA 19-9, scanner, IRM, écho-endoscopie ponction). Nous avons ensuite fait une revue de la littérature sur les travaux fait sur l'étude des éléments circulant (ie ct DNA, CTC, onco-exosomes et tumor educated platelets) et leurs valeurs diagnostique et pronostique dans le CP.

Le second article étayant l'intérêt d'effectuer des biopsie liquide au plus proche de la tumeur. En effet eu égard au caractère rare des éléments circulants plusieurs travaux ont eu comme hypothèse de ponctionner les vaisseaux de drainage des organes atteint. ACet article détaille la littérature concernant l'adénocarcinome canalaire du pancréas du cancer colorectal et la ponction de la veine porte, le carcinome hépatocellulaire et la ponction de la veine porte et des veines sus-hépatiques enfin le cancer du poumon non petites cellules et la ponction des veines pulmonaires.

-Deux articles originaux :

Nous avons conçu un essai clinique prospectif (PANC-CTC# NCT03032913) visant à détecter les cellules tumorales circulantes (CTC), l'ADN tumoral circulant (ADNct) et les onco-exosomes chez les patients atteint de CP et chez les patients d'un groupe témoin sans antécédent de cancer, opérés d'une pathologie bénigne en appliquant

différentes méthodes. Pour les CTCs, il s'agissait de l'enrichissement et détection de CTCs par la méthode CellSearch® (méthode de référence approuvée par la FDA), méthode d'enrichissement de CTCs RosetteSep® et OncoQuick® puis quantification de l'ADN tumoral par dd-PCR. Les exosomes ont été isolés puis caractérisés avec le taux d'expression de Glypican-1 (marqueur spécifique du CP *Melo et al. Nature 2015*). Le statut de mutation *KRAS* des tumeurs primaires, a également été recherché sur les pièces opératoires. Tous les patients de l'étude (groupe témoin et groupe CP) ont eu un prélèvement de sang périphérique, les patients du groupe CP ont eu un prélèvement de

sang portal en peropératoire.

Ainsi la première étude consistait à évaluer les performances diagnostique avec la détection des exosomes GPC1 positif et de l'ADN tumoral circulant. De plus nous avons étudié la corrélation du taux d'exosomes GPC1 positif avec les paramètres pronostique clinique et anatomopathologique.

La seconde étude constistait à aborder les résultats de détection des éléments circulant dans son ensemble. Nous avons ainsi étudié l'intérêt d'une approche « combinée de biospsie liquide » à travers trois méthodes d'enrichissement/détection des CTC et le taux d'exosomes GPC1 positifs.

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## **Revue de la littérature : la biopsie liquide dans l'adénocarcinome canalaire pancréatique**

### **Résumé**

Le cancer du pancréas est un problème de santé publique et médical en raison de son incidence croissante, de l'absence d'outils de diagnostic précoce et de son agressivité. Malgré les progrès récents de la chimiothérapie, le taux de survie à 5 ans demeure inférieur à 5 %. Les biopsies liquides sont particulièrement intéressantes d'un point de vue clinique parce qu'il s'agit de biomarqueurs non invasifs libérés par les tumeurs primaires et les métastases, qui reflètent à distance le fardeau de la maladie. Des études pilotes ont été menées chez des patients atteints de cancer du pancréas afin d'évaluer la détection des cellules tumorales en circulation, de l'ADN tumoral circulant, des exosomes et des « tumor educated platelets ». Il existe une hétérogénéité des méthodes d'isolement des éléments tumoraux circulants ainsi que de la cible utilisée pour leur identification. Les performances pour le diagnostic du cancer du pancréas varient en fonction de la technique mais aussi du stade de la maladie : 30 à 50% des tumeurs résécables sont positives contre 50 à 100% dans les cas localement avancés et/ou métastatiques. Une valeur pronostique significative est démontrée dans 50 à 70% des études cliniques pilotes, quel que soit le type de biopsie liquide. C'est un outil prometteur, mais de vastes études prospectives font défaut, y compris des cohortes homogènes de patients atteints de cancer du pancréas. Une approche possible pourrait être la combinaison de plusieurs méthodes pour détecter les éléments tumoraux circulants.

Mots-clés : adénocarcinome canalaire pancréatique ; cellules tumorales circulantes ; ADN tumoral libre de cellules circulantes ; vésicules extracellulaires ; exosomes ; oncogène KRAS ; biopsie liquide.

## **2- LIQUID BIOPSY APPROACH FOR PANCREATIC DUCTAL ADENOCARCINOMA**

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## 2-1Summary

Pancreatic cancer is a public health and medical problem because of its increasing incidence, the absence of early diagnostic tools as well as its aggressiveness. Despite recent progress in chemotherapy, the 5-year survival remains below 5%. Liquid biopsies are of particular interest for a clinical point of view because they are non-invasive biomarkers released by primary tumours and metastases, remotely reflecting disease burden. Pilot studies have been conducted in pancreatic cancer patients evaluating the detection of circulating tumour cells, cell-free circulating tumor DNA, exosomes and tumor-educated platelets. There is a heterogeneity of the methods for isolation of circulating tumor elements as well as the target used for their identification. The performances for the diagnosis of pancreatic cancer vary depending of the technique but also the stage of the disease: 30 to 50% of resectable tumor are positive versus 50 to 100% in locally advanced and/or metastatic cases. A significant prognostic value is demonstrated in 50 to 70% of pilot clinical studies, whatever the type of liquid biopsy. It is a promising tool, but large prospective studies are lacking, including homogeneous cohorts of pancreatic cancer patients. One possible approach might be the combination of several methods for detecting circulating tumour elements.

**Keywords:** pancreatic ductal adenocarcinoma; circulating tumor cells; circulating cell free tumor DNA; extracellular vesicles; exosomes; *KRAS* oncogene; liquid biopsy.

## 2-2Introduction

Pancreatic ductal adenocarcinoma (PDAC) cancer is a real public health problem and a medical and scientific challenge because of its increasing incidence, the absence of reliable early biomarkers, the absence of preventive screening and efficient therapies to defeat these aggressive and highly heterogeneous neoplasms [1–3]. The only curative treatment is surgery, which is only possible in 15% of cases. For other patients, the tumour is already metastatic at time of diagnosis or locally advanced. Despite application of chemotherapy protocols such as FOLFIRINOX, which increases survival after palliative or adjuvant therapies, the 5-year survival remains below 5% [4–6]. In parallel with the search for new treatments, several challenges must be undertaken to help alleviate the dismal prognosis of PDAC, one of which is to discover biomarkers to ensure early detection, make a meaningful prognosis and help predict recurrences. The final wish will be to address remotely all these questions, without the need of invasive, painful and risky procedures. In this context, real-time liquid biopsy is an emerging tool of particular interest from scientific and clinical points of view because complementary circulating biomarkers are released by the tumour and its metastases and therefore distantly reflect the disease [7–10]. They are obtained in non-invasive approaches and they allow diagnosis and molecular follow-up of patients. They are already used in practice for some epithelial cancers to monitor patients in their therapeutic management[11,12].

The purpose of this review is to address all molecular, technological and clinical aspects and issues of the liquid biopsy applied to PDAC. Circulating Tumour Cells (CTCs), cell-free circulating tumor DNA (cfDNA), circulating tumor extracellular vesicles (e.g. exosomes) and tumor educated platelets (TEPs) are particularly

discussed, because they provide pragmatic perspectives on the application of promising technologies to the manage patients with all stages of PDAC.

### 2-3 Current diagnosis for pancreatic cancer:

PDAC diagnosis usually relies on information obtained using sequential procedures, including imaging data (ultrasonography, computerized tomodensitometry - CT, magnetic resonance imaging - MRI and endoscopic ultrasound - EUS). Blood markers are lacking and the only one used is the carbohydrate antigen 19-9 (CA 19-9) that displays a low sensitivity and specificity to assume the diagnosis of PDAC. If CA19-9 has acceptable performances in advanced and symptomatic tumours (sensitivity  $\approx$  80%; specificity  $\approx$  82%), these performances fall rapidly in small non-metastatic lesions[13]. The different imaging techniques aim to detect a pancreatic tissue mass and to ensure the extension assessment: locally by examining the possible venous and arterial vascular invasion but also at a distance in search of metastases, in particular peritoneal and hepatic. These are sometimes detected by the diffusion. MRI. At the end of this extension assessment, the tumour will be classified as locally advanced non-resectable (25% of patients at the time of diagnosis), metastatic (50%), borderline (10%) or resectable (15%). The performance of the CT and the MRI are generally equivalent for the diagnosis and assessment of pancreatic cancer staging[14]. The CT is more particularly effective for the diagnosis of tumor unresectability. However, in the majority of cases (except for resectable tumors) a pathological confirmation is needed after either fine-needle aspiration of metastasis or EUS-guided fine-needle aspiration (EUS-FNA) of primary tumors. More than 20 years of EUS experience now allows safe guided FNA biopsies of solid pancreatic lesions for cytopathological analysis[3,15]. EUS-FNA is thus now an effective technique to

diagnose and assess the staging of PDAC, especially tumor less than 2 cm in size [16–18]. However, its performances (and those from other imaging techniques) greatly depend upon the operators' experience and certainly on the nature of the PDAC, depending on the importance of stroma. In other terms, more the tumor stroma is abundant, less the carcinomatous cells will be present in EUS-guided microbiopsies samples. Accuracy of EUS-FNA to diagnose malignancy varies widely, with a sensitivity ranging from 65–95% and a mean accuracy of 85%. In addition, the negative predictive value still ranges from 50–70% and the EUS-FNA may be inconclusive or doubtful in up to 20% of cases [16,19,20] of PDAC. Inconclusive specimens can be defined as the presence of coagulum with normal cells or acellular samples. Doubtful samples can be defined by the presence of atypia and/or low-grade dysplasia and/or atypical for malignancy. Recently, technical or clinical improvements to EUS have been developed such as elastography, contrast-enhanced EUS and on-site pathologist. Each procedure may help EUS for tissue characterization [14,21] as well as differentiate between PDAC and neuroendocrine tumor, lymphoma or autoimmune pancreatitis [16,17]. Nevertheless, in case of a highly suspected PDAC, the negative result of EUS-FNA remains problematic. A second EUS-FNA is thus needed that implies a delay in the patient management, which is known to negatively influence the prognosis of PDAC [22]. In addition, there is the problem of the differential diagnosis between PDAC and chronic pancreatitis in its pseudo-tumoural form and, more rarely, with autoimmune pancreatitis. In these cases, histological diagnosis is also crucial to avoid unnecessary surgery.

To assist in cytopathological diagnosis, the research and characterization of new molecular markers is always active. Nevertheless, since the oncogenic point mutation of *KRAS* is a frequent event during PDAC, the identification of this mutation in tumour



tissues may give an aid to the diagnosis. We and others demonstrated the *KRAS*-mutation analysis, when is performed on EUS-FNA materials, appears to be highly accurate at differentiating benign versus malignant pancreatic solid lesions [19,20,23]. In front of solid pancreatic masses, by combining the results of the *KRAS*-mutation assay with cytopathology, one can obtain an increased sensitivity and accuracy compared to cytopathology alone to diagnose [20,24–26]. More important is that the negative predictive value of cytopathology alone for this indication is increased from 67–88% when combined with a *KRAS* mutation assay [20,24–26]. *KRAS* mutation detection has been also performed after extraction of circulating cell free tumor DNA or isolation of exosomes. This hope lies in obtaining a non-invasive, reliable and reproducible "biological witness" of the presence of PDAC, whatever its stage. We will discuss the different liquid biopsy potential applicable to PDAC by trying to identify performance, weaknesses and prospects.

#### 2-4 Circulating tumor cells-based diagnosis of pancreatic cancer:

Clinical utility of CTC detection for cancer patients has regained interest during the late 90's, until being considered as *liquid biopsy* [10]. Progresses made for their capture/identification have relied on technical advances. Numerous CTC detection methods have been described. Shortly, they rely on a first step of CTC capture/enrichment based on their biological (immunological positive or negative selection) or physical properties (size, deformability, cell density), followed by a second detection step based on immunocytochemistry, molecular biology or a functional assay [27]. Liquid biopsy can be performed in several body fluids, but application to pancreatic cancer detection was mainly performed in total peripheral blood.

Originally liquid biopsy applied to pancreatic cancer tested methods combining density centrifugation and RT-PCR detection of tumor markers, which were already known to bear poor specificity as circulating tumor biomarkers. This is exemplified by low detection rate with *CEA* mRNA detection (26%, [28]), cytokeratine 20 (CK20, 34%, [29]) or EpCAM (25%, [30]). These low detection rates could not be justified by higher percentages of patients recruited with early stages ([28,29]), except for *EpCAM* RNA-based detection, since most patients were eligible for up-front surgery (83%, [30]). With increasing available tools allowing for the specific capture of epithelial cells in total blood, numerous studies have tested combined immunocytochemistry detection of tumor markers on isolated cells. First, the CellSearch® system, currently considered as the gold standard method because cleared by the FDA-USA for metastatic breast, colon and prostate cancer, has been tested in several studies. PDAC diagnostic was made for 11% to 48% of patients in cohorts including always at least 53% of patients with locally advanced or metastatic diseases (Table 1)[28–42]. Even when 100% of the patients have advanced disease, rates of CTC detection don't increase (for example 32.3%[38]). Expectedly, when 100% of patients were resectable, the number dropped to less than 7% [41]. These quite disappointing results were usually explained by the fact that the CellSearch® system is based on EpCAM and cytokeratin expression. These epithelial markers are down regulated and might be even lost during the Epithelial Mesenchymal Transition (EMT,[43,44]), which is a known for CTCs [45]. To limit this biased detection, CTC enrichment in blood samples has been tested using CTC physical properties. The Isolation by Size of Epithelial Tumor cells method (ISET,[46]) might be a good alternative for CTC detection, unbiased by the needed presence of cell surface EpCAM. After whole blood cell filtration on a microporous membrane, retained cells are stained by routine histology with or without pan-

cytokeratine immunocytochemistry. CTC identification and counting is performed by pathologists trained to identify the specific morphology of tumor cells [46]. In a cohort with more than 80% of metastatic patients and results available for both methods for 27 patients, the detection rate reached 93% for ISET as compared to only 40% for CellSearch® with higher mean numbers of CTCs (6 vs 26 CTC/7.5mL, Table 1). Another cohort including mainly patients with early stage disease, found 78% of PDAC [36], even if pan-cytokeratin positivity was associated with CTC detection. The ScreenCell® filtration method identified 67% of PDAC patients including 72% of advanced diseases, with cytomorphologic criteria after filtration [37,42]. The latter study included the detection of mutant *KRAS* in CTCs, with high discrepancy between tumor and CTC status. Indeed, whereas 97% of tumors carried mutant *KRAS*, 18% of the CTCs were found to carry *KRAS* wild type allele only. Even CTCs from 5 out of the 12 metastatic tumors were *KRAS*<sup>WT</sup>. So, it is possible that cytomorphological-based CTC identification of filtered cells might falsely consider epithelioid or endothelial cells as epithelial tumor cells, leading to general CTC overestimation. This hypothesis is supported by a report analyzing 171 blood samples from patients with various pancreatic diseases (including 63% of PDAC, [47]) and 9 healthy controls. The 9 healthy controls were free of circulating epithelioid cells (CEC), but of the 115 patients with CECs 25 (15%) had nonmalignant diseases. Morphologic characteristics of malignant CECs were undistinguishable from non-malignant CECs. In addition, CECs were also detected in inflammatory benign colonic diseases [48], suggesting that in specific cases only, these methods might detect cells shed by primary tumors as well as by benign lesions. (We may also argue that looking for *KRAS* mutation, that is detectable very early during PDAC oncogenesis, may help for the early detection of disease, eligible to surgery, before tumors grow too big of even disseminate).

Remarkably, most studies correlate the presence and/or number of CTCs with clinical parameters (Table 1). As expected, high CTC numbers signed metastatic diseases [34] and worse overall or progression free survival [36–38,40,41]. When sensitivity of detection is low (11%), CTC positivity correlates with adenocarcinoma differentiation [49].

Overall CTC-based diagnostic of PDAC is highly specific, since most studies with healthy control groups report close to 0 false-positive results (except for 3.6% in [34]). However, sensitivity suffers from the rarity of CTCs not efficiently captured/enriched by current available methods. Filtration and morphologic-based CTC identification carries the risk of overinterpretation [47]. However, whatever the type of tumor (resected, locally advanced or metastatic) the presence of CTCs has reflected a negative influence on the prognosis of PDAC patients, the presence of metastases or the prediction of recurrence. As shown in Table 1, 12 out of 17 studies concluded that the presence of CTCs had an adverse effect on survival. This is also illustrated in figure 1.

## 2-5 Circulating tumor DNA for diagnosis and prognosis of pancreatic cancer

The ctDNA originates from necrotic or apoptotic cells but also can be actively secreted by cells. CtDNA is highly fragmented with a median size of 170 base pairs, which corresponds to internucleosomal DNA fragments. CtDNA is qualified as cell-free, circulating tumor DNA by the presence of mutations that are specific of cancer cells [9]. Few studies investigated the methylation, microsatellite instability and allelic imbalance [7,50]. In contrast, the detection of *KRAS* mutation in plasma and serum appears the most widely used approach [7,51–73]. Various effective methods have been developed for *KRAS* mutation analysis and replace now direct sequencing or other methods with a pre-amplification step such as RFLP. All these new methods

include q-PCR methods, allele-specific PCR using amplification refractory mutation system technology or co-amplification at a lower denaturation temperature, PCR methods, pyrosequencing approaches and real-time PCR methods that use specific probe technologies, such as peptide nucleic acids [58,74–77]. The last one is digital droplet PCR (dPCR) which displays exceptional sensitivity and a low DNA template requirement. For example, considering the sensitivity of different *KRAS* mutation detection techniques (i.e. ratio mutant/wild type *KRAS*) that of direct sequencing is 10 to 30% while that of NGS is 10% and that of dPCR is 0.01% [78,79].

Numerous studies have been conducted to investigate the role of circulating DNA in patients with PDAC. The main studies that included more than 20 patients are compiled in Table 2 [51–73,80]. Of the 24 studies, only 13 included patients with benign pancreatic lesions or healthy subjects. Most studies have applied the detection of the *KRAS* oncogene mutation to identify circulating tumor DNA. Two questions arise with regard to the detection of circulating DNA: does it have a diagnostic value (and/or detection of early lesions)? does it have a prognostic value?

It seems that mutated *KRAS* detection in blood still has limited value for early tumors or micrometastatic disease detection, arguing either for the lack of ctDNA release at these disease stages, or because the concentration of ctDNA is so low and its composition so degraded, that detection requires more sensitive nucleic acid processing and analysis technologies. In the specific context of the PDAC diagnosis, despite the use of the most efficient techniques such as dPCR, there is the problem of sensitivity of *KRAS* assay. Indeed, concordance studies have been carried out between the presence of the *KRAS* mutation in the primary tumour and the search for the mutation in ctDNA: the concordance varies from 25 to 75 % and finally the sensitivity of this approach depends strongly on the tumour [7,50]. If we analyse the

results displayed in Table 2, the presence of *KRAS* mutation in ctDNA is observed in near 70 to 80 % of locally advanced and metastatic patients while this value ranging from 30 to 68 % for patients with resectable tumours. One simple explanation is that the quantity of ctDNA might depend on the number of PDAC cells as well as metastasis. However, this postulate remains to be proven. In addition, and as discussed above, the sensitivity appears better when applying dPCR (43 to 78%) when compared to classical PCR or sequencing (from 27 to 47%) (Table 2).

Several groups, including ours, have investigated whether the presence or absence of *KRAS* mutation can influence the prognosis of PDAC [81–86]. Biological samples are varied and include tumour tissues, blood, plasma or EUS-FNA. Overall and whatever the type of biological sample, the presence of *KRAS* mutation has a negative influence on the prognosis of PDAC patients whether or not they undergo surgery (with complete tumour resection or locally advanced and/or metastatic PDAC). As shown in Table 2, 16 out of 26 studies concluded that the presence of a *KRAS* mutation had an adverse effect on survival (figure 1).

Some studies also pointed out that the *KRAS* mutational subtype might also negatively influence prognosis *per se* (such as G12D and G12V) [37,60,85]. A different coupling to the downstream-signalling pathways of the *KRAS* protein, depending on the type of mutation, may explain these results [87–89].

On the whole, the role of ctDNA in the prognosis of PDAC is highly probable. However, new prospective studies are needed, especially including control patient groups (i.e. free of pancreatic disease or suffering from chronic pancreatitis) in order to clearly evaluate the sensitivity, specificity and predictive values of this assay for a possible non-invasive diagnosis of PDAC. One more issue is the variability of the detection assay itself in an absence of “universal” threshold and quantification values. To gain

further insight and reach a definitive conclusion, multicentre studies in a larger (i.e. more than 200 patients), homogeneous cohort of patients (to allow strong multivariate analyses) are certainly needed. These studies must include sequential sampling of blood in order to establish the real reproducibility of the methods and to evaluate the potential performances to assess the either the response to treatment or the possible prediction of recurrence.

Beyond mutation burden, methylation of ctDNA has recently emerged as a promising approach for cancer risk assessment and monitoring, especially for the detection of early tumors. This is particularly true for colon cancer patients, for whom five-gene methylation panel can be used to compensate for the absence of patient-specific mutations to monitor tumor burden [90]. While challenging, this strategy has recently benefited from major technological advances [91], and could be proposed soon for assessing pharmacodynamics in clinical trials or when conventional ctDNA detection or imaging have limitations.

## 2-6 Exosomes-based diagnostic for pancreatic cancer

Circulating tumor extracellular vesicles (EVs), including exosomes, are enriched with many bioactive molecules such as RNA, DNA, proteins lipids and metabolites [92]. Once released from parental healthy or cancer cells, the cargoes reflect the status of the original cell, and has the ability to relay signals between cells. For example, pancreatic cancer-derived exosomes loaded with tetraspanin 8 recruit proteins and mRNA cargo that activate angiogenesis-related gene expression in neighboring non-tumor endothelial cells [93]. EVs can also act distantly. For example, Kupffer cells, the resident macrophages in the liver, have been shown to uptake pancreatic cancer derived exosomes containing the macrophage inhibitory factor (MIF). MIF promotes

TGF- $\beta$  secretion by Kupffer cells, which in turns stimulates the neighboring hepatic stellate cells to secrete fibronectin, and creates local inflammation, considered to be the niche of pancreatic metastases [94]. Thus, due to their actual demonstrated activity, the detection of transported biomolecules protected from degradation by external nuclease or proteases presents an opportunity for both diagnosis and prognosis of cancer.

There is currently no universal method for EV isolation/enrichment from body fluids, although recommendations from the International Society for Extracellular Vesicles have been first listed in 2014, and updated in 2018 [95]. Focusing on studies interested in PDAC, several methods have been described, all of them being complemented by PDAC-specific molecular characterization of the enriched samples. For instance, enrichment by ultracentrifugation with or without sucrose gradients and ultrafiltration, or by kit-based precipitation were reported (Table 3). More specifically, antigen-based exosome capture is also available using the CD63 exosome-specific tetraspanin using microfluidic systems or magnetic beads. As EVs are released by any healthy or diseased cell, additional molecular characterization of the obtained vesicles is needed. Authors have been focusing on bioactive molecules carried by EVs such as nucleic acids and proteins.

As for other types of cancers [96] microRNA (miR) identification has been studied in the context of pancreatic cancer. As presented in Table 3, several, distinct miR signatures were reported. Testing four individuals miRs (miR-17-5p, -21, -155 and -196a) reported that the miR-17-5p and -21 had high diagnostic value with sensitivity and specificity between 72% and 95%. They discarded miR-155 and -196a for low levels of expression in cancer exosomes[97]. Conversely, Xu et al described increased abundance of miR-196a, or miR196b, or miR1246 exosomes in PDAC patients with



AUCs ranking between 0.71 and 0.81. Other authors have found an increase in the expression of miR-191, miR-451a and miR-21 in pancreatic cancer and IPMNs. Diagnostic accuracy was better than CA-19.9 for early stages but around 80%[98]. Using a microarray approach on patient exosome samples, miR-1246, -4644, -3976 and -4306 were individually found increased in PDAC samples[99]. Authors did not report individual specificity and sensitivity, which were not 100% according to published figures, but they found that combining all four miRs detected 9% of healthy controls (false positives) and 80% of PDACs (20% of false negatives).

Prognostic value of miR quantification in exosomes was not evaluated by all authors. Unlike miR-21, the expression of miR-17-5p was correlated with the tumor stages[97], whereas, Goto & *al.* did find miR-21 prognostic of overall survival and chemoresistance [98]. Interestingly, miR-451a was associated with patients with mural nodules in the IPMNs [98], which signs malignancy[100]. Quantifying miR-451a in exosome liquid biopsy could help in decision making for surgery of branch duct IPMNs. On the whole, a prognostic value has been found in 5 out of the 11 studies detailed in table 3 (figure 1).

It should be noted that circulating miR detection has superior sensitivity compared to ctDNA in the surgical setting; indeed, pre-operative plasmatic miR-21 was recently found as a sensitive biomarker and independent prognostic factor in patients with pancreatic cancer undergoing surgical resection [101]. Our group has participated in the demonstration that circulating miR sampled from different sources have biomarker value in preclinical models of PDAC and in patients. Briefly, we generated the first signature of salivary miR sampled from patients with locally advanced pancreatic tumors and found that selected salivary miRs, among them miR-23a, miR-23b and miR-21, differentiate pancreatic cancer patients from patients with pancreatitis and

matching healthy controls [101,102]. Interestingly, in mice models of pancreatic cancer, salivary miR-23a, miR-23b and miR-21 increase precedes tumor burden detection by imaging [102]. In addition, in patients treated by gene therapy, we found that a panel of plasmatic miRs is predictive of response to therapy [103]. Since then, our group has partnered with physicists to device novel nanodevices for the detection and quantification of candidate miRs in patients [104].

MiRs are not the only nucleic acid cargo of the exosomes. Unlike ctDNA, which maximal size ranges from 150 to 170 base pairs [105], exosomes contain >10 kb fragments of double-stranded genomic DNA with detectable KRAS and p53 mutations when obtained from PDAC patients [106]. The difference in length observed between ctDNA and exoDNA is explained by the fact that DNA in exosome is protected from nucleases. The relevance of exoDNA is actually a recent concept, and only a few studies have compared ctDNA and exoDNA diagnostic performances. Of note specificity seems impaired by positivity of digital PCR-based detection of mutant KRAS in exoDNA in non-neoplastic patients in two recent reports. Mutation detection rates were 25% (3/12, [74]) and 7.4% (4/54, [107]). *KRAS* mutation has already been described in a non-negligible proportion of plasma from healthy people (14/394, 3.5%, [108]) possibly reflecting spontaneous rare somatic mutation. As for cfDNA, the high rates obtained in exoDNA might be linked to the highly sensitive detection method of *KRAS* mutants, necessitating interpretation considering the mutant allele frequency (MAF, [74]). Diagnostic performance of exoDNA was somewhat disappointing since diagnostic accuracies ranked between 35% and 69% (Bernard and XX, respectively). However, exoDNA showed relevance in PDAC management since a correlation with non-recurrence survival was found in both studies, but limited to patients with

metastatic disease (ref 2 and 10). Thus, although of good prognostic value, exoDNA based on mutant KRAS detection with highly sensitive detection techniques might not be suitable alone for general population screening as it yields high false positive rates [74,107]. Additional biomarkers such as miRs or proteins should be included in the screening plan to gain in specificity.

Since protein cargo of EVs carry their biological function, diagnostic accuracy might be increased by protein marker identification. The membrane protein heparan sulfate proteoglycan glypican 1 (GPC1) is overexpressed in several types of cancers, in particular in primary tumors of pancreatic cancers [109]. In 2015, a promising study proposed the quantification of GPC1-positive exosomes in peripheral blood to diagnose PDAC with a sensitivity and a specificity of 100% [110]. Moreover, this circulating biomarker had strong prognostic value and could detect pre-neoplastic stages. Later on, exosomal GPC1 alone failed to diagnose PDAC [111]. A signature with 5 exosomal surface proteins including GPC1 showed better sensitivity and specificity than each marker taken separately (86% and 81%, [112]). Using an alternating current electrokinetic (ACE) microarray chip that captures nano-objects including exosomes directly from plasma followed by quantification of CD63 and GPC1 retrieved good but not perfect performance (sensitivity 99% specificity 82%) with false negatives and false positives (ref 5 [113]). Pulling down GPC1-expressing exosome did not increase diagnostic performance of miR-196a and miR-1246 [114]. Discrepancies in exosomal GPC1 validity in detecting PDAC might come from methodological differences. However, details in flow cytometry controls and setting published by Melo et al. raise questions on the validity of the published results. This might explain why no independent study confirmed their results with the same method. Besides GPC1, the zinc transporter protein ZIP4 has recently been studied as an exosomal protein

biomarker. Authors did not report diagnostic accuracy, but AUC was 0.89. Thus, as for GPC1, exosomal ZIP4 led to false-positive and false-negative results [115].

In conclusion, although from recent experimental application, utilization of miRs, DNA or protein signature from exosomes did not find routine application, yet. Exosome isolation and characterization is a major challenge in the field to improve and standardize methods [95]. Another obstacle is the choice of the molecular signature. For instance, exosomes are very rich in differentially expressed miRs, as 119 miRs were 5-fold higher in pancreatic cancer than healthy controls [99,116,117]. Among the numerous targets in each category, only a few have been tested, selected by diverse strategies. Of the cited references here, it seems that combining miR-1246, miR-21, GPC1 and ZIP4 might be very relevant to diagnose PDAC.

