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Circulating Tumor Cells and Cell-Free Nucleic Acids as Predictor Factors for Early Pancreatic Cancer Dissemination, A Literature Review

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Abbreviations

CD – Cluster of Differentiation; CK – Cytokeratin; CTC(s) – Circulating Tumor Cells; DEP – Dielectrophoresis; EMT – Epithelial to Mesenchymal Transition; EpCAM – Epithelial Cell Adhesion Molecule; FISH – Fluorescence in Situ Hybridization; GEDI – Geometrically Enhanced Differential Immunocapture; MACS – Magnetic Cell Sorter; MET – Mesenchymal–Epithelial Transition; PC – Pancreatic Cancer; PCR – Polymerase Chain Reaction; PDAC – Pancreatic Ductal Adenocarcinoma; PSMA – Prostate-Specific Membrane Antigen; QMS – Quadrupole Magnetic cell Sorter; cfDNA – Cell-Free DNA; cfNA(s) – Cell-Free Nuclei Acid(s); cfmRNA – Cell-Free mRNA; cfmiRNA – Cell-Free miRNA; ctDNA – Circulating Tumor DNA; ddPCR – Droplet Digital Polymerase Chain Reaction

1. Abstract

1.1. Background: Pancreatic cancer remains as one of the most aggressive and deadliest of cancers largely due to formidable challenges in diagnosis and therapy. Consensus standard treatment for patients with nonmetastatic Pancreatic Cancer (PC) incorporates possible neoadjuvant chemotherapy with timely surgical resection and adjuvant chemotherapy. However, despite all the sophistication of modern radiological and endoscopic techniques, the decision regarding operability is often only made intra-operatively, therefore subjecting a patient to unnecessary surgical intervention, and postponing the possibility of starting early chemotherapy.

1.2. Main Body: Tumors shed various elements of cancerous tissue, such as Circulating Tumor Cells (CTCs) and cell-free nucleic acids (cfNAs) (i.e., cell-free circulating tumor DNA (ctDNA), cell-free mRNA (cfmRNA) and cell-free miRNA (cfmiRNA)), into the circulatory system. Detection and analysis of these biomarkers through "liquid biopsy" has emerged as a novel, minimally invasive approach for early detection, determining prognosis, real□time monitoring and determining recurrence, improved management, and development of targeted therapy in the field of cancer studies. Furthermore, the development of radiological and endoscopic techniques, such as sampling of portal venous blood via endoscopic ultrasound, has enabled obtaining and examining material from localizations typically inaccessible by traditional biopsies. At the moment, a limited number of "liquid biopsy" platforms have been successfully approved, while many are still in preclinical and clinical trial stages and required further research and validation.

1.3. Conclusion: The purpose of this review was to list, systematize and highlight advantages and limitations of different strategies and individual platforms currently used in CTC and cfNA identification, isolation and analysis. This review emphasizes the need for further standardization of different methodologies and accumulation of results under equal conditions in large-scale studies, as well as combining different strategies in single devices to achieve optimal results.

2. Introduction

Pancreatic Cancer (PC) is the 12th most common cancer and the 7th leading cause of cancer-related death worldwide with 495'773 new cases and 466'003 deaths in