## 2-7 Tumor-educated platelets:

The team of Würdinger *et al.* works for many years on how the platelet RNA biomarker signatures can be altered in the presence of cancer [118] and reported an important role of these tumor-educated platelets (TEPs) as *liquid biopsy* in solid tumors (e.g., glioblastoma, non-small cell lung cancer, colorectal cancer, pancreatic cancer, breast cancer, and liver and bile duct carcinoma) [119]. Indeed, it is known that platelets interact with tumor cells affecting tumor growth and dissemination [120]. This interaction affects not only the expression of relevant genes in tumor cells, but also alters the RNA profile of blood platelets called TEPs. Interestingly, tumor-associated biomolecules can be transferred to platelets resulting in their education [118]. External stimuli (e.g., activation of platelet surface receptors and lipopolysaccharide-mediated platelet activation) induce specific splicing of pre-mRNAs in TEPs. Thus, RNA sequencing (thromboSeq technique) performed on 228 blood platelet samples from

patients with different tumor types including pancreatic cancer showed that the location of the primary tumor was correctly identified with 71% accuracy [119]. Moreover, TEPs mRNA profiles could distinguish mutant *KRAS*, *EGFR*, or *PIK3CA* tumors, which is a crucial application in oncology. In conclusion, the ability of TEPs to identify precisely the location of the primary tumor as well as its molecular composition opens a new avenue to use *liquid biopsy* for cancer diagnostic. Finally, an important step will be the demonstration of the clinical utility of TEPs as *liquid biopsy* biosource in large prospective clinical trials.

## 2-8 Conclusions and future directions:

Aims of liquid biopsy is to at least increase the negative predictive value of the gold standard EUS-FNA cytopathology but also to ensure a rapid diagnosis of PDAC in order to early manage and to maintain general status. Others aims are to hopefully identify tools and conditions for a better evaluation of the prognosis as well as a better monitoring of PDAC patients without, before and after surgical resection especially for predicting recurrence. However, there are still some unknowns regarding the use of liquid biopsies in the current practice for PDAC. Indeed, there is an obvious heterogeneity of the methods, regarding either the type of biomarker source "cells, DNA or exosomes" and the molecular target used to identify them. The idea would be to get combined information as an index or an algorithm to get a more precise picture of cancer progression (e.g., quantitative and qualitative). On the other hand, standardization is required before (or after) establishing the real place of liquid biopsies in the management of PDAC. With regard to diagnosis, the results are more convincing in the case of locally advanced and/or metastatic cancers, considering the size of tumour mass and diffusion. Nevertheless, the improvement of detection methods (such

as the dPCR for the detection of the *KRAS* oncogene mutation) and the combination of liquid biopsy types (example of ExoDNA) should allow us to obtain a diagnostic yield that fit to the current clinical practice. Thus, more emphasis on technical validation is required, and projects such as the European CANCER-ID, European Liquid Biopsy Academy (ELBA) and European Liquid Biopsy Society (ELBS) networks or the US-based BloodPAC have been initiated to meet this challenge. These consortia combine the expertise of academic and industry partners and will hopefully lead to the development of robust liquid biopsy assays and inform the design of the trials needed to prove the clinical utility of liquid biopsy testing.

The role of liquid biopsy to assess the prognostic in PDAC is also evident, but in the absence of a significant therapeutic arsenal, the current therapeutic attitude for locally advanced or metastatic cancers will not certainly change. However, this is not to imply that we should sit twiddling our thumbs; our efforts must be doubled to identify biomarkers but also nanodevices for detection in the likely event that progress will be made in the therapeutic management of these advanced forms of the disease.

On the other hand, for patients with a resectable tumor and/or without obvious metastasis, the contribution of liquid biopsies for the diagnosis of small metastases or the prediction of an early recurrence after surgery would be of great benefit. Indeed, surgical decision, the neoadjuvant treatment and the intensification of adjuvant treatment could be thus modified and decided.

In addition, these new non-invasive and repetitive methods of diagnosis could certainly benefit of the combination of several approaches by detecting concomitantly several circulating tumor elements. A representative example is the exosomes that are carriers of tumor DNA, this latter might be representative of tumor spread and metastasis. By this way the “distant activity” of PDAC could be evaluated and hopefully monitored. In

addition, the use of NGS or miR profiling containing specific “molecular signatures” that have been already characterized in pilot studies (as detailed in the present review) may also improve the sensitivity of liquid biopsies in PDAC and could be integrated as a tool for personalized medicine.

The improvement of technology for detection and isolation of CTCs and exosomes will certainly be of importance as well as pilot studies comparing blood samples near the tumor (portal vein in the case of PDAC) and peripheral blood. In addition, isolation of CTC will be also a great model for *in vivo* studies of tumor progression and response to treatment.

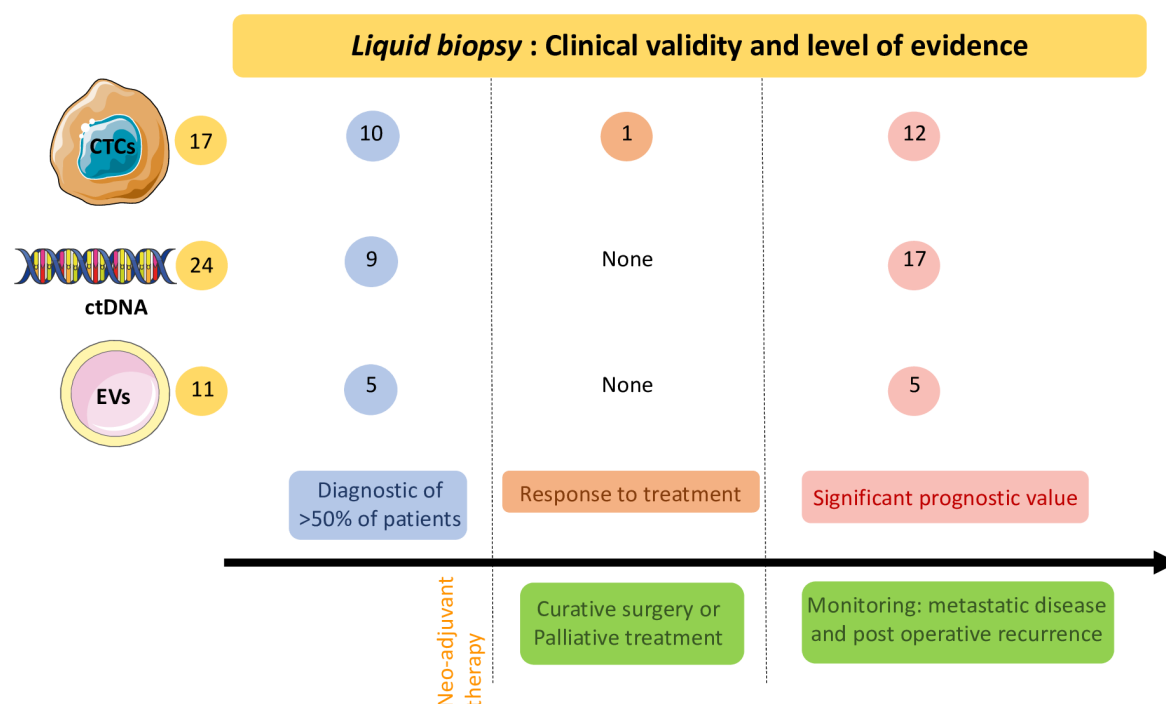
In conclusion, all published studies made the *real-time liquid biopsy* a highly promised clinical tool for the future non-invasive assessment of diagnosis and prognosis of PDAC. Combination of methods will be certainly the key point of these promising modern diagnosis methods.

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**Figure 1: *Liquid biopsy*: Clinical validity and level of evidence**



**Legend for figure 1:**

Clinical validity of circulating tumor elements in pancreatic cancer patients according main published clinical studies. Yellow circles: absolute numbers of publications; Blue circles: number of publications with a significant correlation between diagnosis and the presence of circulating tumor elements; orange circles: publication with a significant correlation between response to neoadjuvant therapy and the presence of circulating tumor elements; light red circles: publications with a significant correlation between prognosis and the presence of circulating tumor elements. (CTCs: circulating tumor cells; ctDNA: circulating tumor DNA; EVs: extracellular vesicles).



## 2-9 TABLES

**TABLE 1:** Results of main clinical pilot studies assessing performances of circulating tumor cells (CTCs) detection in the diagnosis and prognosis of PDAC

PDAC patient number (control)	Type of tumor: Resected, Locally advanced, Metastatic, all	CTC enrichment	CTC detection	CTC count	CTC detection rate in PDAC patients	Prognosis value of CTCs	Reference
20 (15 benign diseases)	All (Samples before treatment)	Density centrifugation	RT-PCR CEA	NA	26%	Positive correlation with recurrence	Mataki et al 2004 [28]
154 (68 benign diseases)	All (Samples before treatment)	Density centrifugation	RT-PCR CK20	NA	34%	Shorter OS (meta.) (p=0.05)	Soeth et al 2005 [29]
25 (15 benign diseases)	All (Samples before treatment)	Immunomagnetic (EpCAM)	RT-PCR: cMET, hTERT, CK20, CEA	NA	80-100% (sens. 100% ; spec. 96%)	Not studied	Zhou et al 2009 [31]
41 (20 HC)	All (Sample before and post treatment)	Immunomagnetic (leukocytes CD45 <sup>+</sup> depletion)	ICC: CK8/CK18 <sup>+</sup> , CA19-9 <sup>+</sup> , CD45 <sup>-</sup>	16	80% before and 20% after chemotherapy	Not studied	Ren et al 2011 [32]
48 (10 CP)	All (Samples before and after treatment)	None	Real-time RT-PCR mRNA EpCAM	NA	25% pre-operative 65% post-operative	No correlation with any outcome	Sergeant et al 2011 [30]
54 (No)	All (Sample time: NA)	Immunomagnetic : ISET and CellSearch®	ISET: Cytology, CD45 <sup>-</sup> ICC: CK <sup>+</sup> , DAPI <sup>+</sup> , CD45 <sup>-</sup>	- ISET: 26 - CellSearch®:6	ISET:93% CellSearch®:40%	No correlation with any outcome	Khoja et al 2012 [33]

79 (No)	LA (Samples before and after chemotherapy)	Immunomagnetic: CellSearch®	ICC: CK <sup>+</sup> , DAPI <sup>+</sup> , CD45 <sup>-</sup>	1 to 15 (only 1 or 2 patients)	11%	Poor differentiation and shorter OS (p=0.01)	Bidard et al 2013 [49]
72 (28 benign diseases)	All (Samples before treatment)	Microfluidic (NanoVelcro)	ICC: CK <sup>+</sup> , DAPI <sup>+</sup> , CD45 <sup>-</sup> , KRAS mutation	0 to ≥ 5 (*)	75%	≥3 CTCs: discriminate metastatic disease (p<0.001)	Ankeny et al 2016 [34]
48 (No)	Metastatic (Samples before treatment)	Immunomagnetic: CellSearch®	ICC: CK <sup>+</sup> , DAPI <sup>+</sup> , CD45 <sup>-</sup> , MUC-1 <sup>+</sup>	23 patients: ≥1 9 patients: ≥2	48%	CTC MUC-1 <sup>+</sup> correlate with a shorter OS (p=0.044)	Dotan et al 2016 [35]
60 (no)	All (40% of the samples performed after neo-adjuvant therapy)	Size based ISET	ICC: CK <sup>+</sup> , ALDH <sup>+</sup> , CD133 <sup>+</sup> , CD44 <sup>+</sup>	Mean: 7.1 Median: 4	78%	CK <sup>+</sup> /ALDH <sup>+</sup> : shorter OS and DFS CK <sup>+</sup> /CD133 <sup>+</sup> /CD44 <sup>+</sup> : shorter DFS	Poruk et al 2017 [36]
58 PDAC (10 HC)	All (samples time NA)	Size based: Screencell©	Cytology KRAS mutation	Range 0-13	67%	> 3 CTC <sup>+</sup> : shorter OS	Kuleman et al 2017 [37]
52 (10 benign diseases)	All (samples time NA)	Size based Screencell©	Cytology	Median 4 Range 0-151	67%	no correlation	Sefroui et al 2017 [42]
65 (15 HC)	LA and Meta. (Samples before treatment)	Immunomagnetic CellSearch®	ICC: CK <sup>+</sup> , DAPI <sup>+</sup> , CD45 <sup>-</sup>	4.9	32.3%	Independent predictor of shorter OS	Okubo et al 2017 [38]
100 (26 benign diseases)	All (32% of the samples after neo-adjuvant therapy)	Microfluidic Nano-velcro	ICC: CK <sup>+</sup> , DAPI <sup>+</sup> , CD45 <sup>-</sup>	NA	78%	Correlated with presence of occult metastasis, shorter DFS and OS	Court et al 2018 [39]
69 (9 benign diseases)	All (10% of the samples after neo-adjuvant therapy)	Immunomagnetic MACS and CellSearch® (n=20)	ICC: CK <sup>+</sup> , DAPI <sup>+</sup> , CD45 <sup>-</sup>	17 patients >1 13 patients > 2	33.3%	Independent predictor of shorter PFS and OS	Effenberg et al 2018 [40]
242 (No)	All (sample time NA)	Immunomagnetic CellSearch®	ICC: CK <sup>+</sup> , DAPI <sup>+</sup> , CD45 <sup>-</sup>	Median 1 Range 1-33	78.5%	Shorter DFS (p<0.001)	Hugenschmidt et al 2018 [41]

CTCs: circulating tumor cells; LA: locally advanced PDAC; Metastatic: metastatic PDAC; All: Resected + Locally advanced + Metastatic PDAC patients; NA: not available; HC: healthy control; CP: chronic pancreatitis; MACS: magnetic activation cell search; ISET: isolation by size of epithelial tumor cells; ICC: immuno-cytochemistry; DAPI: 4', 6-diamidino-2-phénylindole as fluorescent protein linking to thymine and adenine DNA bases; OS: overall survival; PFS: progression free survival; NA: non available; MUC: mucin; EpCAM: epithelial cell adhesion molecule; ALDH: aldehyde dehydrogenase.

CTC count is mostly expressed as mean

(\*) : 18 patients :0 CTC, 54 patients: ≥1 CTC, 39 patients: ≥2 CTCs, 29 patients: ≥3 CTCs, 18 patients: ≥5 CTCs.

**TABLE 2:** The main studies that have investigated the role of ctDNA in diagnosis and/or prognosis of pancreatic ductal carcinoma

PDAC patient number (Control)	Type of tumor: Resected , Locally advanced , Metastatic, all	Site	Target for ctDNA	% of mutations or genetic alterations in PDAC patients	Diagnosis Performances	Positive correlation with a poor prognosis (OS) ( $p$ )*	Reference
44 (60: 37 CP and 23 miscellaneous)	All	Plasma	<i>KRAS</i> mutation Amplified PCR	27	Sensitivity: 27% Specificity: 100%	Yes - <0.005	Castells et al 1999[51]
47 (31: CP)	All	Serum	<i>KRAS</i> mutation Sequencing	47	Sensitivity: 47% Specificity: 87%	No - Ns	Maire et al 2002 [52]
56 (13: CP)	All	Plasma	<i>KRAS</i> mutation PNA-mediated PCR clamping and real-time PCR	36	Sensitivity: 36% Specificity: 100%	No – 0.10	Däbritz et al 2009 [53]
91 (No)	LA + Meta.	Plasma	<i>KRAS</i> mutation Sequencing	33	-	Yes - <0.001	Chen et al 2010 [54]
36 (49: 25 HC and 24 miscellaneous)	All	Plasma	<i>KRAS</i> mutation Cold-PCR combined with an unlabeled-probe HRM	72	Sensitivity: 81% Specificity: 87.5%	-	Wu et al 2014 [55]
27 (No)	LA + Meta.	Plasma	<i>KRAS</i> mutation ARMS PCR	37	-	Yes - 0.003 Yes – 0.014***	Semrad et al 2015 [56]
51 (No)	R	Plasma	<i>KRAS</i> mutation dPCR	43	Sensitivity: 43% Specificity: > 99%	Yes (predictor of disease recurrence) – 0.015	Sausen et al 2015 [57]
45 (No)	All	Plasma	<i>KRAS</i> mutation dPCR	26	-	Yes - 0.001	Earl et al 2015 [58]
110 (25: HC)	All	Plasma	<i>KRAS</i> mutation RFLP + sequ. Two-step enriched-Nested PCR	31	-	No - 0.36	Singh et al 2015 [59]

75 (40: 20 CP and 20 HC)	All	Serum	<i>KRAS</i> mutation dPCR	63	-	Yes - 0.024	Kinugasa et al 2015 [60]
259 (No)	All	Plasma	<i>KRAS</i> mutation dPCR	8 (R), 18 (LA), 59 (M)	-	Yes - < 0.0001	Takai et al 2015 [61]
105 (20 HC)	R	Plasma	<i>KRAS</i> mutation dPCR	31	-	Yes - <0.0001 Yes -< 0.001**	Hadano et al 2016 [62]
40 (10 HC)	All	Plasma and serum	<i>KRAS</i> mutation dPCR	48 (All) 38 (LA) 63 (LA and Meta. Serum)	-	Yes - <0.01	Ako, et al 2016 [63]
188 (No)	Met	Plasma	<i>KRAS</i> mutation dPCR	83	-	Yes - 0.019	Cheng et al 2017 [64]
135 (No)	All	Plasma	<i>KRAS</i> mutation NGS / dPCR	41 (LA and Meta.)	-	LA + Met : Yes - p< 0.001 Resected : Yes – 0.027 ; Yes – 0.03**	Pietrasz et al 2017 [65]
60 (No)	LA + Meta.	Plasma	<i>KRAS</i> mutation BEAMing	65	-	Yes - 0.001 Yes - 0.0022**	Van Laethem et al 2017 [66]
95 (No)	All	Plasma	28 genes Methylation- specific PCR	27 (> 10 hypermethylat ed genes)	-	Yes	Henrikse n et al 2017 [80]
26 (26: 14 CP and 12 HC)	All	Plasma	<i>KRAS</i> mutation dPCR NGS : <i>KRAS</i> , <i>SMAD4</i> , <i>CDKN2A</i> and <i>TP53</i>	NGS : 27 dPCR : 23	-	Yes – 0.018****	Adamo et al 2017 [67]
27 (43 HC)	LA + Meta.	Plasma	<i>KRAS</i> mutation dPCR	70.4	-	No – 0.16 - 0.24***	Del Re et al 2017 [68]
221 (182 HC)	R	Plasma	<i>KRAS</i> mutation PCR Safe- Sequencing System	30	Sensitivity: 30% Specificity: 99.5%	-	Cohen et al 2017 [69]
34 (No)	All	Plasma	NGS: 25 genes (including <i>KRAS</i> )	25 genes: 74 <i>KRAS</i> only: 29	-	Yes - 0.045	Pishvaian et al 2017 [70]
106 (No)	All	Plasma	<i>KRAS</i> mutation dPCR	68(R), 72(LA), 87(M)	Sensitivity: 78% Specificity: 33%	Yes - 0.008 Yes - 0.003***	Kim et al 2018 [71]
65 (20 HC)	All	Plasma	<i>KRAS</i> mutation dPCR	80	-	No - 0.73	Lin et al 2018 [72]

45 (No)	R	Serum	<i>KRAS</i> mutation Real-time quantitative PCR	55	-	Pre-operative samples: No – 0.258 - 0.710** Post-operative samples: Yes – 0.027**	Nakano et al 2018 [73]
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\* A worse prognosis in patients with a mutated *KRAS* vs. wild type in term of overall survival (OS).

\*\* : disease-free survival

\*\*\* : progression-free survival

\*\*\*\* : disease specific survival

CP: chronic pancreatitis; HC: healthy controls; R: resected PDAC; LA: locally advanced PDAC; M or Meta.: metastatic PDAC; All: Resected + Locally advanced + Metastatic PDAC patients; RFLP: restriction fragment length polymorphism; dPCR: digital droplet PCR; NGS: next generation sequencing; BEAM: Beads Emulsion Amplification Magnetic; ARMS PCR: amplification-refractory mutation system polymerase chain reaction; Cold-PCR: co-amplification at lower denaturation temperature-PCR; PNA-mediated PCR: peptide nucleic acid-mediated PCR; HRM: high resolution melt.

**TABLE 3** Results of main clinical pilot studies assessing performances of exosomes detection in PDAC

Patient number (PDAC)	Type of tumor: Resected, Locally advanced, Metastatic, All (treatment)	Molecular Target(s)	Method of isolation	Exosomes detection rate in PDAC patients	Exosomes diagnosis performances	Exosomes prognosis value	Reference
16 (6 HP, 6 CP, 5 cysts, 5 ampullary carcinoma)	All (12 metastatic)	miR-17-5p, -21, -155	Ultracentrifugation RT qPCR	NA	(**)	miR-17-5p correlated with metastasis	Que et al 2013 [97]
131 (64 HC)	All	miR-1246, -4644, -3976, -4306; CD44v6, TSPAN8, EpCAM, MET, CD104	Sucrose gradient, Micro-array, RTqPCR, flow cytometry, latex beads	NA	Sens. 100% Spec. 80%	NS	Madhavan et al 2014 [99]
146 (benign pancreatic diseases 32, 120 HC)	All (Neo-adjuvant: 10)	GPC1	Latex beads Ultracentrifugation Ac GPC1	100%	Sens. 100% Spec. 100%	GPC1+ correlates with worse DFS and OS	Melo et al 2015 [110]
29 (CP 11)	Resected and locally advanced	GPC1 miR-10b, -21, -30c, -181a, -let7a	GPC1 LC-MS/ML RT qPCR	100%	Sens. 100% Spec. 100%	NS	Lai et al 2017 [111]
127 (136 HC)	All	Exo DNA ctDNA	Ultracentrifugation Flowcytometry dPCR	54%	Sens. 54% Spec. 84% PPV 76% NPV 66%	Worse DFS P=0.03 RR: 4.68 441 days vs 127	Allenson et al 2017 [107]
15 (15 HC)	All	miR: R196a, 196b and 1246	ExoKit RTqPCR NGS	Significantly higher for 196a and 1246	AUC: 196a: 0.81 1246: 0.73 196b 0.71	NS	Xu et al 2017 [114]
68 (41 benign pancreatic diseases; 18 HC)	All (Neo-adjuvant: 33)	Signature: EGRF, EpCAM, MUC1, GPC1, WNT2	Ultracentrifugation	89%	Sens. 86% Spec. 81%	NS	Yang et al 2018 [112]
20 (20 benign diseases)	Resected and locally advanced	Protein CD63, GPC1	AC electrokinetics immunofluorescence	Significantly higher in PDAC cohort	Sens. 99 Spec. 82	NS	Lewis et al 2018 [113]
32 (IPMN 29, 22 HC)	All	miR-191, -21, -451a	ExoKit Quick NGS RT qPCR		(*)	miR21 worse OS	Goto et al 2018 [98]
24 (14 CP, 50 miscellaneous, 46 HC)	NA	Protein ZIP4	Exo Kit precipitation	Significantly higher in PDAC	AUC ROC curve 0.89	NS	Jin et al 2018 [115]

194 (25 cysts, 12 HC)	All (123 metastatic)	Exo DNA <i>KRAS</i>	Ultracentrifugation ddPCR	61% metastatic 38% resectable	NS	MAF > 5% Predictor PFS OS	Bernard et al 2019 [74]
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miR : microRNA ; CP: chronic pancreatitis; HC : healthy control patients; IPMN: intraductal papillary mucinous neoplasia; NET: neuroendocrine tumor; NA: not available : NS : not studied ; NGS: next generation sequencing; GPC1: sulfate proteoglycan 1; AUC: area under ROC curves; EpCAM; Epithelial cell adhesion molecule; MUC1 : mucin 1; TSPAN8: tetraspanin8;

(\*): miR-191 : Sens. 71.9%, Spec. 84.2 %, accuracy 76.6% ; miR-21 : Sens. 80.7%, Spec. 81%, accuracy 80.8% ; miR-451a : Sens. 65.8%, Spec. 85.7%, accuracy 73.6%.  
(\*\*) : miR-17-5p : Sens. 72.7%, Spec. 92.6% ; miR-21 : Sens 95.5%, Spec. 81.5%.

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## **Détecter les cellules tumorales circulantes au plus proche de la tumeur pour améliorer les performances diagnostique ?**

La détection des cellules tumorales circulantes (CTC) est un outil prometteur pour le diagnostic du cancer. La détection et la numération des CTC font partie de la pratique clinique courante, en particulier pour le cancer du sein, du côlon et de la prostate. Cependant, leur rareté dans les échantillons de sang périphérique est un obstacle à leur identification. Plusieurs études ont tenté d'améliorer le taux de récupération du CTC en développant des méthodes de détection cellulaire et moléculaire très sensibles. Cependant, le nombre de CTC dans le sang périphérique est encore difficile à détecter. Le taux de récupération du CTC pourrait être augmenté en prélevant des échantillons sanguins sur des vaisseaux proches des territoires de drainage de l'organe envahi, lorsque la situation anatomique est favorable. Cette approche a été testée principalement lors de résections tumorales, lorsque les vaisseaux les plus proches de la tumeur sont facilement accessibles. De plus, des voies radiologiques et/ou endoscopiques pourraient être envisagées pour obtenir des échantillons de CTC à proximité de la tumeur d'une manière moins invasive que les biopsies classiques. Le but de cet article était de résumer les connaissances disponibles sur la récupération de la CCT à partir d'échantillons sanguins prélevés près de la tumeur (c.-à-d. dans les vaisseaux situés dans la zone de drainage de l'organe colonisé). La pertinence de cette approche pour les estimations diagnostiques et pronostiques et le suivi du cancer sera discutée, en particulier pour l'adénocarcinome canalaire pancréatique, l'adénocarcinome colorectal, le carcinome hépatocellulaire et le cancer du poumon non à petites cellules.

Mots-clés : Cellules tumorales circulantes; drainage vasculaire; biopsie liquide ;  
diagnostic du cancer ; pronostic du cancer

### 3- Detecting circulating tumor cells nearest from the tumor to improve liquid biopsy clinical value?

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**Running title:** CTCs nearest from the tumor

**Key Words:** Circulating tumor cells; vascular organ drainage; *liquid biopsy*; cancer diagnostic; cancer prognosis

### **Abbreviations**

**CTC:** circulating tumor cells

**CT:** computed tomography

**PET:** positron emission tomography

**MRI:** magnetic emission imaging

**FNAC:** fine needle aspiration cytology

**CRC:** colorectal cancer

**NSCLC:** non-small cell lung cancer

**HCC:** hepatocolangiocarcinoma

**PDAC:** pancreatic ductal adenocarcinoma

**EMT:** epithelial-to-mesenchymal transition

**PCR:** polymerase chain reaction

**RT-qPCR:** reverse transcription polymerase chain reaction

**EUS-FNA:** endoscopy ultrasound guided fine needle aspiration

### 3-1 Abstract

Circulating tumor cell (CTC) detection is a promising tool for the diagnosis of cancer with prognostic value. CTC detection and numeration have emerged as part of the common clinical practice, especially for breast, colon and prostate cancer. However, their paucity in peripheral blood samples is an obstacle for their identification. Several studies have tried to improve CTC recovery rate by developing highly sensitive cellular and molecular detection methods. However, numbers of CTCs in peripheral blood are still difficult to detect. CTC recovery rate could be increased by obtaining blood samples from vessels close to the drainage territories of the organ invaded, when the anatomical situation is favorable. This approach has been tested mostly during tumor resection surgery, when the vessels nearest to the tumor are easily accessible. Moreover, radiological and/or endoscopic routes could be envisaged to obtain CTC samples close to the tumor in a less invasive way than conventional biopsies. The purpose of this article was to summarize the available knowledge on CTC recovery from blood samples collected close to the tumor (i.e., in vessels located in the drainage area of the colonized organ). The relevance of this approach for the diagnostic and prognostic estimations and cancer follow up will be discussed, particularly for pancreatic ductal adenocarcinoma, colorectal adenocarcinoma, hepatocellular carcinoma, and non-small cell lung cancer.

### 3-2 Introduction

Cancer diagnosis usually relies on information obtained using sequential procedures, including imaging data (CT, PET, MRI, ultrasonography, X-rays), changes in the levels of markers in bodily fluids (e.g., blood, urine), and mainly on the pathology examination of cancer cell or tissue samples, obtained by surgical biopsy or by fine-needle aspiration (fine needle aspiration cytology, FNAC). Biopsy and FNAC are invasive procedures, especially in the case of deeply located tumors, and may present severe complications such as infection, bleeding, or inflammation. More importantly, they also carry the risk of seeding tumor cells around the sampling area. Indeed, detached cells can be cleared by interstitial fluids to lymph nodes, or into the veins draining the tissue, thus entering the circulation. They might then extravasate at distant healthy tissues and contribute to metastasis formation. During fine needle aspiration, cells can be dragged along the needle track, leading to the possibility of increasing the local dissemination (Shyamala, Girish, and Murgod 2014). Moreover, if the amount of tumor cells contained in the biopsy is too low for pathology/molecular analyses, particularly in tumors with strong desmoplastic reaction, repeated sampling is required, possibly delaying tumor management. In addition, biopsies are not recommended for the follow-up of most tumors due to the previously described risks (Shyamala, Girish, and Murgod 2014). Hence, the current methods for disease relapse monitoring are mainly based on imaging methods that often identify metastatic sites only at advanced stages (Robertson and Baxter 2011; Chaffer and Weinberg 2011), when the cancer has become resistant to therapy (de Haas et al. 2011a; Forner, Llovet, and Bruix 2012; Li et al. 2017; Buscail 2017). Besides diagnosis, cancer management would highly benefit from broadening the panel of the available prognostic/predictive markers to better stratify patients in view of precision medicine, and to follow the tumor response

after treatment initiation. For some tumors, for instance pancreatic cancer, the outcome of the patients is highly unpredictable, even if resectability is set and performed, because predictive and prognosis markers are missing (Zhou et al. 2017)

Consequently, there is a need to find effective and reliable biomarkers to help for rapid diagnosis, especially when anatomo-pathologic proof is not available or non-contributive, and with possibility of tumor follow-up after the treatment is started. Primary tumors and metastases release in the blood and other body fluids, tumor-derived elements, such as circulating tumor cells (CTCs), nucleic acids, and exosomes. When identified as tumor-derived, these elements can be considered as a proof of the presence of the tumor (Alix-Panabières and Pantel 2014). The analysis of these circulating tumor-derived elements, called '*liquid biopsy*' (Alix-Panabières and Pantel 2013; Pantel and Alix-Panabières 2010), might represent a non-invasive, safer and faster alternative/complement to tissue biopsy. Tumor element release occurs very early during cancer development. For example, CTCs with metastatic potential shed during the formation of the pancreatic adenocarcinoma primary tumor, before it becomes detectable by histological methods (Rhim et al. 2012). Liquid biopsies can also be used to detect disease progression or treatment resistance before the appearance of the first clinical signs (Riethdorf et al. 2018).

The first proof of CTC was published in 1869 by Thomas Asworth (Ashworth, n.d.). From the 70s, the interest on CTC has progressively increased thanks to the progress in the detection methods based on molecular biology techniques. In the last 20 years, new technologies for CTC enrichment, detection, and characterization with higher sensitivity have been developed, allowing CTC enumeration for different solid cancers (Lianidou, Strati, and Markou 2014). For instance, the US Food and Drug Administration (FDA) has approved the use of the CellSearch® test to detect CTCs in



the clinical routine for metastatic breast cancer in January 2004 (Cristofanilli et al. 2004), and for monitoring colorectal and prostate cancer in November 2007 (Steven J. Cohen et al. 2006; S. J. Cohen et al. 2009) and February 2008 (Resel Folkersma et al. 2010), respectively (Millner, Linder, and Valdes 2013; Riethdorf et al. 2018). Increasing evidence indicates that *liquid biopsy* is a very promising tool for the detection and management of lung cancer, especially non-small cell lung cancer (NSCLC), colorectal cancer (CRC), hepatocolangiocarcinoma (HCC), and pancreatic ductal adenocarcinoma (PDAC) (Hench, Hench, and Tolnay 2018; Pimienta et al. 2017).

Most of the studies have focused on CTC detection and counting in peripheral blood samples obtained by puncture of the median cubital vein. Fewer reports have tested the hypothesis that the chances of capturing and detecting CTCs might be higher in vessels closer to the tumor, especially in the main veins that drain blood from the organ invaded by the cancer. In this review, we first evaluated the CTC yields and the prognostic value of this closer to the tumor approach compared with CTC analysis in peripheral blood. We then discussed its possible future role in the routine cancer management pathway, in lung cancer, colorectal cancer, HCC, and PDAC.

### 3-3 Basis for the analysis of CTC in the main veins close to the tumor site

The primary tumor releases a heterogeneous population of circulating cells. Besides cells with metastatic potential, they also shed apoptotic or necrotic cells cleared by the organism, and live cells that can remain in a latent or dormant state in a distant organ. Most CTCs arise during metastasis formation that depends on the success of tumor cells to complete the metastatic cascade from the primary carcinoma to distant organ colonization (Massagué and Obenauf 2016; Kessenbrock, Plaks, and Werb 2010; Martin et al. 2017). First, epithelial tumor cells in the primary tumor undergo a reversible

phenotypic change, known as epithelial-to-mesenchymal transition (EMT). Consequently, cells detach from the tumor and spread out, using the surrounding fluids to move away, and enter the vessels by extravasation (Tam and Weinberg 2013; Thiery et al. 2009). The first capillary bed that a metastatic cell encounters depends on the blood circulation pattern near the primary tumor. In most organs, the venous circulation leads to the right ventricle of the heart and into the lungs, whereas the gut venous circulation drains into the liver. This explains the high incidence of metastases in lungs and liver (Denève et al. 2013). For this reason, some authors have performed punctures in the vena cava upstream of the liver for metastatic breast cancer (with sample obtained from an implanted vascular device) (Peeters et al. 2011).

### 3-4 Technologies for CTC enrichment and detection

Although CTC release from the primary tumor and/or metastases is deleterious for the patient, it also becomes an opportunity to obtain relevant information for precision medicine using a non-invasive procedure. Several technologies allow the enrichment and the enumeration of CTCs (Alix-Panabières and Pantel 2014). As CTCs are rare events, a first enrichment step is required to detect them correctly. As a first option, CTC physical properties (i.e., size, deformability, density and electrical charges) can be used to differentially enrich CTCs from the numerous surrounding normal circulating blood cells (Alix-Panabières and Pantel 2014; Pantel and Alix-Panabières 2010; Harouaka, Nisic, and Zheng 2013). CTCs can also be enriched and detected using their biological properties. For instance, positive selection-based capture relies on the expression of tumor cell surface markers (most commonly EpCAM). This can be combined with the presence of tumor-specific intracytoplasmic proteins (such as cytokeratin 19, CK19) and the absence of the blood-specific cell surface marker CD45.

These features are the basis of the CellSearch® system. Moreover, a negative selection-based capture is an unbiased CTC enrichment step to eliminate the unwanted white blood cells. Antibodies against cell surface markers of the different blood cell types are used to pull down white blood cells, leaving the remaining supernatant enriched in CD45<sup>(+)</sup> endothelial cells and CTCs. After some enrichment methods, another detection step is needed to confirm the presence of CTCs in the sample (Alvarez Cubero et al. 2017). This can be done using: (1) immunocytological technologies (e.g., the CellSearch® system), (2) molecular (RNA-based) technologies (e.g., RT-qPCR for epithelial mRNA) and (3) functional assays (e.g., EPISPOT assay that detect only viable CTCs) (Alix-Panabières and Pantel 2014).

Despite improvements in methods for CTC enrichment and detection, these cells remain rare in blood samples and difficult to identify. To maximize the chances of CTC recovery, it would seem logical to draw blood close to the primary site of the carcinoma. Indeed, CRC, HCC and PDAC primary tumors are connected to the vascular draining territory of the mesenteric and portal venous system, whereas lung cancer is linked to the pulmonary vein. These vessels are sufficiently large and resistant to allow direct vein puncture. Of note, the portal vein can be accessible by non-invasive ultrasonography puncture (Chapman and Waxman 2016). The pulmonary vein is reachable only during surgery, but it is a good candidate to help capture more tumor elements, with a high prognostic value (Hashimoto et al. 2014). Conversely, in breast cancer and prostate cancer, tumor elements are released mostly in the lymphatic network and the internal iliac vasculature, respectively. As these draining systems cannot be punctured, CTC capture closer to the tumor has not been assessed in these cancer types. Therefore, this review will focus on CTC detection in the draining vessels of primary HCC, PDAC, CRC and NSCLC.

### 3-5Pancreatic ductal adenocarcinoma

PDAC remains one of the deadliest cancers, with increased incidence and mortality due to its late diagnosis and poorly efficient therapies. Moreover, therapeutic onset is often delayed due to difficulties met in proving the presence of malignant lesions. CTC enumeration in the peripheral blood of patients with PDAC has been assessed as a diagnostic option, but rather unsuccessfully due to the low sensitivity (Supplemental **Table 1**). Most of these cohorts were quite small, and included only patients with metastatic or locally advanced tumors, and cohorts including all tumor stages mostly corresponded to metastatic cancers (>80%) (Lianidou, Strati, and Markou 2014). PCR-based or physical-based methods only slightly improved the sensitivity (Supplemental **Table 1**), whereas methods of CTC detection based on the expression of cancer cell markers, such as CK19 or EpCAM, could have missed cells undergoing EMT. The most common site of PDAC spreading is the liver because the pancreas venous blood drains first into the liver (**Figure 1**) (Nieto, Grossbard, and Kozuch 2008; Denève et al. 2013). The liver filters pancreatic CTCs. If they do not stay in the liver, they will become highly diluted in the peripheral blood system (i.e., 1 tumor cell per  $1 \times 10^9$  blood cells, explaining the low detection rates) (Yu et al. 2011). To increase the chances of CTC detection, blood was sampled directly from the portal vein prior to CTC sequestration in the liver (Chapman and Waxman 2016). This approach was first tested in 20 patients with resectable PDAC in whom portal blood could be easily and safely sampled during surgery ((Bissolati et al. 2015), **Table 1**). CTCs were detected (CellSearch®) in nine portal blood samples (45%) and in four peripheral blood samples (20%) from these 20 patients. Twenty five percent of the patients had CTC positivity in the portal blood only, and would have been missed if CTC detection had been performed only in peripheral

blood. CTC presence in peripheral or portal blood did not correlate with long-term overall survival (OS) or disease-free survival (DFS). Conversely, CTC detection in the portal vein sample was associated with higher rate of liver metastases (Bissolati et al. 2015). Another study compared CTC identification in peripheral and portal vein blood samples in 41 patients undergoing upfront surgery for PDAC ((Tien et al. 2016), **Table 1**). CTCs were detected (CellSearch®) in 39% of peripheral and 58.5% of portal vein blood samples. CTC presence in portal blood was a predictive factor of liver metastasis. The short follow-up of this study (only 1 year post-surgery) did not allow assessing the OS and progression-free survival (PFS). In 14 patients with borderline resectable (n=7) or metastatic PDAC (n=7), CTCs (CellSearch®) were identified in 21% of peripheral blood samples and in 100% of portal samples, drawn by pre-operative endoscopy ultrasound-guided fine needle aspiration (EUS-FNA) performed for diagnostic and staging purposes (Catenacci et al. 2015). Moreover, CTC absolute numbers were higher in the portal blood samples (83.2/7.5mL CTCs *versus* 0.4 CTCs in the peripheral blood). No correlation with OS or PFS was reported. The authors also evaluated the suitability of portal vein CTCs for gene expression studies. They found that downregulation of tumor-suppressor genes had a strong prognostic value and could be used to stratify patients eligible for surgery, according to the relapse risk. Similarly, in 29 patients with locally advanced and metastatic tumors ((Liu et al. 2018), **Table 1**), CTCs were detected in 100% of the portal blood samples (obtained by ultrasonography-guided trans-hepatic puncture) and in 54% of the peripheral blood samples, with a higher CTC count in portal *versus* peripheral blood (282 *versus* 21/7.5mL). Moreover, CTC count was correlated with liver metastases, and patients with a portal vein CTC count higher than 150/7.5mL had shorter OS. Finally, portal vein CTCs were used to test drug resistance.

In these studies, while CTC detection rate in peripheral blood was similar to what previously reported (around 50%, as in supplemental **Table 1, Table 1**), in portal blood, it was on average 75% at all stages. More studies with bigger cohorts are needed to determine the value of this approach at early disease stages, particularly in upfront resectable tumors. Interestingly, all studies found a correlation between liver metastases and portal CTCs, including in cohorts with resectable tumors. This suggests that CTC analysis in portal blood samples collected, for instance, during pre-operative EUS-FNA could be used to better select patients for surgery, especially patients with undetectable micro-metastases. Taken together, the results of these pilot studies suggest that liquid biopsy in the portal vein may help improving pancreatic cancer prognosis, and could be associated with tumor sampling during EUS-FNA to improve PDAC management. For instance, CTC detection could be used as a companion diagnostic tool for the molecular/genetic analysis of cancer cells in patients needing neoadjuvant therapy. Indeed, preliminary data showed that CTCs could be useful to stratify patients and adjust the therapeutic options according to the cancer molecular characteristics (Soler et al. 2017). Finally, functional testing of CTCs, such as detection/quantification of tumor-specific secreted factors by isolated cells, is still in the early days, but patient management might benefit of such approaches in future.

### 3-6Colorectal cancer

CRC is the third most common cancer in both sexes. The five-year OS reaches almost 60%. About 50% of patients will develop metastatic disease that accounts for the majority of deaths (de Haas et al. 2011b). After curative resection, approximately 30% of patients who develop metastases eventually die of metastatic disease. Although diagnosis of CRC by colonoscopy is routinely available, good prognostic markers to

stratify patient with CRC according to the metastasis risk are still missing. It has been shown that CTC detection in peripheral blood is a good predictive marker of PFS and OS in patients with metastatic CRC, and in general a poor prognostic factor. Therefore, it could contribute to better tailor the patient general care. However, differently from breast and prostate tumors, CTC release in the peripheral blood by CRC is a rare event, thus making difficult their routine detection (less than 60% of sensitivity) (Alix-Panabières and Pantel 2014; Tan and Wu 2018). PCR-based CTC detection does not improve sensitivity (Supplemental **Table 1**). CTCs shed by CRC are disseminated via the mesenteric venous system that drains in the portal vein. The liver serves as a filter, retaining many CTCs including metastasis-initiating cells, and releasing other CTCs that are then diluted in the peripheral blood (**Figure 1**). A study showed that immediately after tumor resection, CTC numbers decreased in the peripheral blood and in the local main vasculature of the tumor (Jiao et al. 2009) (**Table 2**). This study did not specify the percentage of patients with CTCs detected in the systemic circulation compared with the portal circulation, but the median CTC number before surgery, although very low in general, was higher in the portal circulation and hepatic vein. Moreover, CTC detection rate in the hepatic vein was lower than in the portal vein (17.5% *versus* 35%, respectively, (Rahbari et al. 2012)), underlining the importance of the puncture site for CTC detection. In cohorts that included only patients with metastatic CRC, CTC detection rate was similar in peripheral blood and hepatic vein (46% and 54%, respectively), as well the median CTC count (1 *versus* 2.5, respectively) (Connor et al. 2016) (**Table 2**). OS and PFS were worse in patients with CTC counts >3, suggesting that CTC detection and count could have a prognostic value in patients with metastatic CRC. When the tested population included only 20% of patients with metastatic diseases, CTC detection rate in mesenteric blood was

almost twice higher than in peripheral blood (CellSearch®, 55.9% versus 29%, respectively). However, the small number of patients did not allow testing the correlation between cell count and prognosis (Denève et al. 2013). This study also showed that using an EpCAM-independent CTC enrichment method followed by the functional EPISPOT assay significantly increased CTC detection rate to 55.4% in peripheral blood, and only slightly (to 65.9%) in mesenteric blood samples. CTC counts were significantly higher in mesenteric blood than peripheral blood samples, and more CTCs were detected with the EPISPOT assay than the CellSearch® system. CTC detection (both methods) inversely correlated with the presence of lymphatic emboli, and only the EPISPOT results correlated with the primary CRC differentiation grade. Finally, cancer-related survival was worse in patients with more than 27 CTCs (only with the EPISPOT assay) (**Table 2**).

In conclusion, although CRC-related CTCs are rare, many groups performed studies to test their diagnostic and prognostic relevance. Blood sampling closer to the tumor did not increase significantly CTC detection and enumeration, including in the case of metastatic disease. Thus, for this cancer, CTC detection sensitivity needs to be improved. The promising EPISPOT assay allowed increasing CTC detection in mesenteric blood (Denève et al. 2013). CTC detection correlated with bad prognosis. Studies on larger cohorts are needed to further test the value of CTC detection for CRC management.

### 3-7 Hepatocellular carcinoma

HCC is the sixth most prevalent cancer responsible for one third of all deaths by cancer (Ferlay et al. 2015). When diagnosed early enough, the 5-year OS can reach 50%. Conversely, in stage 4 disease, less than 10% of patients survive the first year after



diagnosis. Thus, tools for early screening are urgently needed, especially in high-risk populations (patients with cirrhosis, hepatitis, and non-alcoholic steatosis hepatitis syndrome) who could greatly benefit from the available treatments in the case of early diagnosis. Currently, screening is based on the use HCC biomarkers, such as alpha fetoprotein and des-gamma-carboxy prothrombin (DCP) (Tateishi et al. 2008). However, these markers show high false-positive rates. Like for other cancers, CTC detection in peripheral blood is sensitive enough to allow HCC diagnosis (Supplemental **Table 1**). Moreover, CTC presence has prognostic value because it is correlated with poor RFS and OS.

Liver is connected with two major vascular systems: the hepatic veins that constitute the efferent pathway, and the hepatic artery and portal veins that compose the afferent pathway (**Figure 2**). CTCs from the primary liver tumor are first disseminated in microscopic portal vessels, and then in the centrolobular veins that drain into the main hepatic veins. Tumor elements can be detected also in the afferent system because HCC has a high propensity to colonize arterial vessels during neo-angiogenesis (**Figure 2**)(Forner, Llovet, and Bruix 2012). We found only one study that compared CTC detection rates in function of the sampling site in patients with localized HCC (**Table 3**)(Sun et al. 2018). Detection rates in peripheral vein or artery blood samples were similar to previously published results (**Table 3**, Supplemental **Table 1**, 68 and 45%, respectively). As expected on the basis of the liver circulation, CTC recovery rate in portal blood and inferior vena cava did not increase compared with peripheral samples. Conversely CTC detection rate in the hepatic vein was very high (80%), because this vessel drains all the microscopic lobular spaces that may receive CTCs. This study did not test the diagnostic/prognostic value of CTC detection in the different vessels. However, intrahepatic recurrence was strongly associated with CTC presence

in peripheral blood samples (artery and vein) and with CTC micro-emboli (or clusters). In addition, high detection rate of CTCs or clusters in the hepatic vein was associated with the presence of lung metastases.

In conclusion, CTC detection in the hepatic vein shows a strong prognostic value, both for disease recurrence and disease dissemination (Fang et al. 2014). It would be also interesting to test whether CTC presence, particularly in the hepatic vein, could be used to stratify patients eligible for adjuvant therapy. CTC detection in peripheral veins could also be a valuable tool for HCC follow-up.

### 3-8 Non-small cell lung cancer

Lung and bronchus cancer remain the first cause of death by cancer in 2017, representing around 25% of all deaths by cancer (Siegel, Miller, and Jemal 2017). NSCLC accounts for 85% of all diagnosed lung cancers. This cancer is the most prevalent cancer in males and the third in females. Its 5-year OS depends on the disease stage, going from 92% for stage IA1 to less than 1% for metastatic stage IV. Overall, the 5-year survival rate is 18% (source: cancer.net). Even after surgery, tumor recurrence with distant metastases occurs in around 25% of patients, reaching approximately 29% in patients with stage I cancer (Goldstraw et al. 2016). After surgery, the currently available clinical imaging and other technologies does not allow the early detection of metastases and of micro- or occult metastases with good sensitivity (Rusch et al. 2011; Uhr and Pantel 2011). Cytotoxic chemotherapy can slightly prolong survival in patients with tumor relapse. Indeed, only 4% to 5% improvement in 5-year survival rates has been reported for stage I-III NSCLC, and prolongation of only few months for stage IV tumors, possibly because tumor recurrence is detected too late (Johnson, Schiller, and Bunn 2014).

NSCLC-derived CTCs disseminate first in the pulmonary vein (**Figure 3**) (Popper 2016). Cancer cells follow the main bloodstream through the heart and join the systemic circulation where metastasis-initiating cells can niche, mostly in brain, bone marrow, adrenal gland, and liver. CTC detection in peripheral blood samples of patients with lung cancer has been evaluated in several studies (Supplemental **Table 1**). The overall sensitivity is quite low at all disease stages (around 53%) when using protein marker-based CTC enrichment methods. CTC detection in patients with metastatic disease is more efficient with PCR-based methods (71%). Most studies reported a strong correlation between peripheral blood CTC detection and OS. Tumor recurrence also was associated with CTC presence (Gallo et al. 2017).

In 2005, CTCs were detected (RT-PCR) for the first time in the pulmonary vein of patients with NSCLC (Bernaudin et al. 2005). Many studies have compared CTC detection rate in peripheral blood and close-to-the tumor vessels. Like for the PDAC and CRC, blood samples were collected close to the tumor drainage territory by puncturing the pulmonary vein. Most of the patients included in these studies had resectable tumors, and only a small percentage had metastatic disease. Overall, peripheral blood samples displayed sensitivity similar to previously published works on peripheral blood only (**Table 4**, average detection rate in peripheral blood about 45% similar to the 53% in Supplemental **Table 1**). Conversely, CTC detection rate in the pulmonary vein was about 91%. Of note, while the peripheral blood detection rates varied among reports (range 6.6%-91.3% mean  $45.1\% \pm 34.1\%$ ), the pulmonary vein detection rate was quite reproducible (range 80%-100% mean  $93.5\% \pm 7.3\%$ ). Similarly, the mean CTC count was higher in the pulmonary vein than in peripheral blood samples (Reddy et al. 2016).

Results on the prognostic value of CTC presence in the pulmonary vein are

heterogeneous. Some studies showed a correlation between CTC and disease progression and OS (Murlidhar et al. 2017; Li et al. 2017; Tarumi et al. 2013), whereas others did not find any correlation with NSCLC clinical features (Okumura et al. 2009; Chudasama et al. 2017; Reddy et al. 2016; Sawabata et al. 2016). One study showed that DFS and OS were associated with high CTC rate in peripheral blood, but not in the pulmonary vein blood (Crosbie et al. 2016).

Some studies focused on the link between CTC detection in lung cancer and the surgery technique. Particularly, it was shown that surgical manipulation significantly increased CTC levels in the pulmonary vein, and that this was associated with lymphatic invasion and a significant reduction of DFS and OS (Hashimoto et al. 2018, 2014). A recent work suggested that intraoperative manipulation contributes to the hematogenous dissemination of tumorigenic CTCs and circulating tumor micro-emboli (Table 5)(Lv et al. 2018). Similarly it was reported CTC rate, including in the pulmonary vein, increases after endoscopic biopsy (Reddy et al. 2016). These data suggest that the pulmonary veins should be ligated before tumor mobilization to minimize tumor cell dissemination.

Taken together, these results show that pulmonary vein puncture greatly increases the chances to detect CTCs originating from lung tumors, but the prognostic value for disease recurrence needs further investigation with better categorization of the disease stages. Moreover, tumor cell dissemination during surgical procedure deserves to be better characterized. But on the high invasivity of this procedure to get CTCs.

### 3-9Future directions

Confrontation of results obtained in different cancer types with similar approaches suggests that combining CTC detection in the tumor-draining vein and peripheral blood at the time of surgery or by ultrasonography-guided puncture could improve the identification of patients at higher risk for cancer recurrence than peripheral CTC detection alone. CTCs as '*a real-time liquid biopsy*' of cancer are clinically relevant. However, additional studies on CTC detection in vessels close to the primary tumors are needed, particularly to obtain crucial information on the tumor biology and the metastatic cascade from the genomic analysis of isolated single CTCs. Indeed, we need to learn more on CTC heterogeneity during their journey in the bloodstream, the selection of CTC sub-clones through specific filtrating organs (e.g., the liver) (Joosse et al. 2018). Based on the hypothesis that CTCs represent cells at the origin of metastases, these cells could also predict the genetic landscape of the new metastatic tumors. For example, in cancers that carry multiple genetic mutations, these alterations may not be homogeneously distributed, and the biopsy sample may not show all the mutations. Conversely, the possibility to detect all mutations is higher in liquid biopsies. Thus, they can contribute to the genetic/molecular characterization of the tumor for prognostic/therapy stratification purposes, and also to the discovery of new biomarkers.

Figure 1

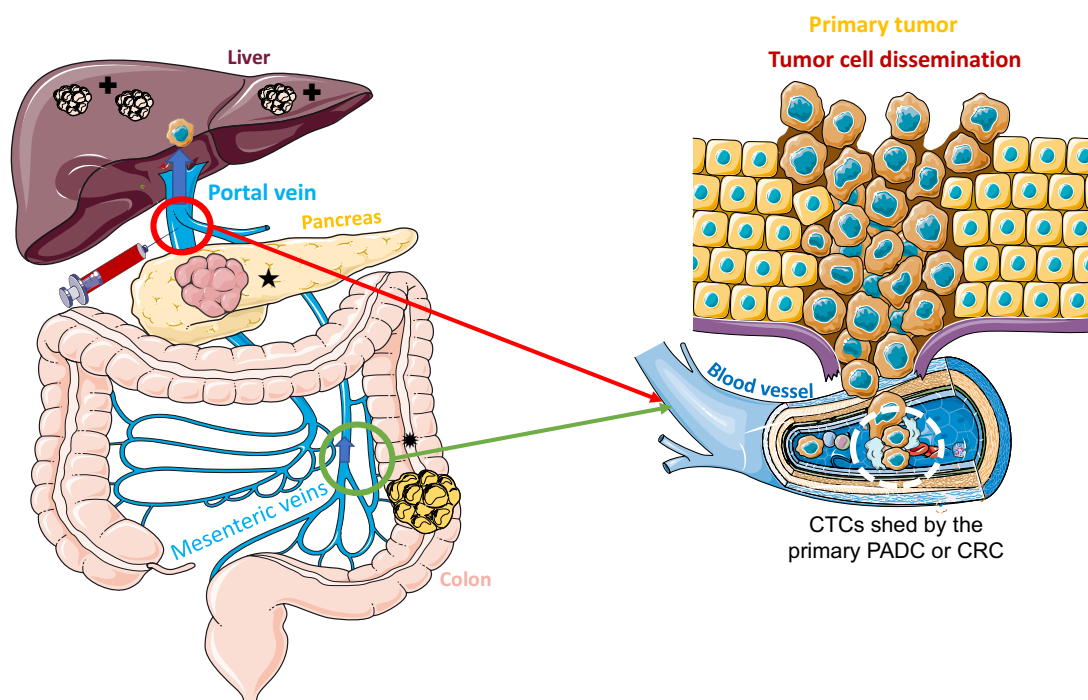


Figure 3

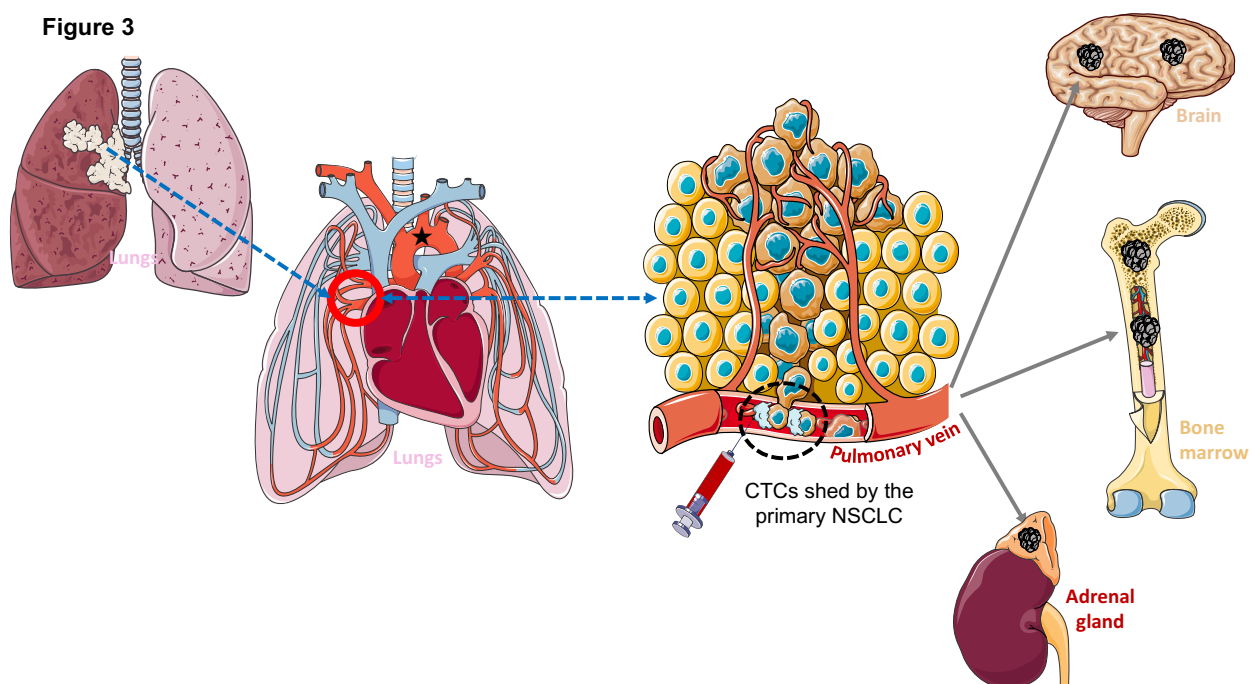
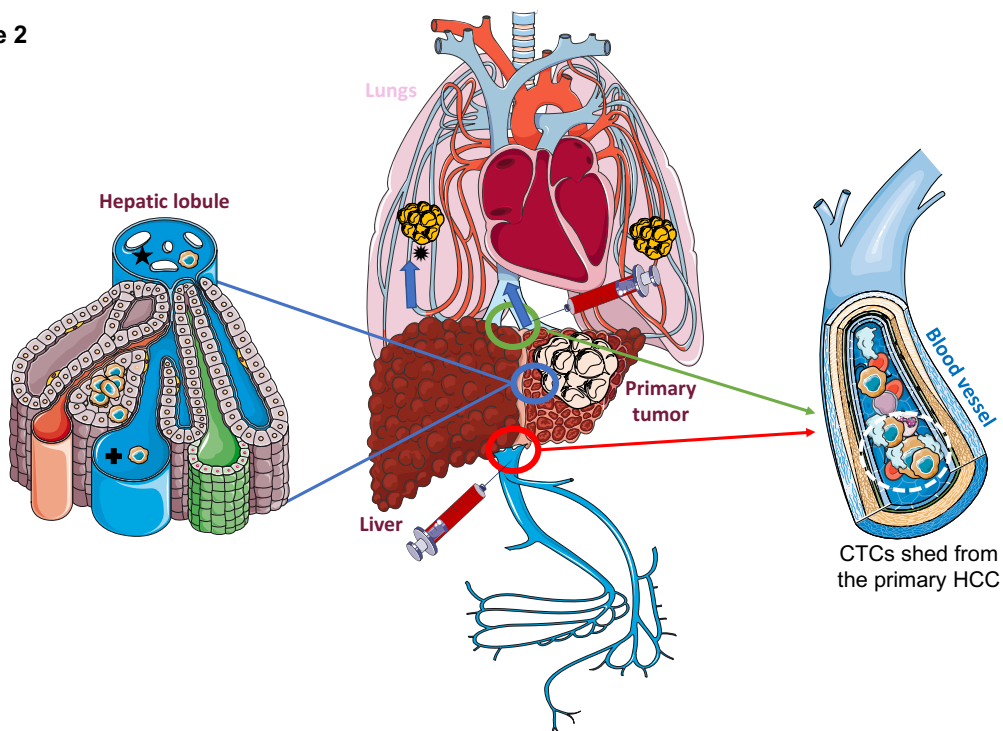


Figure 2



## Figure legends

### **Figure 1. CTC detection in the portal vein for patients with PDAC (★) or CRC (\*).**

Pancreatic cancer and colorectal cancer metastases in the liver (✚) develop through multiple steps. Local invasion by cancer cells is followed by their intravasation into the tumor vasculature. Cancer cells then enter the porto-mesenteric venous system as single cells or clusters that might be coated by platelets. CTCs are released in the superior and inferior mesenteric (green circle) veins for CRC in the right colon and left colon/rectum respectively, and in the portal vein (red circle) for PDAC.

Portal blood flows through the liver and then to other distant organs, after crossing the liver capillaries in portal areas. CTCs follow the same route and might extravasate in the parenchyma of the liver to start colonization. Portal blood sampling before passage in the liver can allow improving CTC recovery rate. The blue arrows show the direction of the blood flow in the veins.

### **Figure 2. Detection of HCC-derived CTCs in the hepatic and portal veins.**

The hepatic circulation is connected to systemic circulation via three major vessels: the hepatic veins (green circle), which serves as the efferent pathway, and the hepatic artery and portal vein (red circle), which function as afferent vessels. HCC-derived CTCs are released in the hepatic lobule (blue circle) in the portal branch (✚) and in the central vein (★) that constitute the hepatic vein system draining into the inferior vena cava. They represent the main intrahepatic and pulmonary metastatic routes (\*). Blood sampling from the hepatic portal veins (red circle) could improve CTC detection.



**Figure 3. NSCLC-derived CTC detection in the pulmonary vein.**

NSCLC metastatic sites are primarily bone marrow, brain and adrenal gland. First, CTCs extravasate in the circulation via the pulmonary veins (black circle). Then, CTCs go into the systemic circulation towards the cerebral capillaries (via the branches of the aortic arch (★)) or the bone marrow sinusoids and other distant sites. The fenestrated structure of bone marrow sinusoid capillaries is permissive to cancer cell infiltration. Brain capillaries are more difficult to penetrate, due to the unique nature of the blood-brain barrier. Based on the features of the pulmonary circulation, CTCs could be retained in the pulmonary vein (black circle), offering an opportunity to increase their detection in blood samples collected from this vein during tumor resection, as already shown by Saintigny *et al.* in 2005.

## Tables

**Table 1.** Comparison of CTC detection in peripheral and portal venous samples in patients with pancreatic ductal adenocarcinoma

Number of patients	CTC enrichment/ Detection methods	CTC count in peripheral blood	CTC count in portal blood	CTC detection rate in peripheral blood	CTC detection rate in portal blood	Prognostic value	Reference
29 $\Delta\theta$	CD45 <sup>+</sup> leukocyte depletion/ ClearBridge®	Mean 21 Med 9 Range 0-74	Mean 281 Med 174 Range 8- 908	31%	100%	n>150 OS 9.2 n<150 OS 19.8	(Liu et al. 2018)
20 $\blacklozenge$	EpCAM <sup>+</sup> CTC selection/CellSearch®	Mean 0.25 Med 0 Range 0-2	Mean 6 Med 0 Range 0- 103	20%	45%	Positive for CTCs: OS 23.1 months Negative for CTC: 26.2 months	(Bissolati et al. 2015)
41 $\blacklozenge$	EpCAM <sup>+</sup> CTC selection/ Immunocytochemistry	Mean 71 Med 40 Range 14-414	Mean 230 Med 60 Range 14- 3579	39%	58.5% $\Sigma$	Correlation between metastatic disease and CTC detection in portal blood	(Tien et al. 2016)
14* $\Delta$	EpCAM <sup>+</sup> CTC selection/CellSearch®	Mean 0.7 Med 0 Range 0-7	Mean 125 Med 68 Range 1- 516	21%	100%	NA	(Catenacci et al. 2015)

**Abbreviations:** CTC, circulating tumor cell; EpCAM, epithelial cell adhesion molecule; PDAC, pancreatic ductal adenocarcinoma; ICC, immunocytochemistry; PCR, polymerase chain reaction; NA, not applicable; NS, not significant; OS, overall survival; SD, standard deviation; Med, median.

Tumor stage in the studied population:  $\blacklozenge$  resectable, \* borderline,  $\Delta$  metastatic/locally advanced;  $\theta$  neo-adjuvant treatment before blood sampling.

$\Sigma$ : statistically significant difference between portal and peripheral sample



**Table 2.** Comparison of CTC detection in portal/mesenteric/hepatic vein and peripheral blood

samples in patients with colorectal adenocarcinoma

Number of patients	CTC enrichment/Detection methods	CTC count in peripheral blood	CTC count in portal blood	CTC count in hepatic vein/central vein	CTC detection rate in peripheral blood	CTC detection rate in portal blood	CTC detection rate in hepatic vein/central vein (vena cava)	References
80Δθ	EpCAM <sup>+</sup> CTC selection/CellSearch®	NA	Mean 1.5 Med 0 Range 0-32	Mean 0.3 Med 0 Range 0-5	NA	35% Σ	17.5% (via central line)	(Rahbari et al. 2012)
75Δ	CD45 <sup>+</sup> leukocyte depletion Epispot®/EpCAM <sup>+</sup> selection/CellSearch®	- Epispot® Med 1.2 Range 0-92 - CellSearch® Med 0 Range 0-142	- Epispot® Med 4 Range 0-247 - CellSearch® Med 2.7 Range 0-286	NA	Epispot 55.4% CellSearch® 29%	Epispot 65.9% Σ CellSearch® 55.9% Σ	NA	(Denève et al. 2013)
29Δ	EpCAM <sup>+</sup> selection/CellSearch®	Open resection: -Arterial Mean 1.82 Med 1 Range 0-6 -Venous Mean 1.45 Med 1 Range 0-3	Open resection: Mean 1.5 Med 0 Range 0-32	Open resection: Mean 126 Med 87 Range 0-500	Open resection: Mean 174 Med 174 Range 0-500	NA	NA	(Jiao et al. 2009)
31	EpCAM <sup>+</sup> selection/CellSearch®	NA	NA	NA	17%	72% Σ	NA	(Wind et al. 2009)
63Δθ	EpCAM <sup>+</sup> selection/CellSearch®	Med 1 Inter-quartile Range : 0-4		Med 2.5 Inter-quartile Range :1-8	46%	NA	54% HV>3 ↓OS Multivariate analysis	(Connor et al. 2016)

Tumor stage of the studied population: Δ metastatic; θ neo-adjuvant treatment before blood sampling.

Prognostic value not evaluated except for [46]

Σ: statistically significant difference between portal and peripheral sample

**Table 3.** Blood sampling in different sites for CTC detection in patients with hepatocellular carcinoma (Sun et al. 2018)

Number of patients	CTC enrichment/Detection methods	Detection rate: pvCTC	Detection rate: paCTC	Detection rate: CTC hepatic vein	Detection rate: CTC portal blood	CTC recovery in inferior vena cava	Prognostic value
3*	EpCAM <sup>+</sup> selection/CellSearch <sup>h</sup> ®	68.49%	45.2%	80.82%	58.9%	39.72%	Intra hepatic recurrence: Univariate PaCTC+Pv CTC Multivariate: PvCTC with CTM

#### Abbreviations:

pvCTC, CTCs in the peripheral venous blood; paCTC, CTCs in the peripheral artery blood;  
CTM, circulating tumor micro-emboli;

\*11% of patients had metastatic disease and none received neo-adjuvant therapy

CTC count:

-Peripheral vein: median 2, range 0-26

-Peripheral artery: median 0, range 0-11

-Hepatic vein: median 6, range 0-31

-Portal vein: median 1, range 0-8

Differences in CTC detection rate between portal and peripheral venous samples statistically significant:

-Peripheral vein vs hepatic vein; peripheral vein vs inferior vena cava

-Peripheral artery vs hepatic vein; peripheral artery vs inferior vena cava

-Hepatic vein vs portal vein

**Table 4.** CTC detection in pulmonary vein and peripheral vein samples in patients with non-small lung cancer

Number of patients	CTC detection methods	CTC count in peripheral blood	CTC count in pulmonary vein blood	CTC detection rate in peripheral blood	CTC detection rate in pulmonary vein	Prognostic value	Reference
36	Oncobeam®	Med 1.5 Range 0-15	Med 7.5 Range 0-10	69.4%	83.3% $\Sigma$	Shorter PFS associated with CTC clusters	(Murlidhar et al. 2017)
30 $\Delta$	Veridex®	Mean 0.8 Med 0 Range 0-16	Mean 1195 Med 81 Range 0-10034	16.7%	96.7%	NS	(Okumura et al. 2009)
23	MACS+flow cytometry	Med 5 Inter-quartile range 3-9	Med 28 Inter-quartile range: 3-9	91.3%	95.7% $\Sigma$	High CTC count associated with lower DFS	(Li et al. 2017)

10	ScreenCell®+immunochemistry	Mean 22	Mean 65	80%	100%	NS	(Chudasa
	Analysis of 549 human lung cells	Range 0-100	Range 8-200				ma et al. 2017)
23 Δ	ScreenCell®+hematoxylin-eosin method	Cluster( CTC>4)n=6 Single CTC n=1	Cluster n=15 Single CTC n=4	30%	93%	NA	(Sawabata et al. 2016)
30	CellSearch®	CTC≥1/7.5ml n=6 [1-4]	CTC≥18/7.5ml n=23	22.2%	100% Σ	High CTCs in peripheral blood associated with DFS and OS	(Crosbie et al. 2016)
32 Δ	EpCAM-based microfluidic chip	Mean 3.1 CTCs/7.5mL	Mean 544 CTCs/7.5mL Σ	NA	NA	NS	(Reddy et al. 2016)
15 θ	EpCAM-based microfluidic chip	NA	Mean 95.7 Range 0-855	6.6%	80%	Correlation with neo-adjuvant therapy IT<SA PV CTC	(Tarumi et al. 2013)

**Table 5.** CTC count after tumor mobilization in pulmonary vein and peripheral vein samples in patients with non-small lung cancer

Number of patients	CTC detection methods	CTC count in pulmonary vein	CTC detection rate in peripheral blood	CTC detection rate in pulmonary vein	Prognostic value	Reference
30 Δ	CellSearch®	Med 60	6.7%	73.3%	NA	(Hashimoto et al. 2014)

30	Cellsearch®	Increase $\Delta$ CTC	No sample	80%	DFS OS metastasis correlated with $\Delta$ CTC	(Hashimoto et al. 2018)
32	Cellsearch®	Mean 617 Med 18 Range 1-8000	25%	90.6% $\Sigma$	NA	(Lv et al. 2018)

#### Table 4 and 5 Abbreviation and comments

Tumor stage of the studied population:  $\Delta$  metastatic;  $\theta$  neo-adjuvant treatment before blood sampling

$\Sigma$ : statistically significant difference between pulmonary vein and peripheral sample

IT: induction chemotherapy, SA: surgery alone, PV CTC: pulmonary vein CTC



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## Supplemental data

### Supplemental data

**Supplemental Table 1.** Main studies for CTC detection in the peripheral blood of patients with PADC, CRC, NSCLC and HCC.

Cancer types	Methods	Studies(n)	Nb Patients (with cancer)	Disease stage	Results Mean%+/- SD	References
PDAC	CellSearch®/ICC	4	194	All stages	49.6+/- 29.9	(Kurihara et al. 2008; Bidard et al. 2013; Earl et al. 2015; Khoja et al. 2012)
	Filtration	4	180	All stage	72.1+/- 18.3	(Iwanicki-Caron et al. 2013; Cauley et al. 2015; Bobek et al. 2014; Kulemann et al. 2015)
	PCR-based	7	344	All stage	68.5+/- 21.7	(Funaki et al. 1996; Miyazono et al. 1999; Chausovsky et al. 1999; Zhang et al. 2005; Soeth et al. 2005; Ishizone et al. 2006; Hoffmann et al. 2007)
KCR	Cellsearch®/ICC	11	1893	Metastatic	35.63+/- 19.85	(S. J. Cohen et al. 2009; Schoppmeyer et al. 2006; Steven J. Cohen et al. 2006; Hiraiwa et al. 2008; Königsberg et al. 2010; Matsusaka et al. 2011; Papavasiliou et al. 2010; Tol et al. 2010; Aggarwal et al. 2013; Seeberg et al. 2015; Sastre et al. 2013)
		3	878	Non-metastatic	23.33+/- 15.27	(Bork et al. 2015; Sotelo et al. 2015; P. Gazzaniga et al. 2013)

	PCR-Based	7	309	Metastatic	43.28+/- 17.43	(Vlems et al. 2003; Paola Gazzaniga et al. 2010; Rahbari et al. 2011; Koch et al. 2005; Staritz et al. 2004; Wyld et al. 1998; Yen et al. 2009)
HCC	Cellsearch®/ICC	12	963	NA	57.83+/- 18.03	(Kelley et al. 2015; Vona et al. 2004; S. Liu et al. 2013; Fan et al. 2011; W. Xu et al. 2011; Y.-M. Li et al. 2013; Schulze et al. 2013; Sun et al. 2013; Fang et al. 2014; Guo et al. 2014; Jun Li et al. 2014; Mu et al. 2014)
	PCR-Based	8	881	NA	43.75+/- 19.06	(Matsumura et al. 1999; Cillo et al. 2004; Jeng, Sheen, et Tsai 2004; Witzigmann et al. 2002; Mou et al. 2002; Yao et al. 2013; Choi et al. 2015; Kong et al. 2009)
NSCLC	Cellsearch®/ICC	4	483	Non-metastatic	53.5+/- 10.63	(Sawabata et al. 2007; Hofman, Bonnetaud, et al. 2011; Hofman, Ilie, et al. 2011; Bayarri-Lara et al. 2016)
		6	312	Metastatic	53.33+/- 24.4	(Krebs et al. 2011; Muinelo-Romay et al. 2014; Y. H. Xu, Zhou, et Pan 2015; Juan et al. 2014; Punnoose et al. 2012; Isobe et al. 2012)
	PCR-Based	3	250	Non-metastatic	39.66+/- 19	(Yamashita et al. 2002; Yoon et al. 2011; Jian Li et al. 2014)
		5	288	Metastatic	71.8+/- 7.15	(Du et al. 2014; Nieva et al. 2012; L. Liu et al. 2008; Sher et al. 2005; Chen et al. 2007)

Abbreviations: CTC, circulating tumor cell; PDAC, pancreatic ductal adenocarcinoma; CRC, colorectal cancer; NSCLC, non-small cell lung cancer; HCC, hepatocarcinoma;



ICC, immunocytochemistry; PCR, polymerase chain reaction; NA, not applicable; NS, non-significant.

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**La quantification des exosomes Glypican 1 positif dans le sang portal et périphérique ne peut pas être utilisée seul comme outil de diagnostic pour les cancers du pancréas résécable d'emblée.**

## **Résumé**

Les vésicules extracellulaires (VE) libérées par la tumeur contiennent une cargaison spécifique de la tumeur qui les distingue des VE sains et les rend utilisable comme biomarqueurs circulants. La pertinence de la détection des exosomes glypican 1 positif (GPC1) comme éléments de biopsie liquide est encore débattue. Nous avons mené une étude prospective pour quantifier les exosomes GPC1 positifs dans le sérum de patients atteints d'adénocarcinome canalaire pancréatique (PDAC) résécable d'emblé, avec un groupe contrôle de volontaire sans pathologie néoplasique. Les sérums ont été enrichis en VE et les exosomes ont été enrichie avec des billes magnétiques couplées anti-CD63. Les pourcentages de billes positives GPC1 déterminés par cytométrie de flux étaient significativement plus élevés dans la PDAC que dans le groupe témoin. La sensibilité était de 64 % et la spécificité de 90 %, lorsque les résultats du sang périphérique et du sang porte étaient combinés. En association avec les résultats de l'écho-endoscopie ponction, la valeur prédictive négative était de 80% contre 33% pour l'écho-endoscopie ponction seule. Cette approche, bien qu'elle ne soit pas suffisante pour diagnostiquer le cancer du pancréas, est pertinente en tant que valeur ajoutée aux outils de diagnostic déjà disponibles.

Mots-clés :

Exosomes Glypican 1 positifs, biopsie liquide, adénocarcinome pancréatique canalaire, biomarqueurs circulants, sang veine porte.



#### **4- Glypican 1 positive exosome quantification cannot be used as a sole diagnostic tool to detect all early pancreatic cancer even in portal blood liquid biopsies**

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**Running title:** GPC1 diagnosis pancreatic ductal adenocarcinoma

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### **Highlights:**

- PDAC patients show higher levels of GPC1-positive exosomes in serum than healthy controls
- GPC1 positive exosome quantification in serum is not a perfect diagnostic tool for PDAC as previously proposed
- GPC1 positive exosome quantification with EUS-FNA and/or CA19-9 show very good diagnostic performances
- Patients with negative for GPC1 positive exosome in peripheral blood show less disease recurrence

### **Key Words:**

Glypican 1 positive exosomes, Liquid biopsy, Pancreatic Ductal Adenocarcinoma, Circulating Biomarkers, portal blood.

#### 4-1 Abstract

Tumor-released extracellular vesicles (EVs) contain tumor-specific cargo distinguishing them from healthy EVs, and making them eligible as circulating biomarkers. Glypican 1 (GPC1)-positive exosome relevance as liquid biopsy elements is still debated. We carried out a prospective study to quantify GPC1-positive exosomes in sera from pancreatic ductal adenocarcinoma (PDAC) patients undergoing up-front surgery, as compared to controls including patients without cancer history and patients displaying pancreatic preneoplastic lesions. Sera were enriched in EVs, and exosomes were pulled down with anti-CD63 coupled magnetic beads. GPC1-positive bead percentages determined by flow cytometry were significantly higher in PDAC than in the control group. Diagnosis accuracy reached 78% (sensitivity 64% and specificity 90%), when results from peripheral and portal blood were combined. In association with echo-guided-ultrasound-fine-needle-aspiration (EUS-FNA) negative predictive value was 80% as compared to 33% for EUS-FNA only. This approach, although not sufficient to diagnose PDAC, is clinically relevant as a companion test to the already available diagnostic tools, since patients with GPC1-positive exosomes in peripheral blood showed decreased tumor free survival.

#### **Abbreviations:**

EUS-FNA, endoscopic ultrasound-guided fine needle aspiration; EVs, extracellular vesicles; GPC1, Glypican 1; PDAC, pancreatic ductal adenocarcinoma; CA19-9 carbohydrate antigen19-9; CEA, Carcinoembryonic antigen; IPMN, intraductal papillary and mucinous neoplasm; ddPCR digital droplet polymerase chain reaction;

CT-scan, computerized tomography scanner; MRI, magnetic resonance imaging; FSC, forward side scatter; SSC, side scatter.

## 4-2 Introduction

Pancreatic ductal adenocarcinoma (PDAC) is discovered at advanced stages because its clinical presentation is preceded by non-specific symptoms. The only curative treatment is surgery but only 20% of the patients are eligible for tumor resection [1,2]. Several diagnostic tools such as imaging are needed, making diagnostic long and costly [3,4]. PDAC management is complicated by the fact that the onset of neo-adjuvant or palliative therapies depends on the required histological proof of the presence of the tumor. This proof, routinely obtained by conventional biopsies, is risky for the patients. For example, endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) may result in cancer cell dissemination along the needle track, may provoke pancreatitis, and its predictive negative value is low [3]. When EUS-FNA is negative, the procedure is repeated leading to delayed patient management, which worsens the prognosis [5]. The CA19-9 (Carbohydrate Antigen) or CEA (Carcinoembryonic Antigen) plasma biomarkers have poor sensitivity and specificity and are not recommended for PDAC diagnosis [6–8]. Finally, even when resection is possible and complete, a high percentage of patients develop metastatic disease, without possible identification before surgery by predictive markers [9,10]. Thus, efforts aim at finding new diagnostic, predictive and prognostic tools for PDAC.

In 2015, a major advance was published by Melo et al. identifying PDAC by quantifying circulating tumor-specific exosomes enriched in the membrane protein heparan sulfate proteoglycan glypican 1 (GPC1) [11]. GPC1 is overexpressed in PDAC primary tumors [12] and supports tumor cell proliferation and migration [13]. Moreover, heparan sulfate

proteoglycans, including GPC1 are involved and remain in exosome internalization [14]. GPC1 was found membrane bound to exosomes isolated from several cancer cell lines where it was also up-regulated as compared to healthy fibroblasts [11]. Based on this observation, a flow-cytometry test distinguished perfectly PDAC over healthy donors or patients with benign pancreatic diseases in exosome-enriched sera. Since this proof of principle, studies trying to confirm this crucial advance have reached various degrees of validation. In 2017, exosomal GPC1 quantified by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS), using a GPC1 specific peptide, did not identify PDAC patients over controls (healthy donors or chronic pancreatitis (CP), [15]). Instead, a 5-microRNA signature with high miR-10b, -21, -30c, and 181a and low let7a differentiated PDACs from controls. In the same way, a signature of 5 proteins (EGFR, EpCAM, MUC1, HER2, GPC1 and WNT2), found by EV (extracellular vesicle)-based protein marker profiling identified PDAC patients with a sensitivity of 86% and a specificity of 81% [16]. In this report, EV-GPC1 used alone did not distinguish PDAC patients from controls. In 2018, alternating current electrokinetic (ACE) microarray chip, capturing exosomes directly from plasma, followed by immunofluorescent detection and quantification of CD63 and GPC1 did identify PDAC over healthy patients [17]. This is the only independent study confirming the possibility to diagnose PDAC patients by quantifying CD63<sup>high</sup>/GPC1<sup>high</sup> exosomes. Thus, according to conflicting results in published data, a key question to answer is whether reported findings on GPC1-positive exosomes identifying PDAC can be validated independently in early stage patients.

We used a prospective cohort of patients who underwent up-front surgery without neo-adjuvant treatment for localized PDAC, because they are the population of patients for whom rapidity in diagnostic is crucial to avoid delay, and lower cancer progression risk.

We pulled down GPC1-positive exosomes from sera from cancer patients and non-cancer controls, with anti-CD63 coupled magnetic beads and quantified them by flow cytometry. Importantly, we tested the hypothesis that GPC1-positive exosomes might be more numerous in portal blood drained from the primary tumor. We assessed the diagnostic accuracy of this method as compared to CA19-9 quantification, endoscopy ultrasound guided fine needle aspiration (EUS-FNA) tumor cell identification and *KRAS* circulating tumor DNA amplification.

### **4-3 Materials and methods**

#### Patients demographics:

Patients eligible for pancreatic surgery with suspicion of pancreatic cancer or IPMN (intraductal papillary and mucinous neoplasm) with worrisome features as determined by CT-scan (computerized tomography scanner) and or MRI (magnetic resonance imaging) without metastasis were enrolled at the department of hepatobiliary surgery of Bordeaux university hospital between February and November 2017. This prospective study was conducted according to the Declaration of Helsinki, the French rules (Law for Bioethics November 2016) and the recommendations of CNIL (Comité National Informatique et Liberté), and was approved by an Institutional Review Board. The biological collection was declared to and approved by the “comité de protection des personnes sud ouest outremer” under the number 2016-A00431-50 and the database was registered in Clinical Trials under the number: NCT03032913. Informed consent was obtained from patients and before surgery. Patients did not receive neoadjuvant therapy. A control group enrolled patients, with informed consent, undergoing surgery for benign pathologies, and with no history of cancer. Patient follow-up, completed until December 1, 2018, evaluated survival and disease recurrence.

### Blood sampling:

Two samples of 7.5 ml of blood were collected from the portal vein in BD vacutainer collection tubes without additives (SST tubes, Becton Dickinson, Le Pont de Claix, France), after laparotomy, before manipulation of the tumor. Two samples of 7.5ml were collected during surgery in the median cephalic vein in BD vacutainer collection tubes without additives. A sample of 7.5ml in BD vacutainer collection tube containing EDTA was collected for complete blood count, to determine neutrophil over leukocyte ratio in the patient group (DXH automated counter, Beckman Coulter, Villepinte, France). Median cephalic vein was punctured in the control group, to collect sample in vacutainer collection tubes without additives. Tubes were centrifuged quickly at 2000g for 15 minutes to collect sera. Sera from patients were immediately used to determine CA19-9 concentration (Architect automated instrument, Abbott). Then sera were frozen at -80°C until they were further processed. In addition, one tube (Cell free DNA collection tube®, Roche, Meylan, France) was collected in a peripheral vein for all patients and controls, and also in the portal vein for IPMN and PDAC groups for ctDNA analysis.

### Exosome isolation and flow cytometry

Sera were enriched in extracellular vesicles (EVs) using the Total Exosome Isolation kit (Thermofisher, Courtaboeuf, France), according to the manufacturer's instructions. Briefly, serum samples stored at -80°C were thawed on ice and centrifuged at 2000g for 30 minutes to remove cells and debris. Sera supernatants were incubated for 30 minutes with Total Exosome Isolation reagent at 4°C and centrifuged for 10 minutes at 10,000g. EV-enriched pellets were resuspended in Phosphate Buffered Saline (PBS), and stored at -20°C. The Exosome-Human CD63 Isolation/Detection Reagent (Thermofisher) was used to pull down sera exosomes. Magnetic bead-coupled exosomes were stained with anti-CD63-FITC (Biolegend, London, UK) to validate

exosome isolation. Flow cytometry analysis confirmed that 98 to 99% of the beads were CD63-positive (not shown). Anti-CD9-PE antibody (Biolegend) and anti-GPC1 primary antibody (PIPA528055, Thermofisher) revealed with Alexa Fluor 647 donkey anti rabbit IgG (Biolegend) were used together. All staining steps and washes were carried out in PBS1X/ BSA 0.1%. Samples were examined on a BD FACS CANTO II apparatus using unstained beads and beads stained with isotype controls (PE mouse IgG1 Kappa isotype control clone MOPC-21 (Biolegend) and rabbit igG isotype control Alexa Fluor 647 conjugate (OZYME, Montigny-le-Bretonneux, France)) to setup quantification areas on dot plots. Data were collected on FSC and SSC linear parameters (645V and 520V, respectively) and on a logarithmic scale for Alexa 647 and PE (455V and 400V, respectively). Data were analyzed with BD FACS Diva software (BD Biosciences, Le Pont de Claix, France). Each sample was analyzed twice in independent experiments.

#### Western Blot Analysis

After EV extraction, proteins were purified from sera as already described [18]. We used the same primary antibodies as in flow cytometry analysis. Densitometry quantification was carried out with Image J 1.52a software [19].

#### ctDNA quantification

Plasmas were collected in Cell free DNA collection tube and were subjected to DNA extraction (RSC ccfDNA plasma kit, Maxwell (Promega, Charbonnières-les-Bains, France)). Tissue DNA was extracted from FFPE specimens using Maxwell® RSC DNA FFPE Kit (Promega) according to the manufacturer's instructions. *KRAS* mutant alleles were detected by droplet digital PCR (ddPCR) with the *KRAS* G12/G13 Screening Kit (Biorad, Marne la Coquette, France).

#### Statistics



Qualitative and quantitative data were analysed with usual statistic tools (including Student's-test, Chi-Square and Fisher exact tests) using GraphPad-Instat and GraphPad-Prism 8.0 software (GraphPad Software Inc. San Diego, CA, USA). The Spearman correlation coefficient was used to assess the correlation between different variables. Group differences were tested using the nonparametric Wilcoxon-Mann-Whitney test for two groups. Receiver operating curves (ROC) were obtained to describe the accuracy of detecting cancer. The cutoff points were selected using Youden's index, which maximizes the sum of sensitivity and specificity. ROCs and Youden's index calculation were performed using SAS software (version 9.4, SAS institute, Cary, NC, USA).

#### **4-4 Results:**

##### Patient characteristics

From February to November 2017, 72 patients underwent surgery for PDAC of which 32 had an up-front surgery for presumed PDAC without neo-adjuvant therapy (Figure 1). Among them, two patients were excluded because metastatic disease was discovered during surgery, and 8 patients were excluded from the cancer group and switched to the control group after definitive pathology analysis, because of non-invasive intraductal papillary mucinous neoplasm (IPMN) diagnostic. Thus, control group included 20 control patients operated in our surgical unit without neoplasia and without a history of cancer (including two chronic pancreatitis who underwent surgery for symptomatic reasons) and 8 IPMN (Figure 1). Demographic and clinico-pathological characteristics are shown in Table 1. The groups were similar according to all clinical features (Table 1). Mean tumor size was 31 mm. All tumors were early stages.

### GPC1 positive exosomes in peripheral blood

To test the validity of GPC1 levels in EVs, we used EV-enriched sera. Western blot analysis found expected signals for the exosomes markers CD63 and CD9 (Figure 2A) and GPC1 (Figure 2B). Densitometry analysis showed that CD9 expression levels were similar in PDAC and control groups. By contrast CD63 signals tended to be higher in the PDAC group by almost 3 fold ( $p=0.07$ ,  $n=4$ ). GPC1 protein levels were similar in both groups.

Previous data reported that PDAC exosomes overexpressed both CD63 and GPC1 as measured by alternative current electrokinetic (ACE) [17]. In their working model the authors used both biomarkers to discriminate PDACs from healthy donors. Our total EV GPC1 levels were similar in both groups by western blot, so we hypothesized that pooling down CD63-positive EVs followed by GPC1 detection would distinguish cancer patients from non-cancer controls. First, anti-CD63 magnetic beads were used to obtain CD63-positive exosomes from peripheral blood. As expected, they were positive for the exosome specific CD9 marker with no difference between PDAC and control groups, including healthy volunteers and patients with IPMNs ( $p=0.5$ , Figure 3A and 3B, Table 2). Interestingly, mean percentages of GPC1-positive beads were significantly higher in the PDAC group as compared to non-cancer patients ( $23.7 \pm 7.31$ ,  $n=22$  and  $5.7 \pm 3.88$ ,  $n=20$ , respectively,  $p=0.04$  by unpaired student's t test, Figure 3A and 3C, Table 2). The IPMN group was not significantly different from both other groups ( $9.71 \pm 7.50$ ,  $n=8$ ,  $p>0.05$  by unpaired student's t test). The positivity threshold for GPC1 exosomes calculated using the Youden index was 5%. Fifty of the patients were above this threshold.

### GPC1 positive exosomes in portal blood

We hypothesized that circulating tumor exosomes could be more numerous in a blood sample drawn from the portal vein, near the tumor, as compared to peripheral blood drawn from the median cephalic vein. Portal samples were obtained only in the operated patients. GPC1-positive bead percentages were not significantly different in peripheral and portal samples ( $16.33 \pm 6.19$ ,  $n=22$  and  $7.3 \pm 6.31$ ,  $n=8$ , respectively,  $p=0.42$ , Figure 3D), even if they tended to be lower in the portal samples. Next, as we had both portal and peripheral blood samples of the 31 patients we assessed whether GPC1-positive bead percentages matched between samples (Figure 3E). Three patients presented discordant results, but overall, percentages were correlated with Pearson's test ( $\rho=0.59$ ,  $p=0.037$ ).

#### Diagnostic performance

Diagnostic performance was evaluated by determining accuracy, sensitivity and specificity (Table 3). Overall, diagnosis accuracy of GPC1-positive exosomes was better in peripheral blood than in portal blood. A total of 63% (14 out of 22) of patients were detected for GPC1 positive exosomes in portal and/or peripheral blood. Both sampling sites showed better performance than CA19-9. EUS-FNA carried strong specificity, but poor negative predictive value, as expected. Interestingly, combining all three diagnostic tools led to high sensitivity and specificity (82% and 86%, respectively), and highest diagnosis accuracy (84%, Table 3). ROC curves showed that GPC1-positive exosomes in peripheral blood displayed higher area under the curve (AUC) than CA19-9 in peripheral blood (Figure 4).

#### ctDNA detection

All patients and controls were negative for circulating *KRAS* mutant DNA in peripheral or portal blood, whereas both our metastatic patients had detectable *KRAS* mutant alleles (data not shown and mutant allelic frequency in peripheral blood: 1.6% and

0.6%; in portal blood 0.41% and 0.4%). Thus, ctDNA did not distinguish PDAC patients from controls.

Importantly, all primary tumors were *KRAS* mutant with a mean mutant allele frequency of 26.15 (Table 1).

#### Correlation of exosome levels to clinico-histopathologic risk factors and prognostic

The median follow-up was 18 months (range 2-23). All data were included until December 2018. A low positive correlation between the percentage of GPC1-positive exosomes and the tumor size was found (Pearson's test  $p=0.39$ , Figure 5A), with a  $p$  value close to statistical significance ( $p=0.07$ ). Exosome rate did not correlate with other histopathological parameters, including tumor stage, the number of invaded lymph nodes and carcinoma differentiation. Despite the short follow-up period (median 554 days; mean 532 days; range 74-718 days) and the small size of the cohort, we analyzed the exosome results in the context of the clinical follow-up. Among the 22 patients, 14 developed tumor recurrences (64%), including metastatic relapses in the liver and other organs. Four patients died (18%). Kaplan-Meier analysis indicated that patients with of GPC1 positive exosome in peripheral blood above the threshold relapsed sooner than patients with GPC1-positive exosomes below the threshold ( $p=0.01$ , Figure 5B). GPC1-positive exosomes did not prognostic value combined with portal results for disease recurrence or for overall survival (figure 5C, D).

#### **4-5 Discussion:**

Tumor EVs carry sufficient information to control the activity of recipient cells, whether they are tumoral [20] or healthy [21], leading to responses supporting tumor growth or dissemination. Thus, they seem to be very relevant material as circulating tumor biomarkers carriers, such as nucleic acids, proteins or lipids. In this way, the identification of GPC1-positive exosomes as highly specific and sensitive biomarkers

to diagnose PDAC was very exciting [11]. In fact, only few reports have been published since to test this potentially powerful diagnostic tool. Our study aimed at confirming this breakthrough finding in a group of PDAC patients eligible for up-front surgery, considered detected early in the course of the disease [22]. Moreover, as for circulating tumor cells, it is possible that such patients might release less tumor-related elements in the blood circulation as the disease is less advanced [23], making their detection even more challenging.

First, we detected GPC1 protein at the expected size in total sera by western-blot and found no difference in signal intensity between patients and healthy controls. We tested very few samples ( $n=4$  in each group) as the main aim of our work was to quantify GPC1-positive exosomes. Increasing the number of samples might identify significant differences in both groups as published for serum GPC1 levels measured by ELISA [24]. This latter work however, found that serum GPC1 was inferior to CA19-9 in terms of diagnosis accuracy, suggesting that this approach is not relevant for PDAC.

The first publication proposing GPC1-positive exosomes for identifying PDAC reported perfect 100% sensitivity and 100% specificity [11]. This exceptional result was not confirmed by further studies. In particular, Yang et al. [16] described an EV protein signature (EGFR, EpCAM, MUC1, HER2, GPC1 and WNT2), by measuring antibody-linked fluorescence intensity of ultracentrifuged plasma EVs, identifying PDAC with a diagnostic accuracy of 84%, a sensitivity of 86% and a specificity of 81%. Detecting GPC1 alone showed a lower diagnosis accuracy of 67%, a sensitivity of 82% and a specificity of 52% ( $n=43$ ). We found a similar accuracy of 72% but a lower sensitivity (50%) and a higher specificity (90%). In addition, Lai et al.[15] did not report diagnostic performance for GPC1-positive exosome but did not find significant difference in levels of GPC1 protein in exosomes from PDAC versus controls, in small groups ( $n=3$  and

n=6, respectively), and using LC-MS/MS. A recent publication identified PDAC patients with alternating current electrokinetic (ACE) microarray chip, capturing nanoscale objects, including exosomes, directly from plasma, followed by immunofluorescent detection and quantification of CD63 and GPC1. Although high sensitivity and specificity (99% and 82%, respectively) were achieved, false positive and false negative results were obtained [17]. Thus, GPC1 positive exosomes quantification as originally described by Melo et al.[11] with 100% sensitivity and specificity was not confirmed by us or other groups. The discrepancies might originate from different technical approaches to measure exosome-GPC1 levels. Ultracentrifugation is not easily applicable in clinical routine [25]. Moreover, we tested ultracentrifugation followed by latex beads coupling and did not distinguish PDAC from controls (n = 7 PDACs n = 20 controls, not shown). Thus, as the first enrichment step in EVs, we used a density-based separation kit coupled to magnetic beads decorated with anti-CD63, limiting the analysis to the exosome population [26]. We used the same antibody as Melo, because this parameter seemed crucial in reproducing their results. This approach improved PDAC patient identification but did not reach perfection as published by Melo [11]. Instead our results are similar to already published results [15,16].

As blood from portal vein is enriched in circulating tumor cells [27,28], we hypothesized that GPC1-positive EVs were more numerous in portal samples. To our knowledge, this series is the first testing GPC1-EVs in portal vein samples. In fact, we found that although not significantly different, GPC1-positive EVs tended to be less concentrated in portal blood as compared to peripheral blood. Sensitivity and specificity were similar but positive predictive value was higher (91% vs 79%) suggesting that that portal sample can improve PDAC identification. Moreover, combined results found better

AUC in ROC curves (Figure 4). In the same way, combining peripheral and portal blood analysis led to better diagnostic performance (Table 3). Noticeably, this approach is valid since portal blood sampling is feasible. Indeed, authors reported sampling of portal vein for 18 patients during EUS FNA for circulating tumor cells enrichment [29]. As already published, ctDNA did not identify early stage PDAC as we only detected our two metastatic patients [23]. Recently, it was reported that quantifying tumor DNA in exosome might not be more useful since only 25 to 29 % of PDAC patient had *KRAS* or *TP53* mutant DNA in exosomes [16].

Our cohort is prospective and homogenous since composed of patients all eligible for up-front surgery, which was confirmed by histopathological analysis. Previous studies identifying circulating tumor elements in PDAC included mainly advanced stages or metastatic patients. At this stage of the disease, our control group is interesting because it consists of heterogeneous pathologies, two patients with chronic pancreatitis and eight patients with pre-neoplastic cystic lesions. Nevertheless, the cohort presents limits. In particular, the number of patients is small and conclusions need to be validated in a larger prospective cohort. This is especially true when we analysed correlations between exosomes levels and clinico-histopathologic data. Pearson correlation coefficient showed a low positive correlation between exosomes levels and tumor size and p value was 0.07. It is probable that increasing cohort size would reach statistical significance.

Traditional tools for the diagnosis of PDAC include CA19-9 and histologic proof currently obtained by EUS-FNA [10]. Patients with early-stage disease are more difficult to diagnose by EUS-FNA. Cytopathological specimens, may reach high sensitivity (75%-98%), specificity (71%-100%), but are not devoid of post-procedure morbidity, especially pancreatitis. The presence of pancreatitis decreases sensitivity to

74% as compared to 91% with normal surrounding pancreatic parenchyma. Current data show that the negative predictive value of EUS-FNA actually ranks between 33 and 85% [3,10,30]. This is probably due to the histological nature of the tumor, rich in fibrosis and often poor in tumor cells. Our series presented the most difficult diagnostic conditions since patients were all at an early stage with small lesions. Moreover, PDAC management tends now to perform neoadjuvant therapy even for patients eligible for up-front surgery. So, the low negative predictive value of EUS-FNA is becoming problematic [5]. Quantifying GPC1 positive EVs in the peripheral and/or the portal blood as a companion test might improve the diagnostic leading to a negative predictive value of 80% (as compared to 33% for EUS-FNA alone).

CA19-9 had sensitivity and specificity as low as 70% and 68% respectively, which is in agreement to recommendations to not use it routinely for diagnosis [7,8]. In the same way, CA19-9 is not a good prognostic marker for early stages [9]. Accordingly, we found a very low sensitivity (37%) and a specificity of 87%. Interestingly however, combining all three diagnostic tools, i.e. GPC1-positive EVs, CA19-9 and EUS-FNA improved all diagnostic performance parameters (Table 4), and displayed the best diagnosis accuracy (84%). Consequently, it would be very interesting to test in a larger cohort a patient early management strategy including GPC1-positive exosome quantification. Indeed, liquid biopsy is a very low risk procedure and additional cost is manageable.

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## **Author's contributions**

EB: conception, design, analysis, assembly and interpretation of data, financial support, manuscript writing.

AC: collection and assembly of data, interpretation of data.

PQ: collection and assembly of data, interpretation of data.

OD: collection and assembly of data, interpretation of data.

CB: Statistical analyses

IL: collection and assembly of data.

IM: collection and assembly of data.

CC: design, collection of data, interpretation of data.

SV: design, collection of data, interpretation of data.

AB: design, final approval of the manuscript

MM: analysis and interpretation of data, final approval of the manuscript.

LC: design, patient inclusion, final approval of the manuscript.

CL: design, patient inclusion, final approval of the manuscript.

VV: interpretation of data, final approval of the manuscript.

FMG: design, financial support, final approval of the manuscript.

ABe: conception, design, analysis, interpretation of data, final approval of the manuscript.

SD: conception, design, collection, analysis, assembly and interpretation of data, financial support, manuscript writing.

### **Conflict of interest**

The authors declare no conflict of interest.

### **Figures legends**

#### **Figure 1: Patient selection criteria**

PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary and mucinous neoplasm

#### **Figure 2: GPC1 is present in patient and control EV-enriched sera**

Proteins extracted from EV-enriched patient and control sera were analysed by western blotting for the presence of CD63 and CD9 exosome markers and for GPC1 proteins. Protein sizes of marker are indicated by arrow heads in kDa. Glyceraldehyde phosphodehydrogenase (GAPDH) detection was used as a loading control. A student's t test was used to compare densitometry values.

#### **Figure 3: GPC1-positive exosomes partially identify PDAC patients**

(A) Representative dot plots of PDAC and controls for the quantification of positive exosomes for the exosome specific marker CD9, and the GPC1 protein. Top three panels show bead detection according to physical criteria (size, FSC and granularity,

SSC, left panel), rabbit IgG isotype control Alexa Fluor 647 control stain (medium top panel) and PE mouse IgG1 Kappa isotype (right top panel). Three medium panels show representative results for a PDAC patient and lower three panels show representative results for a control. Percentages of stained beads are indicated in each dot plot. GPC1/CD9 results correspond to % of GPC1-positive beads within the CD9-positive population. Individual results are plotted for each group of participants, for CD9-positive exosome counts in peripheral blood (B), GPC1-positive exosome counts in peripheral blood (C), GPC1-positive exosome counts in portal blood (D). Correlation of GPC1-positive exosome counts in peripheral blood versus portal blood has been plotted in (E). The dashed lines delineate the positivity threshold. Ns: not significant. \*:  $p < 0.05$ .

#### **Figure 4: ROC curves for GPC1-positive exosomes and CA19-9**

The ROC curves were built with data obtained from peripheral blood for markers used alone, or for GPC1-positive exosome measured in portal blood, or for combined GPC1-positive exosomes measured in portal and peripheral blood; for each pair, we took the lowest %GPC1<sup>+</sup> exosomes of PDAC and the highest lowest %GPC1<sup>+</sup> exosomes of controls. AUC, area under the curve.

#### **Figure 5: Analysis of GPC1-positive exosome quantification according to clinical and pathological criteria**

(A) Pearson correlation between peripheral blood positive GPC1 exosomes and tumor size ( $\rho$ , Pearson correlation coefficient). (B-C) Tumor free survival according to presence of GPC1-positive exosomes in peripheral blood (B); in peripheral and portal blood (C). (D) Overall survival according to presence of GPC1-positive exosomes in peripheral blood. Positivity threshold for GPC1 positive exosomes =5% as determined by Youden's index.



## TABLES

Table 1: Clinical and histological features of the cohort

Variables	PDAC group (n=22) N (%)	Control Group (n=28) N (%)
Age, yr, (median; range)	68.8 (69.5; 57-81)	58.3 (61;22-73)
Male gender, n (%)	13 (59)	8 (28)
Serum CA19-9 (n=19 PDAC group)		
Normal	16 (84)	23 (82)
Elevated	3 (16)	5 (IPMN) (18)
NLR mean (med; range)	7.19 (3.7;0.69-21)	
EUS-FNA	Total n=18 Positive n=8 Negative n=10	
Pathology: Macroscopic		
Tumor size(mm) mean (med; range)	31 (30;11-50)	
Tumor stage (mean)	Stage 1a: 1 (5) Stage 1b: 4 (18) Stage 2b: 11 (50) Stage 3: 6 (59.2)	
Glandular Differentiation (%)	Well 3 (13.6) Moderately 11 (50) Poorly 8 (36.4)	
<i>KRAS</i> status all primary tumors were positive for <i>KRAS</i> :		
Mutant allele frequency mean (med; range)	26.15 (17.45;0.35-77.6)	

IPMN (n=8) are included in the control group. med, median; PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary and mucinous neoplasm; EUS-FNA endoscopic ultrasound-guided fine needle aspiration; NLR, neutrophil to lymphocyte ratio; med, median; Note that CA19-9 dosages for 3 PDAC patients were uninterpretable because of jaundice.

Table 2: Numeric results of exosome quantification in PDAC patients and control group

Sample	PDAC group	Control group
Portal Samples (n)	22	8 (IPMN)
Peripheral samples (n)	22	28
CD9 positive (n (%)) peripheral blood	22 (100%)	28 (100%)
CD9 positive beads rate peripheral blood mean (med; range)	31.4 (19.45;1-87.1)	42.7 (41.2;1-90.3)
CD9 positive beads (n (%)) portal blood	22 (100%)	8 (100%)
CD9 positive beads rate portal blood mean (med; range)	22.5 (12.45;1-94)	36.4 (40;1-91.8)
GPC1 positive (n (%)) peripheral blood	11 (50%)	3 (10,7%)
GPC1 positive beads rate peripheral blood mean (med; range)	23.7 (3.45;1-96.5)	-IPMN:9.71 (2.65;0.3-62.1) -Control (without-IPMN n=20): 5.7 (0.7;0-77.3) -Control (with IPMN n=28): 7.02 (1.35;0-77.3)
GPC1 positive (n (%)) portal blood	10 (46%)	1 (12%)
GPC1 positive beads rate portal blood mean (med; range)	16.33 (3.6;1-92.6)	7.3 (0.7;0.6-51.4)
GPC1 positive portal and peripheral n (%)	14 (63%)	3 (10.7%)

med, median; PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary and mucinous neoplasm

Table 3: Diagnosis values of GPC1 positive exosomes, CA19-9 and EUS-FNA

Test	Sensitivity (95% CI)	Specificity (95%CI)	Positive predictive value (95%CI)	Negative predictive Value (95%CI)	Diagnosis accuracy (95%CI)
EVs GPC1 portal vein	46 (27-66)	88 (53-99)	91 (63-99)	36 (20-59)	57 (50-64)
EVs GPC1 peripheral vein	50 (31-70)	90 (77-99)	79 (58-98)	70 (54-82)	72 (65-78)
*EVs GPC1 peripheral and/or portal vein	64 (43-81)	90 (73-97)	83 (59-94)	76 (59-88)	78 (72-83)
CA19-9	37 (19-59)	87 (72-95)	63 (36-85)	69 (54-82)	68 (61-74)
EUS FNA (n=18; PDAC n=15; IPMN n=3)	60 (36-81)	100 (31-99)	100 (60-99)	33 (13-65)	66 (59-73)
*EVs GPC1 and CA19-9	72 (52-87)	93 (78-99)	89 (68-99)	81 (65-92)	84 (78-89)
CA19-9 and EUS-FNA	50 (31-70)	92 (78-99)	86 (58-98)	70 (55-83)	74 (67-80)
*EVs GPC1 and EUS FNA	73 (52-87)	86 (69-95)	80 (59-92)	80 (63-91)	80 (74-85)
*EVs GPC1 + CA19-9+ EUS FNA	82 (62-93)	86 (69-95)	82 (62-93)	86 (69-95)	84 (78-89)

PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary and mucinous neoplasm; EUS-FNA endoscopic ultrasound-guided fine needle aspiration. \*EVs GPC1: quantification in peripheral and portal vein.

FIGURES

Figure 1

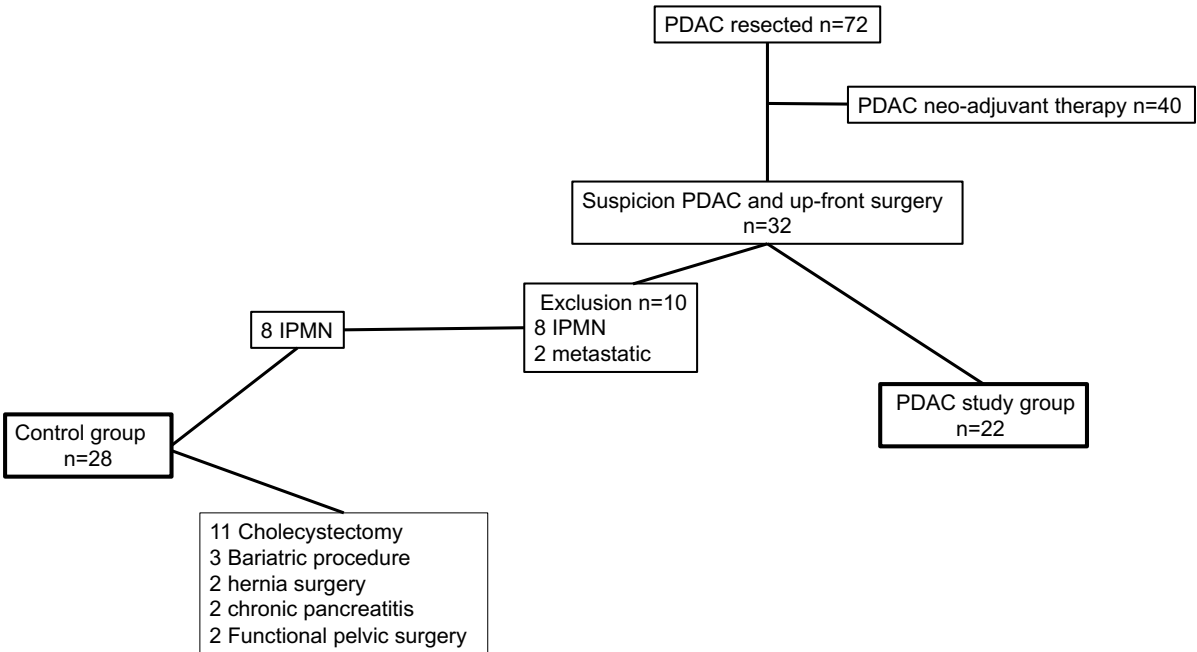
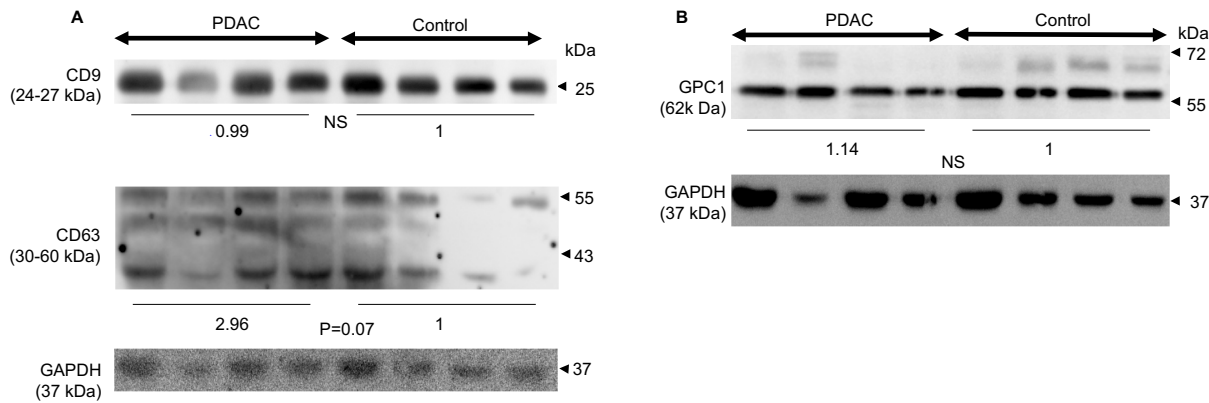


Figure 2





**Figure 3**

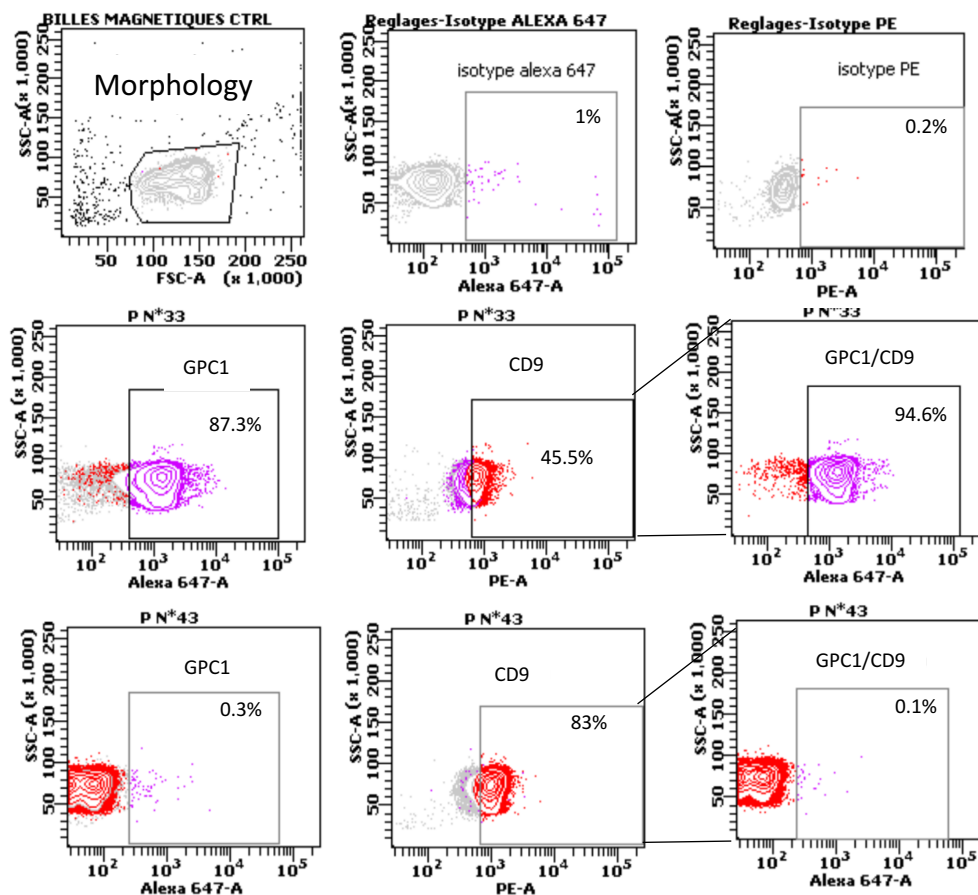
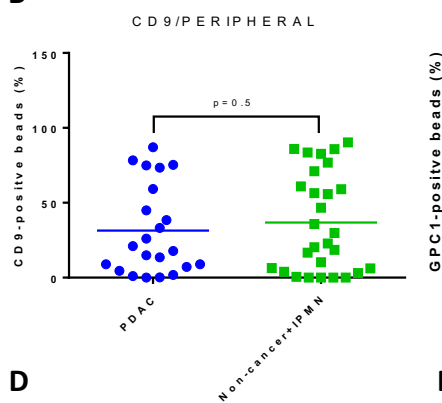
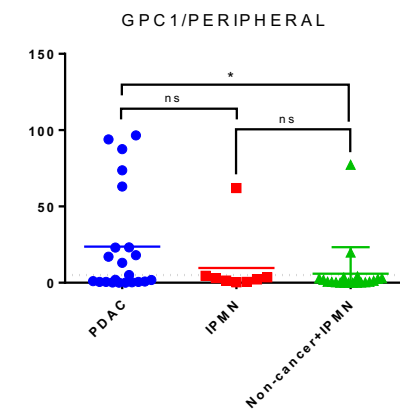
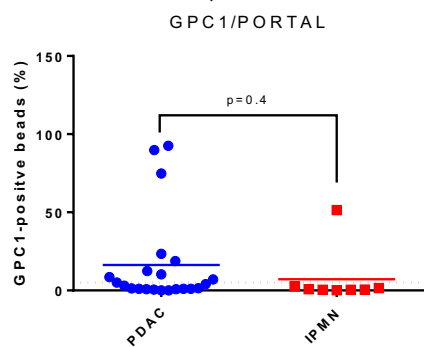
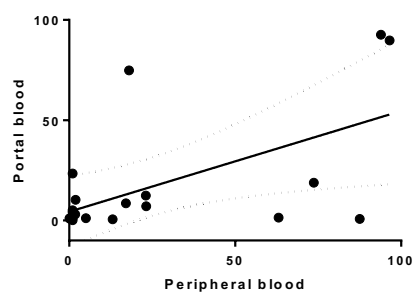
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Figure 4

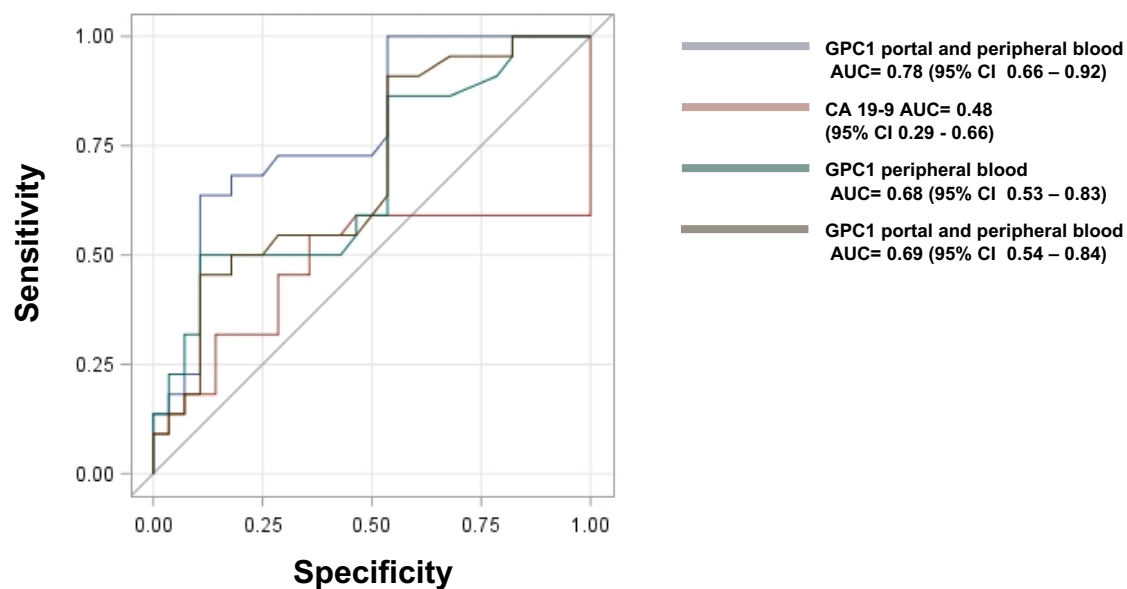
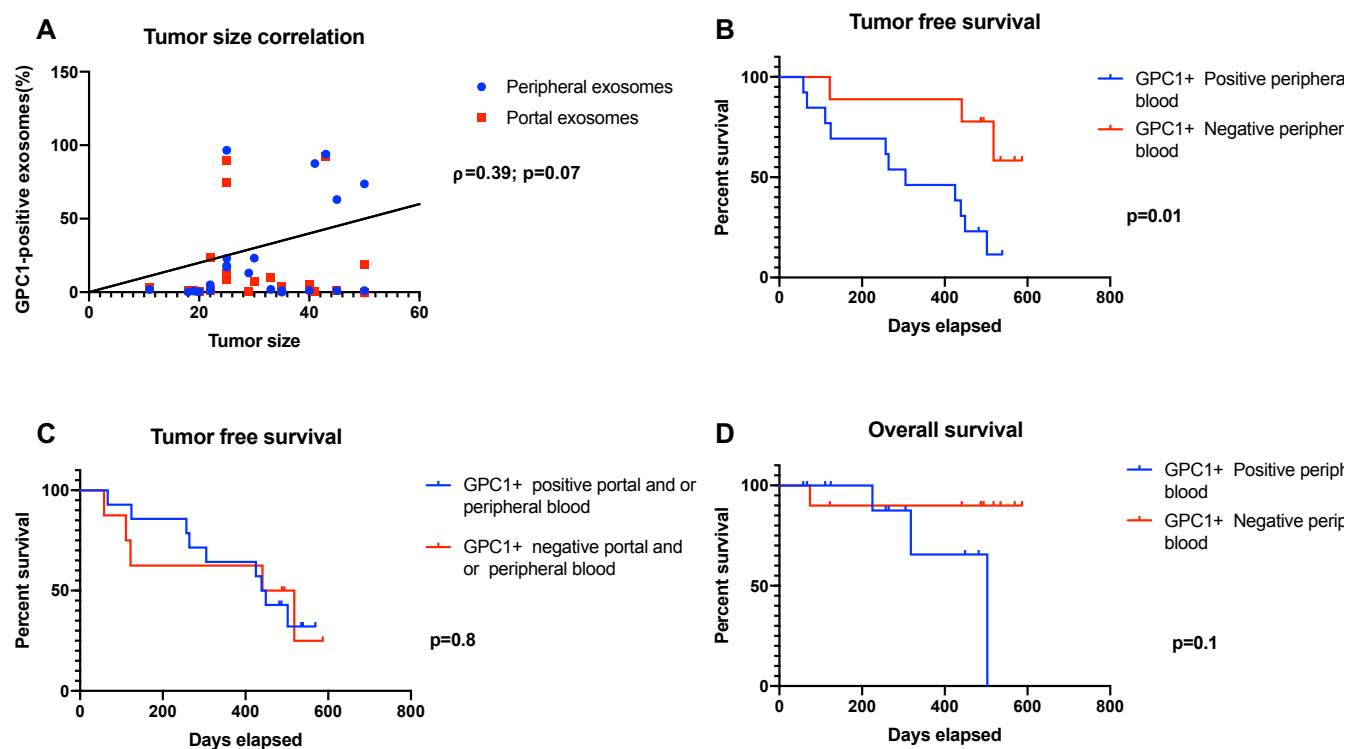


Figure 5





## **L'approche « combinée de biopsie liquide » per-opératoire dans l'adénocarcinome canalaire du pancréas résécable : un outil diagnostique et pronostique prometteur.**

### **Introduction :**

Un des problèmes du cancer du Pancréas (CP) est le temps de latence entre la suspicion du CP et la mise en place des traitements, notamment néo-adjuvants qui nécessitent une preuve histologique. Les méthodes de biopsie liquide pourraient accélérer la mise en évidence d'éléments tumoraux et le diagnostic.

### **Objectif :**

L'objectif principal de l'étude était de comparer la performance diagnostique de plusieurs techniques de biopsie liquide chez des patients atteints d'un CP résécable d'emblée sans traitement néo-adjuvant. L'objectif secondaire était la corrélation entre la quantification des paramètres de la biopsie liquide et le taux de récurrence post-opératoire.

### **Matériel et méthodes :**

Nous avons conçu un essai clinique prospectif (PANC-CTC# NCT03032913) visant à détecter les cellules tumorales circulantes (CTC), l'ADN tumoral circulant (ADNct) et les onco-exosomes chez les patients atteints de CP et chez les patients d'un groupe témoin sans antécédent de cancer, opérés d'une pathologie bénigne en appliquant différentes méthodes. Pour les CTCs, il s'agissait de l'enrichissement et détection de CTCs par la méthode CellSearch® (méthode de référence approuvée par la FDA), méthode d'enrichissement de CTCs RosetteSep® et OncoQuick® puis quantification de l'ADN tumoral par dd-PCR. Les exosomes ont été isolés puis caractérisés avec le taux d'expression de Glypican-1 (marqueur spécifique du CP *Melo et al. Nature 2015*). Le statut de mutation *KRAS* des tumeurs primaires, a également été recherché sur les pièces opératoires. Tous les patients de l'étude (groupe témoin et groupe CP) ont eu un prélèvement de sang périphérique, les patients du groupe CP ont eu un prélèvement de sang portal en peropératoire.

## Résultats :

De février à novembre 2017, 22 patients atteints de CP résécable et 28 patients témoins ont été inclus (dont 8 TIPMP non dégénérées et 2 pancréatites chroniques calcifiantes). Au total 22 patients (100%) ont été détectés positifs par au moins une méthode. Des CTC ont été détectés chez 2/22 patients (9%) avec la méthode CellSearch® dans le sang périphérique et dans 5/11 (45%) échantillons de sang du portail, suggérant que CellSearch® était plus susceptible d'identifier les CTC avant le premier passage hépatique. La détection des CTCs par RosetteSep™ a permis d'identifier 13/22 patients (59%) et les exosomes GPC1 étaient positifs pour 14/22 patients (64%) dans le sang périphérique et/ou portal, sans différences de taux de détection selon le site de prélèvement. . Il est important de noter que la combinaison des trois biopsies liquides présentait une sensibilité de 100 % et une spécificité de 80 %, avec une valeur prédictive négative de 100 %. Des taux élevés d'exosomes GPC1+ et/ou de présence de CTC étaient significativement corrélés avec la survie sans progression et aussi avec la survie globale lorsque des « clusters » de CTC étaient présent

**Résultats :** De février à novembre 2017, 22 patients atteints d'un cancer du pancréas résécable et 28 témoins ont été inclus. Des CTC ont été détectés chez 2/22 patients (9%) avec la méthode CellSearch® dans le sang périphérique et dans 5/11 (45%) échantillons de sang du portail, suggérant que CellSearch® était plus susceptible d'identifier les CTC avant leur filtration par le foie. La détection du CTC par RosetteSep® a permis d'identifier 13/22 patients (59%) et les exosomes positifs GPC1 étaient positifs pour 14/22 patients (64%) dans le sang périphérique et/ou portique, sans différences de taux de détection selon le site de prélèvement. Il est important de noter que la combinaison des trois biopsies liquides présentait une sensibilité de 100 % et une spécificité de 80 %, avec une valeur prédictive négative de 100 %. Des taux élevés d'exosomes GPC1+ et/ou de présence de CTC étaient significativement corrélés avec la survie sans progression et aussi avec la survie globale lorsque le groupe CTC a été trouvé.

**Conclusion :** Cette étude est la première à évaluer des biopsies liquides combinées à base de CTC et d'onco-exosomes. Cette étude pilote suggère que la biopsie liquide peut être un outil prometteur à fois diagnostique et pronostique dans le CP à un stade

précoce. De plus, la combinaison de la recherche de CTC et celle des onco-exosomes circulants permettrait de prédire la récurrence post-opératoire.



### **5-Combined liquid biopsies show high diagnostic value for pancreatic ductal adenocarcinoma patients eligible for up-front surgery.**

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## 5-1 ABSTRACT

### **Introduction:**

Efforts to expedite the diagnosis of Pancreatic Ductal Adenocarcinoma (PDAC) are likely to benefit care management. The available imaging and circulating biomarker tools often need to be completed by invasive biopsy, especially for starting treatments that require histological evidence. Liquid biopsy, which evidences the presence of the tumor by detecting circulating complex tumor elements might be valuable to help diagnose PDAC. The main objective of the study was to evaluate the combined diagnostic performance of several liquid biopsy techniques in patients with resectable pancreatic cancer.

### **Methods:**

We designed a prospective clinical trial (PANC-CTC# NCT03032913) to detect circulating tumour cells (CTC) and onco-exosomes in portal and peripheral blood of patients with PDAC and in peripheral blood of a non-cancer control group using different methods. CTCs were counted using the gold standard CellSearch® method. Alternatively, the RosetteSep® and OncoQuick® CTC enrichment methods followed by the quantification of KRAS mutant alleles by droplet digital PCR. Onco-exosomes were quantified by Glypican-1 (GPC1)-positive exosome level determination.

**Results:** From February to November 2017, 22 patients with resectable pancreatic cancer and 28 controls were included. CTCs were detected in 2/22 (9%) patients with the CellSearch® method in peripheral blood and in 5/11 (45%) portal blood samples, suggesting that CellSearch® was more likely to identify CTCs before they are filtered by the liver. RosetteSep®-based CTC detection identified 13/22 (59%) patients and the GPC1-positive exosomes was positive for 14/22 (64%) patients in peripheral and/or portal blood, without differences of detection rates according to the sampling site. Importantly, combining all three liquid biopsies displayed 100% of sensitivity and 80% of specificity, with a negative predictive value of 100%. High levels of GPC1<sup>+</sup>-exosomes and/or CTC presence were significantly correlated with progression free survival and also with overall survival when CTC cluster were found.

**Conclusion:** This study is the first to evaluate combined CTC- and onco-exosome-based liquid biopsies, proving to be very promising the diagnosis of early stage pancreatic cancers. This could provide a rapid decision-making tool to initiate neoadjuvant treatments and could be of strong interest for monitoring disease relapse.

## 5-2 INTRODUCTION

Whereas overall survival of pancreatic ductal adenocarcinoma (PDAC) is less than 10%, survival can reach around 20% when surgery is possible, giving the best chance to the patients[1]. The diagnosis of PDAC can be challenging, especially for patients eligible to up-front surgery. Imaging is the first diagnostic tool used to decide resectability in patients who are strongly suspected to have pancreatic cancers[2]. Patients with small lesions, hypertrophied pancreatic head, isoattenuating tumors and focal fatty infiltration of the parenchyma might necessitate further investigations. Echoendoscopy ultrasound guided–fine-needle aspirations (EUS-FNA) are strongly recommended[3], as they represent the sole tool able to diagnose the malignancy of the lesion. However, conventional biopsies show heterogeneous diagnostic performance because of the intrinsic nature of the tumors with low cellularity associated with high stromal content. Moreover, it is operator-dependent. These difficulties lead to non-informative analysis of the tumor and even to false-negative diagnosis, with a negative predictive value ranking between 33 and 85%. Overall, this test may be inconclusive or doubtful in up to 20% of cases [4]. The alternative circulating biomarkers, such as the protein serum markers, CEA and CA19.9 are used to monitor early recurrences in patients affected by PDAC, but their low sensitivity and specificity prevent any use as screening or diagnosis tools[5].

Primary tumors release in the blood and other bodily fluids tumor-derived elements, such as circulating tumor cells (CTCs), nucleic acids, or exosomes. When identified, these elements could be considered as a proof of the presence of the tumor for various cancers, including PDAC[6]. Liquid biopsy[7] might represent a non-invasive, safe and fast companion test to tissue biopsy. CTC detection has been carried out with diverse non-equivalent approaches that could be complementary in improving CTC detection rate. In particular, the most popular method is the CellSearch® system because it has been approved by the United States FDA to monitor prostate, breast and colorectal cancers [8–10]. Based on the presence of EpCAM overexpression by solid tumor cells, CellSearch® cannot identify CTCs which have completed the Epithelial to Mesenchymal transition (EMT). So, alternative methods have been developed, based on CTC-enrichment. The density gradient centrifugation with OncoQuick® resulted in

higher relative tumor cell enrichment than Ficoll density gradient centrifugation [11] and provided in good detection rate of EpCAM-negative breast cancer CTCs[12]. Another EpCAM unbiased approach is to negatively enrich blood samples with CTCs by using immune cocktails to withdraw the blood mononuclear cells [13,14]. CTC-enrichment methods must be followed by molecular identification such as the detection of mutant KRAS, present in >92% of PDACs[15].

The tumor-released exosomes raised high interest as circulating biomarkers, because they carry the physiopathological signature of the emitting cell, not only via molecules expressed in their membranes, but also via components they cargo[16]. In particular, PDAC onco-exosomes carry the membrane protein Glypican-1 (GPC1) that detected 100% of patients with PDAC and distinguished patients with precancerous pancreatic lesions from those with benign diseases [17]. Thus, they are potentially valuable because they can identify early stage patients.

In general, studies with high diagnostic value of liquid biopsy include a majority of patients with advanced disease. In this study, we aimed to assess whether combining methods for CTC detection and PDAC exosomes was efficient for PDAC diagnostic and carried prognostic value, in a homogeneous group of patients with early stage disease, all eligible for up-front surgery. In addition, portal blood was previously found to contain numerous CTCs as compared to peripheral blood in patients with advanced disease [18,19], and even in patients with resectable tumors[20,21]. To increase chances of circulating CTC and/or exosomes, we analyzed peripheral and portal blood samples.

## **5-3 MATERIALS AND METHODS**

### Study design

Patients eligible for pancreatic surgery with suspicion of pancreatic cancer or IPMN (intraductal papillary and mucinous neoplasm) with worrisome features as determined by CT-scan (computerized tomography scanner) and or MRI (magnetic resonance imaging) without metastasis were enrolled at the department of hepatobiliary surgery of Bordeaux university hospital between February and November 2017. All patient underwent standardized staging, including CT-scan, MRI (in case of doubt on liver

metastasis) and CA 19-9 and evaluation in a multidisciplinary board. Exclusion criteria were borderline or locally advanced disease with an indication of neo-adjuvant therapy[2], metastatic disease, or history of other malignancies. The control group was composed of patients who underwent surgical procedure in our department for non-neoplastic pathology and without a history of solid cancer or hematologic malignancy. This prospective study was conducted according to the Declaration of Helsinki, the French rules (Law for Bioethics November 2016) and the recommendations of CNIL (Comité National Informatique et Liberté), and was approved by an Institutional Review Board. The biological collection was declared to and approved by the French Ministry of Research under the number 2016-A00431-50 and the database was registered in Clinical Trials under the number: NCT03032913. Informed written consent and information was obtained from patients before surgery. Patients did not receive neoadjuvant therapy. A control group enrolled patients operated for benign pathology with no history of cancer. Patient follow-up was done until December 1, 2018. A scan was performed 3 months after surgery and then 6 months after with CA19-9 dosage each time. Postoperative data were also collected.

#### Surgical procedure, blood sampling and tumor staging:

After laparotomy, we inspected and palpated liver and peritoneal cavity to identify metastasis. Biopsy was performed for suspicious lesions and resection was abandoned if intra operative specimens were positive for metastatic adenocarcinoma. Pancreaticoduodenectomy (Whipple procedure) was started with isolation and division of the common bile duct, and then the portal vein was exposed. For left pancreatectomy, a retro-isthmus portal puncture of the portal vein was performed. Two samples of 7.5 ml of blood were collected from the portal vein in BD vacutainer collection tubes without additives (SST tubes, Becton Dickinson, Le Pont de Claix, France), before manipulation of the tumor. For the patient group two samples of 7.5ml were collected during surgery in the median cephalic vein in BD vacutainer collection tubes without additives. A sample of 7.5ml in BD vacutainer collection tube containing EDTA for complete blood count to determine neutrophil over leukocyte ratio in the patient group (DXH automated counter, Beckman Coulter, Villepinte, France) was also obtained. Median cephalic vein was punctured in the control group, to collect vacutainer collection tubes without additives. Tubes were transferred quickly in the laboratory and were centrifuged at 2000g for 15 minutes to collect sera. Sera from

patients were immediately used to determine CA19-9 concentration (Architect automated instrument, Abbott). Sera were frozen at -80°C until they were further processed. In addition one tube (Cell free DNA collection tube®, Roche, Meylan, France) was collected in a peripheral vein for all patients and controls, and in the portal vein for IPMN and PDAC groups for ctDNA analysis. Tumor staging was performed according to the TNM AJCC2017, 8<sup>th</sup> version and histological analyses were performed by a single specialized pathologist [22]. Progression free survival (PFS) and Overall survival(OS) was defined by the time from surgery to progression based on CT staging.

### CTC identification

First, with RosetteSep™ (Stemcell technologies, Grenoble, France) or OncoQuick® (Greiner Bio One SAS, Les Ullis, France) capacity to recover tumor cells from total blood samples was tested by the CAPAN-2 cell line spiking experiments. Total blood from healthy volunteers was obtained from the Etablissement Français du sang (EFS, Pr. Jeanne, convention 16PLER023). CAPAN-2 cells were first transduced with the pSIN-EF1aL-eGFP-IRES-Puro lentivector (Vect'UB, Bordeaux, France), and the subpopulation of green fluorescent cells was sorted on a FACSARIA II (BD Biosciences, Le Pont de Claix, France). A known number of fluorescent cells (15-42 for RosetteSep™ and 11-44 for OncoQuick®) were spiked. CTC enrichment was carried out according to the manufacturers' protocols. Cell pellets were recovered in a minimal volume of cell medium 60-well plates (Thermofisher, Courtaboeuf, France) in order to recover all the cells in a single well. Fluorescent cells were counted under an inverted Nikon Microscope (Eclipse Ti Nikon, Champigny sur Marne, France). Pictures were processed with the NIS-Elements Nikon software, connected with a video camera. Patient and control total blood samples were processed in the same way. Cell pellets were further analyzed for the presence of KRAS mutations by droplet digital PCR (ddPCR, with the KRAS G12/G13 Screening Kit (Biorad, Marne la Coquette, France) after total DNA extraction with the RSC ccfDNA plasma kit, Maxwell (Promega, Charbonnières-les-Bains, France).

The CellSearch® semi-automated platform with the Circulating Epithelial Cell Kit and the CELLTRACKS ANALYZER II System was used for CTC detection (Menarini Silicon Biosystems Inc., Castel Maggiore, Italy). Blood samples were drawn 7.5 mL blood from a peripheral vein and portal vein into 1 CellSave tube and kept at ambient temperature

until processing within 36 hours at Laboratory of Rare Human Circulating Cells, University Medical Centre of Montpellier, EA2415, Montpellier, France. After automated EpCAM-based immunomagnetic sorting, all objects presented on the CellSearch screen were analyzed by a certified technician. All cells meeting the CellSearch analysis standard for CTC, that is, all DAPI positive, CK positive, EpCAM positive and CD45 negative with a cellular shape and visible nucleus, were analysed and the final diagnostic approval was done by a single experienced Biologist. The presence of 1 CTC/7.5 mL was considered positive as previously described [18,20,23].

#### CRISPR/Cas9 –driven cut of KRAS WT allele

Ribonucleoprotein complexes (RNPs) containing 104pmol of Cas9 and 120pmol of the WT KRAS specific guide RNA (5'GGAACTTGTGGTAGTTGGAGC GUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUU GAAAAAGUGGCACCGAGUCGGUGCUUUUU 3', [24]) in 5µL, were prepared at the final concentration of 1µg/µL of Cas9. DNAs extracted from RosetteSep™-enriched circulating cell pellets (see above) were treated with 1µg of RNPs for 18h at 37°C. The cut DNA (2µL) was then amplified by conventional PCR (GoTaq®, Promega, Charbonnières-les-Bains, France, 45 cycles at 50°C, forward primer 5'-GGTGAGTTTGTA TTA AAA GGT ACT GG-3' and reverse primer 5'-TCCTGCACCAGTAATATGCA-3'), followed by ddPCR of 50ng of PCR product, with the KRAS G12/G13 Screening Kit, according to the manufacturer's instruction

#### Exosome analysis

Sera were enriched in extracellular vesicles (EVs) using the Total Exosome Isolation kit (Thermofisher), according to the manufacturer's instructions. EV-enriched pellets were resuspended in Phosphate Buffered Saline (PBS), and stored at -20°C. The Exosome-Human CD63 Isolation/Detection Reagent (Thermofisher) was used to pull down sera exosomes, which were stained with anti-GPC1 primary antibody (PIPA528055, Thermofisher) revealed with Alexa Fluor 647 donkey anti rabbit IgG (Biolegend) on a BD FACS CANTO II apparatus (BD Biosciences, Le Pont de Claix, France). Percentages of GPC1-positive beads were determined with BD FACS Diva software (BD Biosciences).

#### Statistics



Characteristics of the two groups were compared using Fisher exact tests or Wilcoxon-Mann-Whitney tests according to the type of data (qualitative or quantitative, respectively). The OS and PFS were first determined by the Kaplan-Meier method. A log-rank test was then used to assess the associations between various covariates and OS. All statistical analyses were performed using the GraphPad-Instat and GraphPad-Prism 8.0 software programs (GraphPad Software Inc. San Diego, CA, USA). A  $p$ -value  $<0.05$  was considered significant.

## 5-4 RESULTS

### Cohort characteristics

Seventy two patients underwent surgery for PDAC from February to November 2017. Upfront surgery was performed for 32 patients for presumed PDAC without neo-adjuvant therapy (Figure 1). Among them, two metastatic patients were excluded. Eight patients were excluded from the cancer group and switched to the control group after definitive pathology analysis, because of non-invasive intraductal papillary mucinous neoplasm (IPMN) diagnostic. Thus, control group included 20 control patients operated in our surgical unit without neoplasia and without a history of cancer (including two chronic pancreatitis who underwent surgery for symptomatic reasons, eleven cholecystectomy, three bariatric procedure, two hernia surgery, and two functional pelvic floor surgery) and 8 IPMN. Demographic and clinico-pathological characteristics were similar between groups and are shown in Table 1. Patients underwent 20 Whipple procedures and two left pancreatectomies. IPMN control group consisted seven Whipple procedures and one left pancreatectomy. Mean tumor size was 31 mm. All tumors were early stages: 22.5% were stage I, 50% stage IIb and 27.5% stage were III. 77.5% of patients had positive lymph nodes (Table 2).

### Cell spiking experiments

CTC counting with CellSearch© is limited to the identification of EpCAM+ pancreatic tumor cells. To increase our chances of detecting EpCAM negative cells, we tested two CTC enrichment methods followed by KRAS mutant DNA detection by ddPCR. First, the pancreatic tumor cell line CAPAN-2 was transduced with a lentivector carrying a recombinant GFP gene. Fluorescent CAPAN-2 cells were spiked into 7.5mL

of healthy donor total blood samples. Percentages of spiked cell recovery were determined by fluorescent cell counting under a microscope, after spiked blood samples were processed with RosetteSep<sup>TM</sup> or OncoQuick® to obtain blood cell pellets enriched with CTCs (Figure 2A). All experiments allowed for the isolation of at least one tumor cell, but percentages of recovery were higher in OncoQuick® as compared to RosetteSep<sup>TM</sup> (Figure 2B, 67.5% ± 3.5%, n=59 and 50.7% ± 3.5%, n=65, respectively, p<0.001). However, cell enrichment was 10 times lower, as determined by total cell count after recovery (not shown), leading to high levels of contamination mainly with PBMCs (Figure 2A). Molecular detection of mutant *K-RAS* alleles by ddPCR after RosetteSep<sup>TM</sup> enrichment was 3- to 4-fold more sensitive than after OncoQuick® (Figure 2C).

Thus, OncoQuick® was superior to RosetteSep<sup>TM</sup> in recovering tumor cells, but RosetteSep<sup>TM</sup> was more sensitive in detecting tumor DNA. Considering that both methods might be complementary, we used them both on patient and control sera. Of note, all primary tumors displayed mutant *KRAS* alleles by ddPCR (not shown).

#### Diagnostic values of single liquid biopsies performed in peripheral and portal blood

Each patient was subjected to CTC detection by 3 independent methods. Interestingly, CellSearch® identified 5 out of 11 patients (46%) for whom we had portal blood samples and only 2/22 (9%) when peripheral blood was considered. Positive patients were distinct between portal and peripheral blood, suggesting the complementary performance of portal and peripheral blood sampling. Thus, CTC detection with CellSearch® showed an expected low sensitivity of 32% and a very strong specificity of 100% (the IPMN group was considered the control group, as the CellSearch® technique was not performed on the non-cancer group) (Table 3, figure 1 and figure 3). Of note, patients (41 and 50, 2/11) displayed cell clusters in portal blood.

Direct *KRAS* mutant detection by ddPCR after Oncoquick® or RosetteSep<sup>TM</sup> CTC enrichment displayed low PDAC identification rate (2/22, 9%) with RosetteSep<sup>TM</sup> enrichment in peripheral blood, only. OncoQuick® was negative for all patients regardless of the sampling site. All controls were negative for both methods. However, when analyzing the raw ddPCR data, we found that 17/22 PDAC patients had MAFs borderline to the detection threshold in at least one sample (portal or peripheral) after RosetteSep<sup>TM</sup> but not OncoQuick® enrichment (Figure 4). Thus, we hypothesized that

increasing the sensitivity of the PCR might better identify the patients, especially because we knew that PBMCs, bearing the wild type (WT) KRAS allele, contaminated the CTC-enriched pellets. For that, depleting the WT allele with CRISPR/CAS9-directed specific double-stranded cut was a good option to increase chances to detect mutant alleles [24], see materials and methods section for detailed protocol). All DNAs extracted after RosetteSep<sup>TM</sup> enrichment (PDAC patients and controls) were analyzed again after CAS9-cut PCR. Out of the 17 samples 11 became frankly positive (Figure 4 and Figure 5). Two previously negative PDAC samples were positive after CAS9 treatment. Thus a total of 13/22 patients (59%, 11 in peripheral blood and 10 in portal blood, Figure heatmap, venn) were identified using CAS9-cut PCR/KRAS ddPCR. Four out of 8 IPMN samples were found positive after CAS9-cut PCR treatment (3 in peripheral blood and 1 in portal blood, 50%), while 2 non-cancer controls (10%) became positive (Figure 5). Finally, gaining in sensitivity, specificity was affected by the positivity of 4 IPMNs (50%). In conclusion, RosetteSep<sup>TM</sup>-based CTC detection (in portal and/or peripheral blood) was better than CA 19-9, with higher diagnostic accuracy (75% versus 68%), a better sensitivity of 59% as compared to 37%, and same specificities 75% (Table 3). EUS-FNA carried strong specificity, but poor negative predictive value, as expected.

Similarly to CTC detection, a total of 64% (14 out of 22) of patients had GPC1-positive exosomes in portal and/or peripheral blood (Figure 6). Both sampling sites showed similar performances (Table 3), and GPC1-positive exosomes in peripheral and/or portal blood displayed a diagnostic accuracy of 78%, similar to RosetteSep<sup>TM</sup>-based CTC detection (75%), and higher than CA 19-9 (68%) or EUS-FNA (66%).

#### Diagnostic value of combined liquid biopsies

Overall, single liquid biopsy showed higher diagnostic performance than the routinely available tools (CA 19-9 and EUS-FNA). Combining results from individual tools, in both sampling sites, proved to increase the number of detected patients, better than combining the traditional tools CA 19-9 and EUS-FNA (Table 3: combined sensitivity). RosetteSep<sup>TM</sup>-based CTC detection and quantification of GPC1-positive exosomes displayed a very high sensitivity of 96%, with a high negative predictive value (96%). Addition of CA 19-9 and/or EUS-FNA did not improve the performances (Table 3). Noticeably, combining all three liquid biopsies (quantification of GPC1-positive

exosomes, RosetteSep™ and CellSearch®-based CTC detection) identified all the PDAC patients, showed a negative predictive value of 100%, and an overall diagnostic accuracy of 91% (Table 3). As 4 out of 20 non-cancer controls were positive with either RosetteSep™-based CTC detection or quantification of GPC1-positive exosomes, the specificity was 80% and the positive predictive value was 85% (Figure 3C). Importantly, all the patients who were not diagnosed by EUS-FNAC were identified by one or more liquid biopsy (Figure 3A).

#### Combined liquid biopsies and prognostic performances

The progression free survival (PFS) and median overall survival (OS) were 365 days (range 58-587) and 503 days (range 74-718), respectively. Individually, positive liquid biopsies were not prognostic. Interestingly however, patients with >20% GPC1-positive exosomes in peripheral blood, which is 4 times the median value, and/or CellSearch® positive clusters in portal blood had shortened PFS and OS (Figure 7). The tumor burden, in particular, tumor stage, node status or tumor size, did not correlate with any individual liquid biopsy (Figure 3). Furthermore, there was no correlation between the tumor stage or the PFS or OS and the number of positive liquid biopsies.

### **5-5 DISCUSSION**

Our experimental design aimed to test the diagnostic performance of several liquid biopsies to identify PDAC in a group of patients eligible for up-front surgery.

Taken individually, our CTC detection rates varied from 10% to 59% and were in accordance with published results. In particular, CellSearch® previously found detection rates ranging from 11 to 48% in cohorts comprising at least 53% of patients with locally advanced or metastatic diseases[23,25]. Including patients with advanced diseases only did not increase rates of PDAC identification [26]. Fewer studies analyzed patients with early stages disease, such as ours, and also found very low rates of PDAC identification. For example, 6.8% (2/37) with resectable tumors were detected in peripheral blood [27]. It was previously reported that CTCs are more numerous in portal blood, before they are filtered by the liver. One hundred percent of patients with advanced or metastatic diseases had detectable CTCs in portal blood using either CellSearch® or the similar ClearBridge® systems[18,19]. When resectable patients were considered, numbers dropped to 49% and 58.5% [20,21].

This is in agreement with the identification of 5/11 (45%) patients by CellSearch® in the portal blood in the present study. Thus, CellSearch® has low capacity to detect PDAC patients in peripheral blood but is valuable when portal blood samples are considered, even in resectable patients considered to present early stage disease [28].

Next, we tested two alternative CTC detection methods, based on the molecular identification of *KRAS* mutant allele in CTC-enriched peripheral or portal blood samples. After RosetteSep™-driven mononuclear blood cell depletion we could identify only 2 samples with CTCs in peripheral blood. However, after depleting samples with WT *KRAS* allele, about 50% of the samples displayed detectable mutant *KRAS*, regardless of the sample type. OncoQuick® enrichment failed to detect any patients in both sampling sites, probably because of high concentrations of WT DNA from mononuclear cells.

The last liquid-biopsy-based PDAC identification tool we used was the quantification of GPC1-positive onco-exosomes. The publication from Kalluri's group reported 100% sensitivity and specificity, which is far from what we found here (about 50% and 90%, respectively, regardless of the sampling site). Moreover, Melo et al's excellent results were not replicated by others who found detection rates close to ours. In particular, Yang et al. measured exosomal GPC1 levels by antibody-linked fluorescence intensity of ultracentrifuged plasma EVs, and found a sensitivity of 67% and a specificity of 82%. They preferred a 5-protein signature to increase their diagnostic accuracy [29]. Similarly, Lai et al abandoned GPC1-based onco-exosome quantification and reached 100% sensitivity and specificity with a combined approach of several microRNA signatures with GPC1 detection [30].

Thus, various performances were reached with individual methods and we tested whether combination of several methods could improve PDAC detection. Indeed, it is likely that early stage, resectable tumors release less circulating elements, such as CTCs and exosomes. Combining all three liquid biopsies (quantification of GPC1-positive exosomes, RosetteSep™ and CellSearch®-based CTC detection) identified 100% of the PDAC patients. The specificity was 80% because of a few false-positive controls (discussed below). Importantly, the negative predictive value was 100%, as compared to the poor negative predictive value of EUS-FNA (varying from 36 to 80% depending on the studies, and confirmed here, 33%[4]). The high performance of

combined liquid biopsy is interesting in light of current clinical practice evolution. If patients present locally advanced disease, it is crucial to establish as soon as possible resectability to avoid unneeded and even deleterious surgery for patients with undiagnosed metastatic stages [28]. Moreover, it is essential to increase the rate of complete (R0) resection with rapid and effective neoadjuvant therapy. Furthermore, neoadjuvant treatments, requiring histological and/or diagnostic evidence of tumor malignancy, are now being considered, even when patients are directly resectable [31].

Diagnostic performance, especially specificity, has been reduced by false-positives in the control non-cancer group. In particular, 4 out of 8 IPMN samples were found positive with RosetteSep<sup>TM</sup>-based CTC enrichment and CAS9-cut ddPCR (50%) and 2 out of 20 non-cancer controls (10%). This was not linked with age or smoking status of the patients. Previous studies using the ddPCR for identification of *KRAS* mutant alleles reported false-positive rates in exoDNA varying from 7.4% (4/54) to 20.7% and 25% (17/82 and 3/12)[32,33]. This might be partly explained by the fact that spontaneous somatic mutations are believed to occur in the normal population[34] and the high sensitivity of the PCR-based methods. The IPMN group showed interesting results, especially with RosetteSep<sup>TM</sup>-based CTC enrichment, because the positive patients were those with high grade dysplasia. Similarly, a previous report showed that CTC counts (by Isolation by Size of Epithelial Tumor Cells) were higher in patients with high-grade dysplasia IPMN and were qualified as circulating epithelial cells (CECs)[35]. It would be very interesting to search for *KRAS* mutations in such CECs. The presence of false-positives is a limit of our study but it does not invalidate the value of our combined approach. Indeed, the aim was to identify PDAC patients entering the care process with PDAC suspicion and not to screen the general population. In the same way, due to the low lifetime risk of pancreatic cancer (around 1%), population-based screening of unselected individuals is not recommended for this tumor[36]. Two non-cancer controls and 1 IPMN were false-positive for GPC1<sup>+</sup>-exosome quantification. Very interestingly, all were carriers of autoimmune pathologies (lupus and rheumatoid arthritis). It is possible that high levels of auto-antibodies interfere with the antibody-based test. Further investigations need to be carried out to test this hypothesis.

Besides us, only a few authors investigated the value of combining several liquid biopsy techniques applied to PDAC. A combination of CTC detection (filtration-based

method) and cell free circulating tumor DNA (ctDNA) quantification, in association with CA 19-9, identified 78% of PDACs, with a low negative predictive value (53%)[37]. Another recent report evaluated combined detection of ctDNA and exosomal DNA (exoDNA, *KRAS* mutant allele) on a prospective cohort of 168 patients with a majority of metastatic patients (60%). This approach identified 37.3% of metastatic PDACs and only 9.1% of patients with localized tumors [33], suggesting that ctDNA approach is not suitable for resectable PDAC diagnosis. Instead, quantification of exoRNA (*KRAS* mutant) might be of high interest as it was recently reported for detecting mutant *EGFR* in lung cancer [38].

The detection rate of CTCs after RosetteSep<sup>TM</sup>-enrichment step yielded similar efficiency than that observed by CellSearch® in portal blood [20,21]. The same observation was made for onco-exosome quantification, which was similar in both sample types (50% and 46%). So, unlike CellSearch®, these two detection methods were not impacted by the sampling site, suggesting that molecular-based detection methods suffer less from tumor-element dilution after liver filtration.

One of the strengths of our study is the homogeneity of the cohort reflecting current clinical practice, especially the trickiest diagnostic situations. Few studies have produced homogeneous cohorts of patients with blood samples collected before any surgical and/or neo-adjuvant therapy, but all the recruited patients presenting advanced or metastatic diseases [23,30,39]. Our strength is also a limit since the size of the cohort is small. However, we consider it as a pilot study, worthy of further validation in bigger cohorts. Additionally, it would be of interest to test the combined methods for the diagnosis of all stage diseases and longitudinal monitoring of on-treatment.

The presence of CTCs in the peripheral blood has been associated with a reduced PFS and OS in PDAC [6]. Taken individually, none of the liquid biopsy method was associated with clinical outcomes, such as tumor stage, tumor size and survival. Interestingly however, the combination of CTC detection by CellSearch and GPC1<sup>+</sup>-exosome quantification in peripheral blood was correlated to disease recurrence and the high levels of onco-exosomes combined with the presence of clusters was associated to OS. The presence of clusters was already of worse prognostic in other solid cancers [40,41].

Echo-endoscopy with puncture is now the gold standard for histologic proof and formal diagnostic but has certain limitations. It carries variable negative predictive value, largely operator-dependent for both the endoscopic ultrasound guided-fine needle aspiration biopsy and the pathological analysis. It is invasive and of high-risk morbidity, with possible induction of acute iatrogenic pancreatitis, sometimes compromising surgical management. With resectable disease, combined liquid biopsies could contribute to decision making, in particular, for triggering neo-adjuvant and adjuvant treatment.

In conclusion, our results suggest that combining liquid biopsies from peripheral and portal blood might represent a highly valuable diagnostic tool for patients with resectable PDAC. Concomitant detection of several circulating tumor elements, i.e. CTCs and exosomes carried high diagnostic value and identified patients at risk of early disease relapse or fatal outcome. This approach might greatly accelerate the diagnostic, which might in turn improve clinical outcomes and care experience. Adopting triple liquid biopsy combination, with a negative predictive value of 100% might help decision making.

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## FIGURE LEGENDS

### Figure 1: Study design, blood samples and liquid biopsy methods

A) PDAC Patients and patients with IPMN had both peripheral and portal samples for CTC-enrichment detection/count and quantification of GPC1 positive exosomes (blue rectangle and arrows). B) Control group had peripheral samples for CTCs enrichment detection (RosetteSep<sup>TM</sup>, Oncoquick®) and quantification of GPC1 positive exosomes (green rectangle and arrows). EVs: extracellular vesicles; CTC: circulating tumor cell; IPMN: intraductal papillary and mucinous neoplasm; GPC1: Glypican 1.

### Figure 2: *KRAS* detection after cell-spiking experimentation

A-60-well plate pictures after fluorescent cell spiking and recovery images obtained after OncoQuick® enrichment (a bright field, b fluorescence) and RosetteSep<sup>TM</sup> enrichment (c bright field, d fluorescence) all images with original magnification X100. B Recovery efficiency for two methods. \*\*\*:  $p < 0.001$ : Cell count and recovery after cell spiking experiment. Statistically greater count in Oncoquick® compared with RosetteSep<sup>TM</sup> paired analysis performed by Wilcoxon test ( $p < 0.001$ ). C Mutant allele frequency determined by ddPCR after enrichment according to spiked cells: Molecular detection of mutant *K-RAS* alleles by ddPCR after RosetteSep<sup>TM</sup> enrichment was 3- to 4-fold more sensitive than after OncoQuick®.

### Figure 3: Heat map analyses of liquid biopsy elements

Heat maps gathering liquid biopsy results for PDAC patients (A) IPMN patients (B) and non-cancer control individuals (C). White cell: negative result, blue cell: positive result, crossed cell: not done. In PDAC heat map, the bottom ladder indicates adenocarcinoma stages ranking from 1 to 3 according the stage of the disease. In IPMN heat map, the bottom ladder indicates dysplasia ranking from 0 for low grade dysplasia to 1 for high grade dysplasia.

### Figure 4 Representative ddPCR results for *KRAS* detection.

Individual droplet PCR fluorescence results are plotted as two-dimensional dot plots (left). Grey dots correspond to empty droplets. Green dots correspond to droplets containing wild-type copies of *KRAS*. Blue dots correspond to droplets containing one mutant *KRAS* allele. Orange dots correspond to droplets containing WT and mutant

alleles the red lines indicate the positivity threshold. Patient 36 (A) became positive and patient 39 (B) was negative before and after Cas9.

**Figure 5 MAF of *KRAS* mutation by ddPCR after RosetteSep™ CTC enrichment.**

Greater median MAF in CTC-enriched samples after CRISPR/Cas9 cut of the wild type *KRAS* allele as compared to uncut DNA in peripheral(A) and portal blood(B). Higher median MAFs in patients compared with the control group tended toward significance ( $p=0.06$  by Mann–Whitney test). MAF: mutant allele frequency

**Figure 6 Venn diagrams recapitulating rates of CTC detection by CellSearch® or RosetteSep™-based enrichment and GPC1 positive exosome quantification**

(A) peripheral blood samples (B) portal blood samples (C) combined peripheral and portal blood samples

**Figure 7 Analysis of GPC1-positive exosome quantification and CellSearch® positive CTC count and clusters according to clinical criteria**

Kaplan-Meier curves, with p values (log Rank) for comparison between A) PFS for patients with GPC1- positive exosomes and/or CellSearch® positive and GPC1-negative exosomes and/or CellSearch® negative in peripheral blood B) OS for patients with >20% GPC1-positive exosomes (4 times the median value) and/or with CTC clusters and patient with < 20% GPC1-positive exosomes and/or CellSearch® without CTC clusters.

## TABLES

Table 1: Clinical features of the cohort

Variables	PDAC group 22 N (%)	Control Group 28 N (%)
Age, yr, (median; range)	68.8 (69.5; 57-81) *	58.3 (61;22-73) *
Male gender, n (%)	13 (59)	8 (28)
BMI	24	26.5
ASA score		
1-2	16 (72)	19 (67)
≥3	6 (28)	9 (33)
Smoking status	5 (22)	12 (42)
First symptoms		IPMN (n=8)
Acute pancreatitis	0 (0)	2
Jaundice	13 (59)	0
New onset of diabetes	0 (0)	1
Weight loss	5 (22)	0
Diarrhea and/or steatorrhea	0 (0)	0
Abdominal pain	3 (13)	3
No symptoms	4 (18)	3
Serum CA 19-9 n =30(n=22 PDAC group, n=8 IPMN;)		
Normal	16 (72)	23 (77)
Elevated	6 (28)	7 (23)
EUS-FNA	Total n=15	
n=18	Positive n=7	Positive n=0
(metastatic n=2; IPMN n=3)	Negative n=6	Negative n=3

\* Statistically significant ; IPMN are included in the control group

med, median; PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary and mucinous neoplasm; EUS-FNA endoscopic ultrasound-guided fine needle aspiration;

Table 2 Details for pancreatic surgery and pathologic features (N=30)

Variables	PDAC group (N=22) N (%)	IPMN (N=8) N (%)
Procedures		
Whipple	20 (91)	7 (87)
Left pancreatectomy	2 (9)	1 (13)
Vascular reconstruction	4(18)	0(0)
Post-operative complications		
Dindo-Clavien III-IV	3 (13)	2 (25)
Dindo-Clavien V	0 (0)	0 (0)
Pathology: Macroscopic		
Tumor size(mm) mean (med; range)	31 (30;11-49)	
Tumor stage	Stage 1a 1 (4.5) Stage 1b 4 (18) Stage 2b 11 (50) Stage 3 6 (27.5)	In situ carcinoma n=0 High grade dysplasia n=6 Low grade dysplasia n=2
Nodes status	Positive n=17 (77.5) Negative n=5 (22.5)	

Glandular Differentiation	Well 3 (13.5) Moderately 11 (50) Poorly 8 (36.5)	
<i>KRAS</i> status all primary tumors were positive for <i>KRAS</i> : % mutation mean (med; range)	26.15 (17.45;0.35-77.6)	

med, median; IPMN, intraductal papillary and mucinous neoplasm

Table 3: Diagnosis values of GPC1 positive exosomes, CTCs detection by CellSearch®, CTCs quantification by RosetteSep™, CA 19-9 and EUS-FNA

Test	Sensitivity (95% CI)	Specificity (95%CI)	Positive predictive value (95%CI)	Negative predictive Value (95%CI)	Diagnosis accuracy (95%CI)
Conventional tools					
CA19-9	37 (19-59)	87 (72-95)	63 (36-85)	69 (54-82)	68 (61-74)
EUS FNA (n=18; PDAC n=15; IPMN n=3)	60 (36-81)	100 (31-99)	100 (60-99)	33 (13-65)	66 (59-73)
Single liquid biopsy					
CellSearch® peripheral and/or portal vein (n=30)	32 (15-49)	100	100	35 (18-52)	50 (32-68)
Rosette sep portal vein (n=30)	46 (28-64)	75 (59-90)	84 (71-97)	34 (17-51)	54 (36-72)
Rosette sep peripheral vein (n=42)	50 (35-65)	90 (81-99)	85 (74-96)	63 (48-78)	70 (56-84)
Rosette sep peripheral and/or portal vein (n=52)	59 (46-72)	87 (78-96)	77 (66-88)	75 (63-87)	75 (63-87)
EVs GPC1 portal vein	46 (27-66)	88 (53-99)	91 (63-99)	36 (20-59)	57 (50-64)
EVs GPC1 peripheral vein	50 (31-70)	90 (77-99)	79 (58-98)	70 (54-82)	72 (65-78)
EVs GPC1 peripheral and/or portal vein	64 (43-81)	90 (73-97)	83 (59-94)	76 (59-88)	78 (72-83)
Combined diagnosis methods					
CA19-9 and EUS-FNA	50 (31-70)	92 (78-99)	86 (58-98)	70 (55-83)	74 (67-80)

*Rosette sep+ EVs GPC1	96 (90-100)	70 (55-83)	70 (55-83)	96 (90-100)	81 (70-93)
*Rosette sep+ CA19-9+ *EVs GPC1	96 (90-100)	68 (54-83)	68 (54-83)	96 (90-100)	79 (67-92)
*Rosette sep+ *EVs GPC1+ EUS FNA	96 (90-100)	70 (55-83)	70 (55-83)	96 (90-100)	81 (70-93)
*Rosette sep +CA19-9+ *EVs GPC1+ EUS FNA	96 (90-100)	68 (54-83)	68 (54-83)	96 (90-100)	79 (67-92)
*CellSearch®+ *RosetteSep+ *EVs GPC1	100	80 (68-93)	85 (75-96)	100	91 (83-99)

PDAC, pancreatic ductal adenocarcinoma; IPMN, intraductal papillary and mucinous neoplasm; EUS-FNA endoscopic ultrasound-guided fine needle aspiration. \*EVs GPC1; \*RosetteSep; \*EVs GPC1: quantification in peripheral and portal vein.

## FIGURES

Figure 1 Study design and patient selection

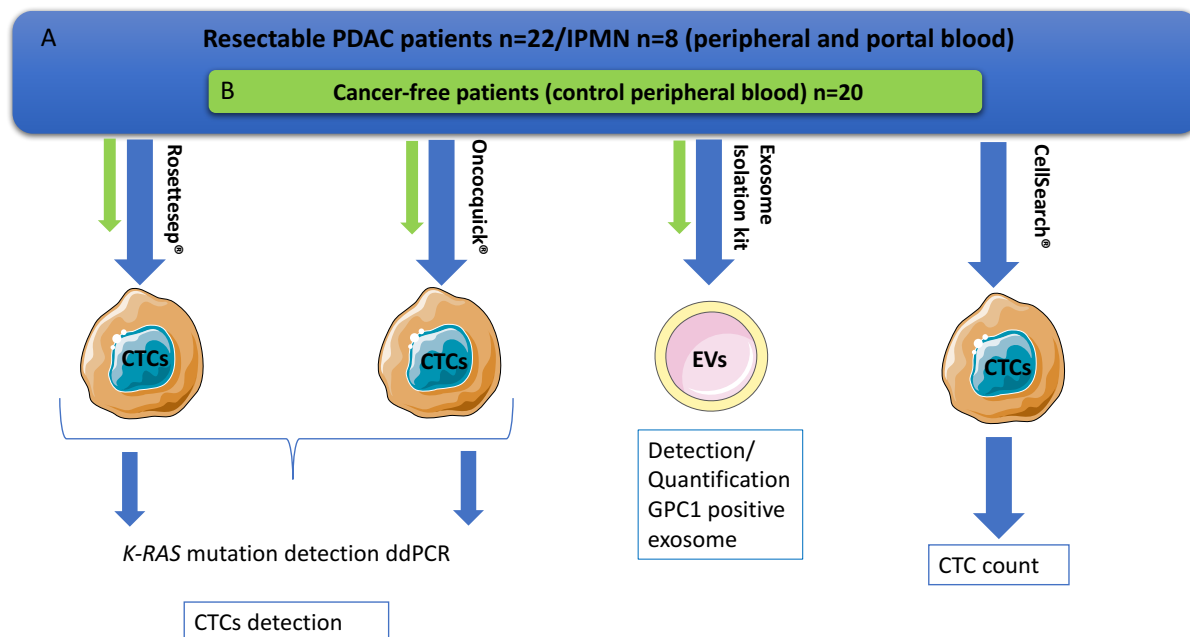
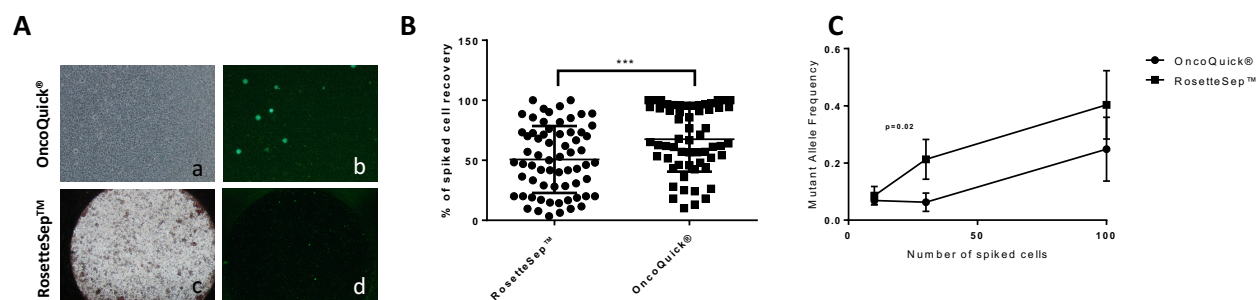


Figure 2





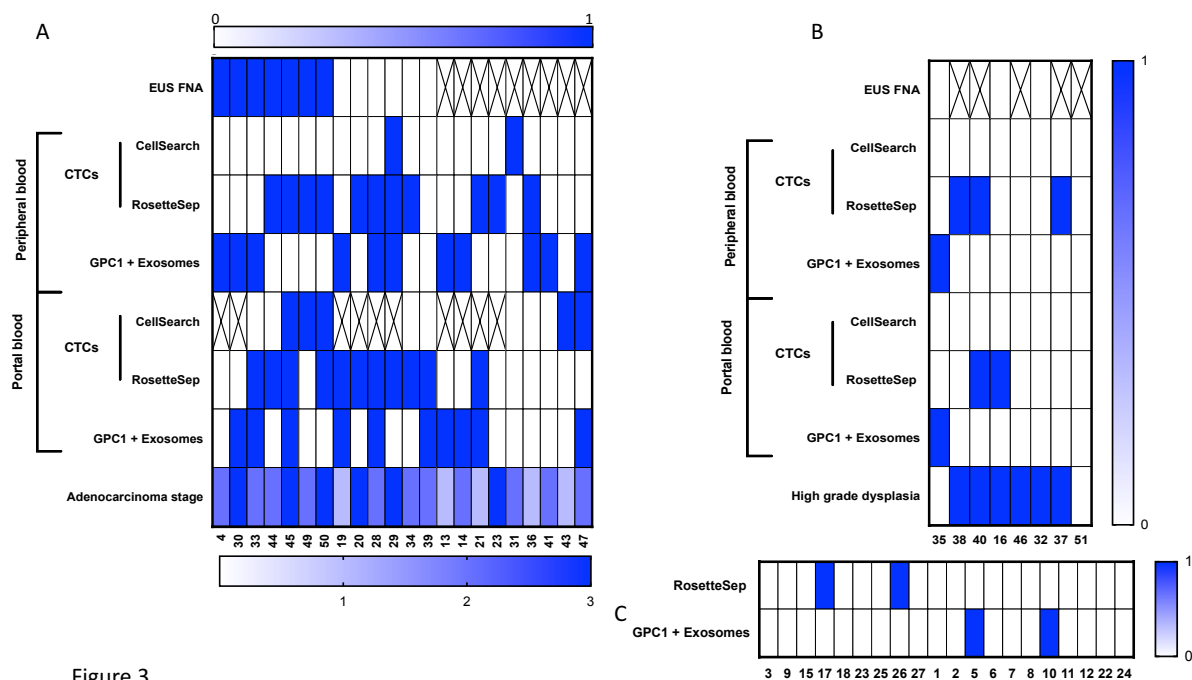


Figure 3

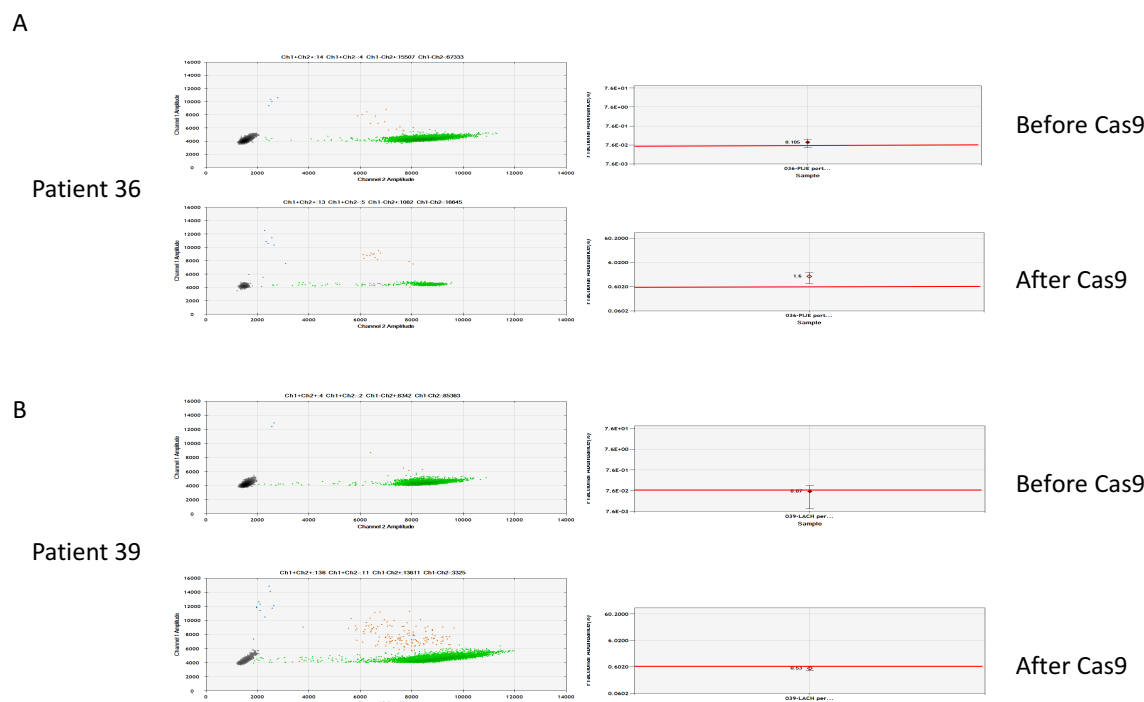


Figure 4

Figure 5

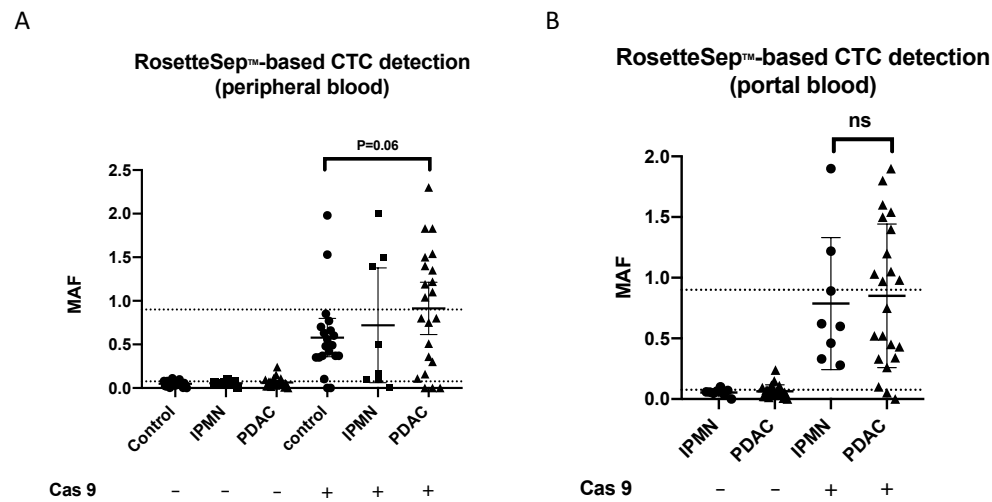


Figure 6

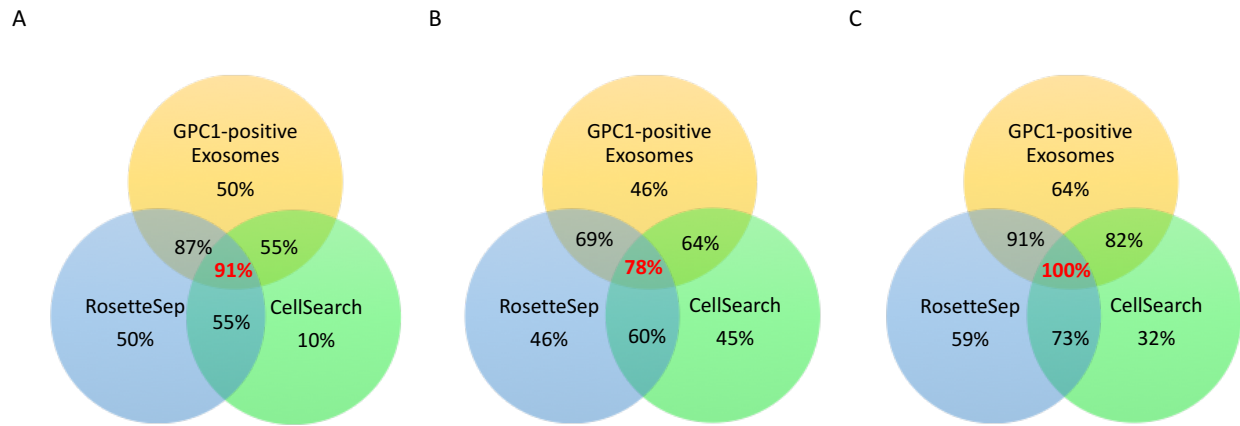
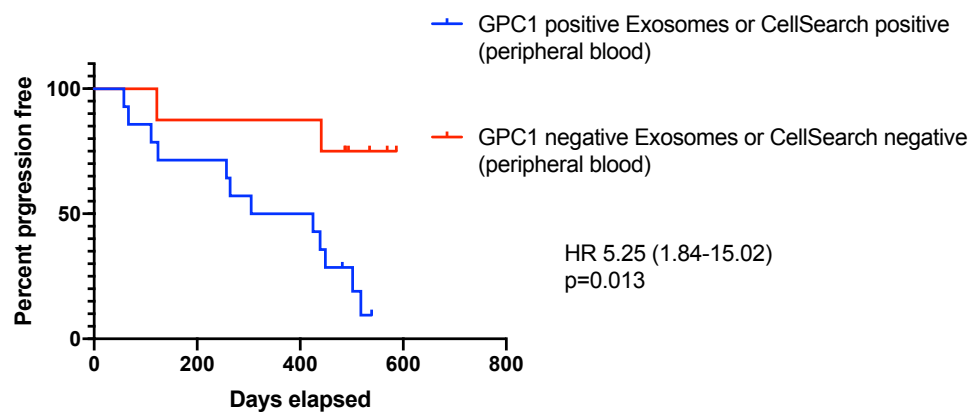
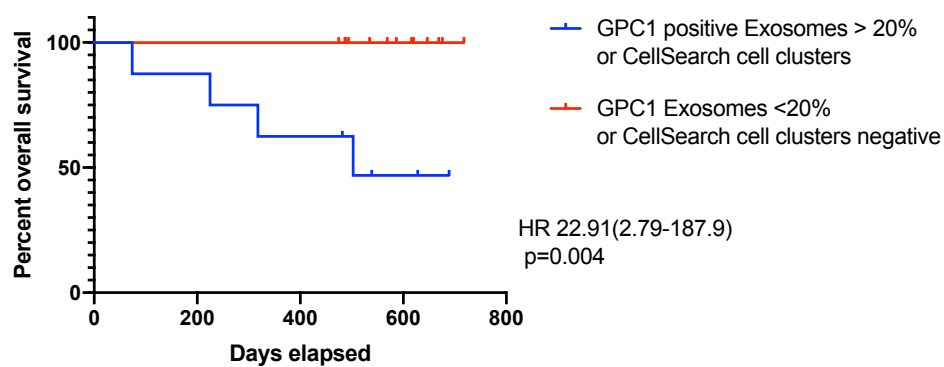


Figure 7

A



B



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## 6-Perspectives

### 6-1 La biopsie liquide combinée dans la stratégie diagnostique et thérapeutique

Les résultats prometteurs de nos travaux permettraient après validation sur de plus larges cohortes d'accélérer la prise en charge du CP pour améliorer le parcours du patient à la phase du diagnostic avec des méthodes peu invasives permettant d'obtenir du matériel moléculaire et/ou cellulaire tumoral. La biopsie liquide remplit le cahier des charges de ces exigences.

Les indications de traitements néo-adjuvants sont de plus en plus fréquentes et un diagnostic formel est nécessaire avant tout traitement. L'écho-endoscopie avec ponction est aujourd'hui l'examen de référence mais présente certaines limites : une valeur prédictive négative très moyenne, un caractère invasif, et opérateur-dépendant, une morbidité avec risque de pancréatite aiguë iatrogène allant jusqu'à compromettre la prise en charge chirurgicale. L'approche combinée de biopsie liquide pourrait être un outil diagnostique d'accompagnement d'avenir et pallier à la mauvaise valeur prédictive négative de l'écho-endoscopie ponction.

L'approche combinée pourrait également être une aide lors de la prise en charge des tumeurs intracanalaires papillaires et mucineuses du pancréas (TIPMP). En effet la décision opératoire repose sur des faisceaux d'arguments d'imagerie et pour certains opérateur-dépendant (i.e écho-endoscopie et IRM pancréatique). Les différentes séries chirurgicales montrent que ces lésions sont dégénérées dans 50% des cas. En pratique clinique cette prise de décision revient à proposer une chirurgie morbide pour des lésions potentiellement non dégénérées (*European guidelines GUT 2018*). De façon intéressante nos travaux rapportent une détection de CTC par la méthode RosetteSep™ chez les patients avec une TIPMP en dysplasie de haut grade. Des résultats similaires, décrit par *Poruk et al*, ont montré une association significative entre

la présence de cellules épithéliales circulantes (CEC) et les lésions en dysplasie de haut grade (*Pouruk et al Pancreas 2017*).

Pour évaluer la valeur diagnostique de l'approche combinée nous avons construit un projet (protocole ESPOIR financé en attente de CPP) consistant à effectuer une prise de sang et une ponction de sang portal en même temps que l'écho-endoscopie ponction.

## 6-2 La biopsie liquide combinée dans le monitoring de la maladie

La biopsie liquide combinée pourrait être un outil intéressant à deux niveaux. Tout d'abord dans l'évaluation de la réponse aux traitements néo-adjuvants et s'inscrire dans l'algorithme décisionnel pour la prise en charge chirurgicale. En effet des résultats prometteurs ont déjà été décrit par l'équipe de P Hammel et la détection des CTC avant et après traitement néo-adjuvant et leurs corrélations avec la survie globale (*Bidart et al 2013*).

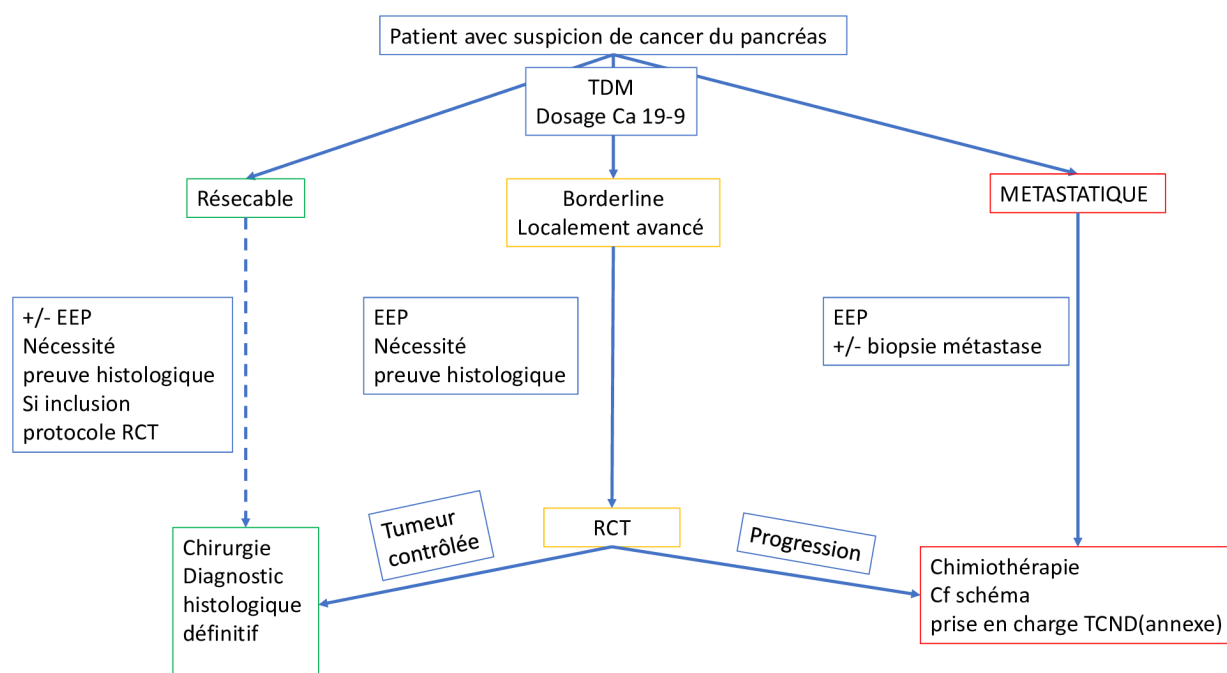
L'approche biopsie liquide combinée pourrait ensuite permettre le suivi sur le long terme des patients opérés d'une part mais aussi des patients métastatiques comme le suggère les travaux de *Bernard et al* qui ont montré une valeur pronostique significative grâce à une approche combinée avec détection de l'ADN exosomal et le ctDNA.

Nos résultats montrent une corrélation entre la survie sans récurrence et l'association entre les exosomes GPC1 positif et la présence de CTC avec la méthode CellSearch dans le sang périphérique. Et de façon très intéressante une association avec la survie globale lorsque des clusters de CTC sont retrouvés au plus proche de la tumeur comme précédemment décrit pour le cancer du poumon (*Murlidhar et al 2017*).



En conclusion la validation d'une nouvelle approche innovante de biopsie liquide combinée pour le CP permettra :

- Si très spécifique, on évite l'écho-endoscopie ponction aux patients positifs si résultats de biopsie liquide négatif réalisation d'une EEP (cf. schéma 1-2-3 en annexe).
- Sensibilité importante donc prise en charge plus rapide pour les vrais positifs améliorant le pronostique.
- D'évaluer sa valeur pronostic en effectuant des prélèvements répétés lors du suivi des patients.



Prise en charge diagnostique du cancer du pancréas ; EEP,écho-endoscopie ponction ; TDM tomodensitométrie

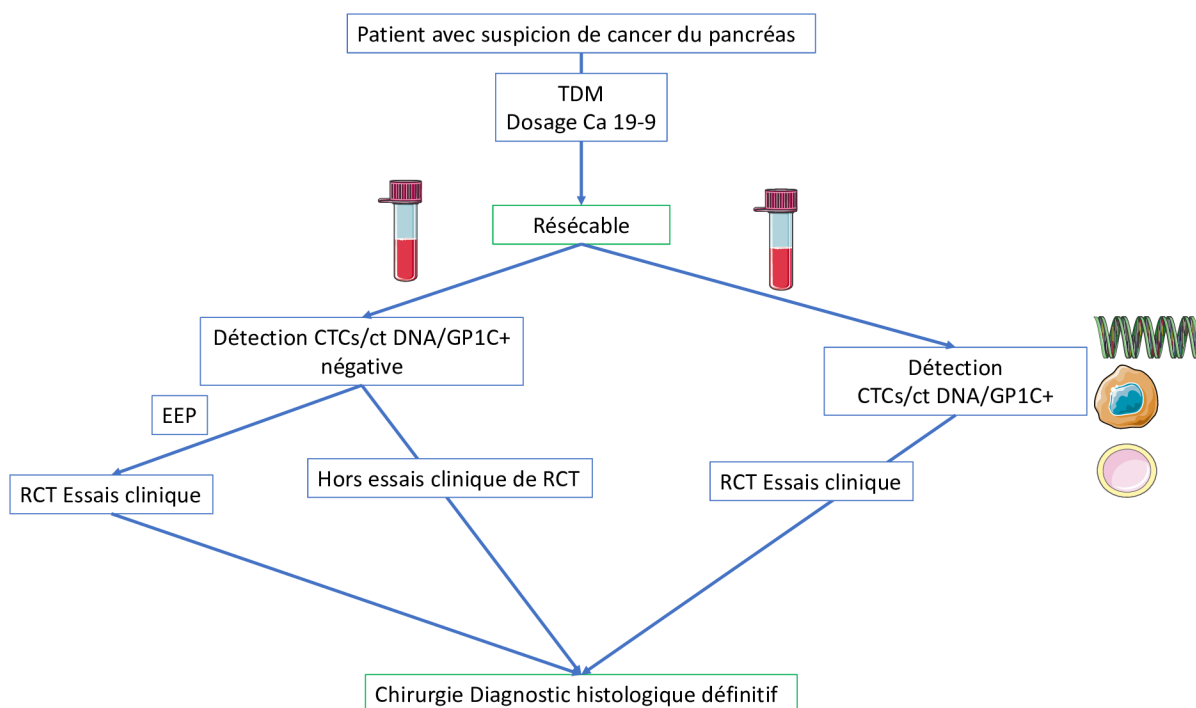


Figure 1 : Perspective des biopsies liquide combinée chez les malades résécables d'emblée : La biospie liquide combinée s'intégrant dans le schéma diagnostique des patients résécable d'emblé et pouvant s'inscrire dans la décision d'un traitement néo-adjuvant lors d'inclusion dans un essais clinique.

RCT,radio-chimiothérapie ; EEP,écho-endoscopie ponction ; ct DNA,ADN tumoral circulant, CTCs cellules tumorales circulantes ; GPC1+, exosomes GPC1 positif.

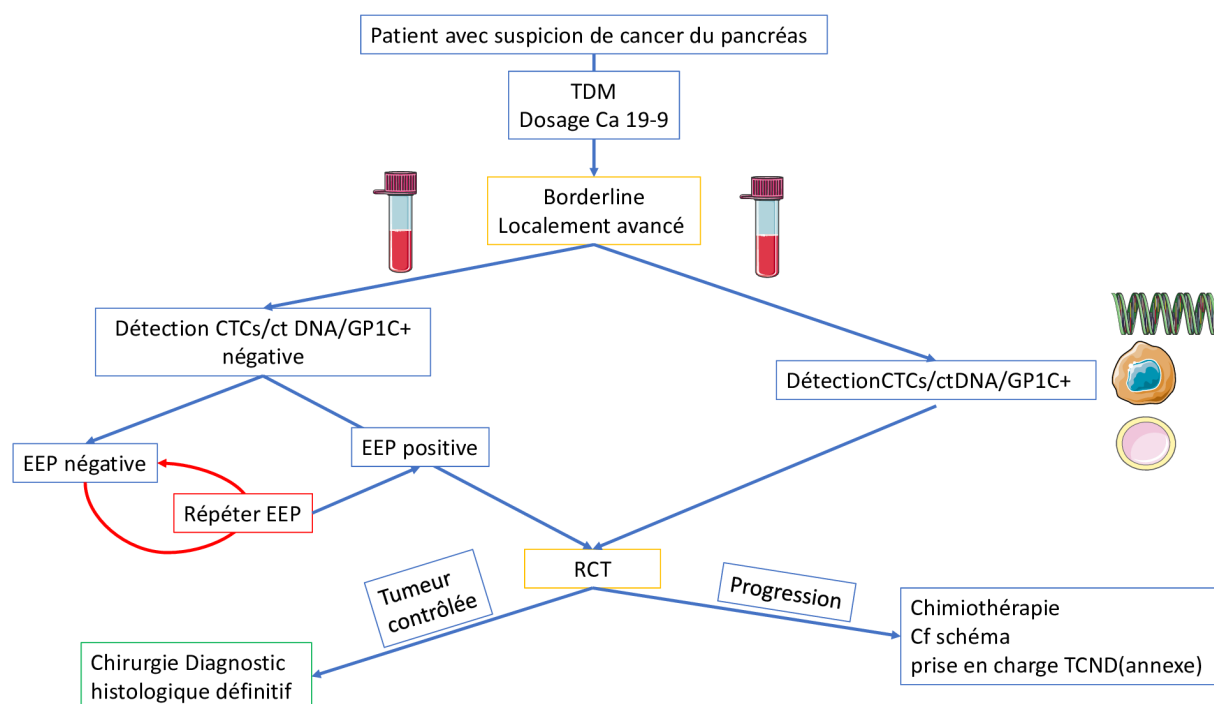


Figure 2 : Perspective des biopsies liquide chez les malades borderline/localement avancé

La biopsie liquide combinée s'intégrant dans le schéma diagnostique des patients borderline/localement avancé et pouvant s'inscrire dans la décision d'un traitement néo-adjuvant. De plus si les résultats de notre étude pilote se confirme la biopsie liquide combinée pourrait être un argument quant à la décision du type de traitement adjuvant.

RCT, radio-chimiothérapie ; EEP, écho-endoscopie ponction ; ct DNA, ADN tumoral circulant, CTCs cellules tumorales circulantes ; GP1C+, exosomes GP1C positif.

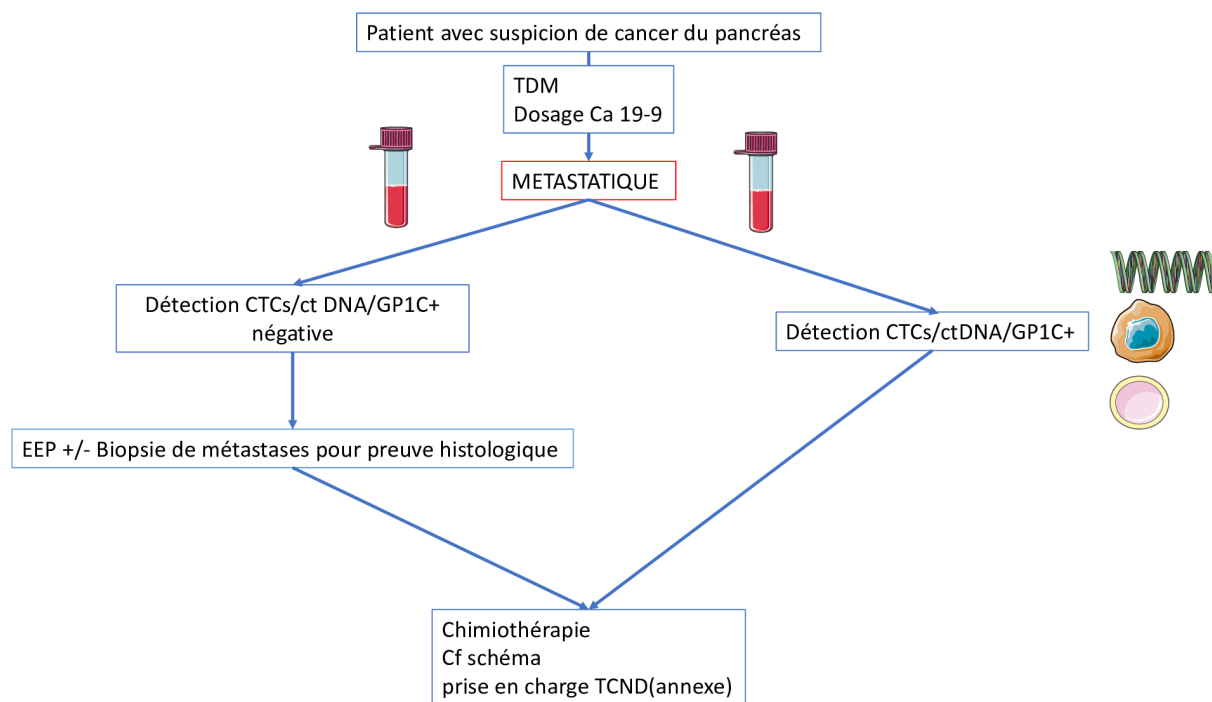
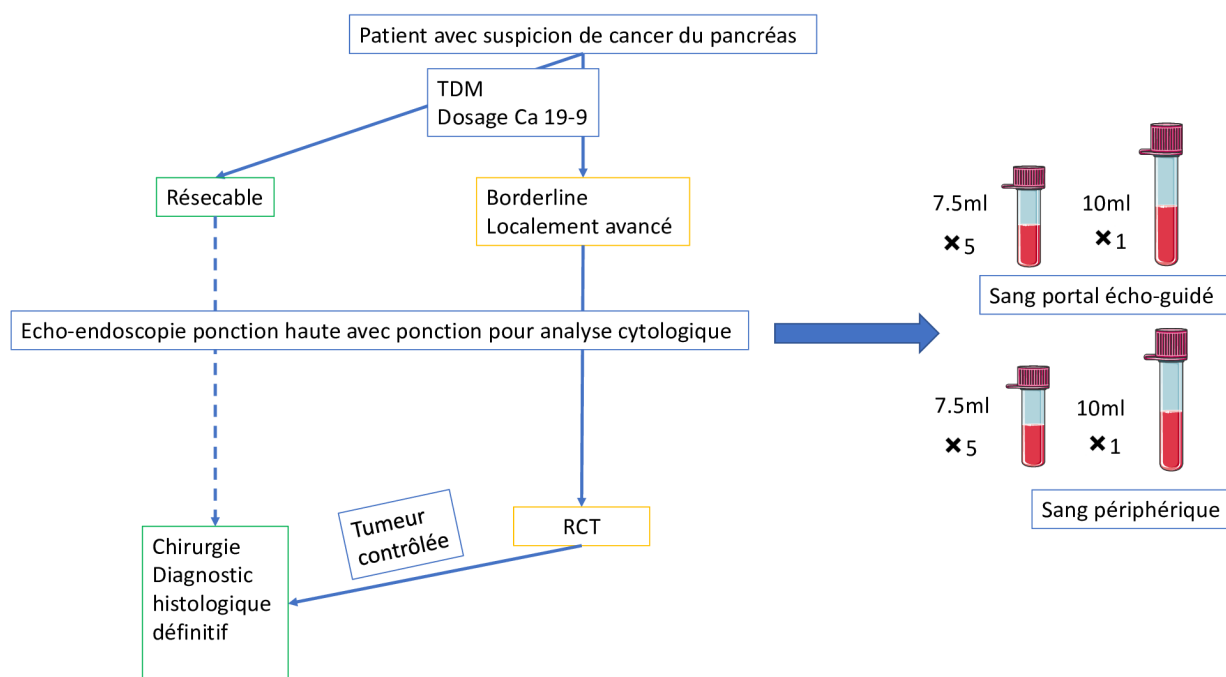


Figure 3 : Perspective des biopsies liquide chez les malades métastatiques

La biopsie liquide combinée s'inscrit dans la démarche diagnostique et pourrait éviter une ponction radiologique et /ou écho-endoscopique afin de mettre en place un traitement par chimiothérapie. ct DNA, ADN tumoral circulant, CTCs cellules tumorales circulantes ; GPC1+, exosomes GPC1 positif.



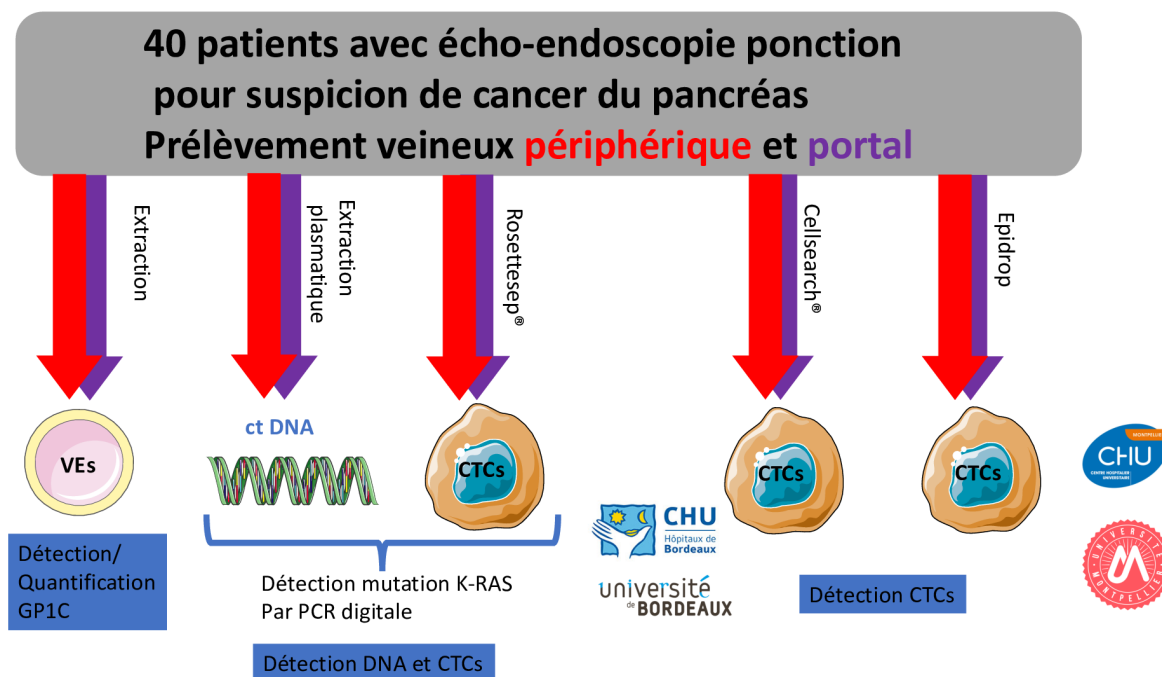
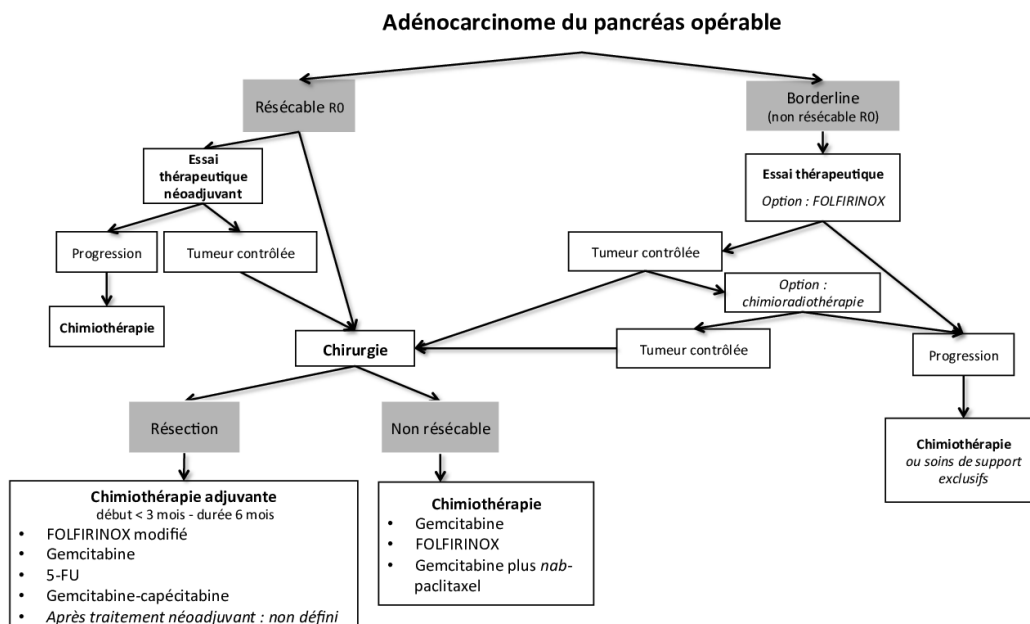


Figure 4 et 5 Schéma expérimental du protocole ESPOIR :

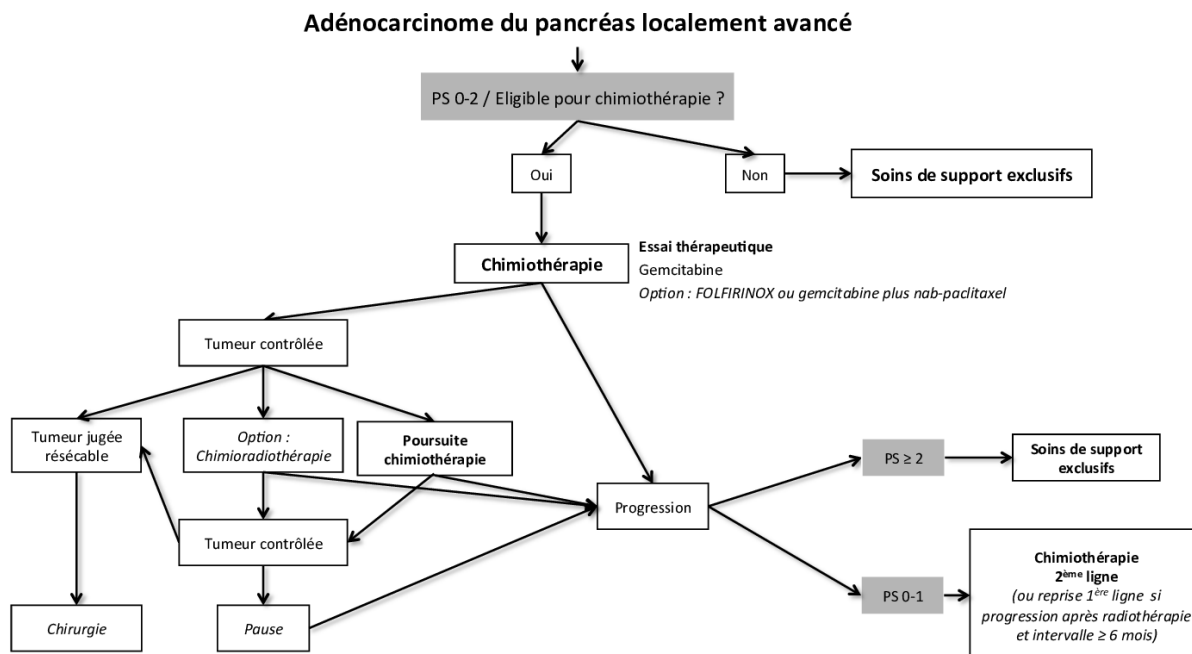
Réalisation d'une approche combinée de biopsie liquide au moment de l'écho-endoscopie ponction avec prélèvement de sang portal écho-guidé.

**Annexe** : Schématisation de la stratégie thérapeutique des adénocarcinomes du pancréas en 2019 (*Théaurus National de Cancérologie Digestive cancer du pancréas chapitre 9 2018*)

### Algorithme 1 - Traitements pour cancer résecable ou borderline

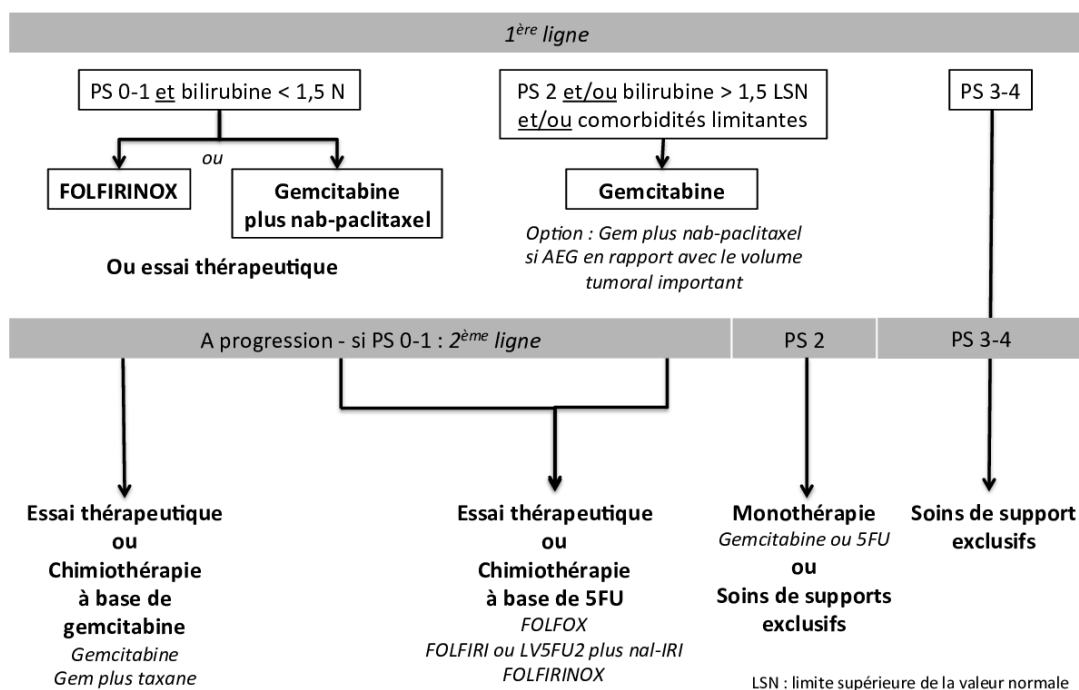


### Algorithme 2 - Traitements pour cancer localement avancé



### Algorithme 3 - Traitements pour cancer métastatique

## Adénocarcinome du pancréas métastatique



Schématisation de la stratégie thérapeutique des adénocarcinomes du pancréas en 2019 (Théaurus National de Cancérologie Digestive cancer du pancréas chapitre 9 2018)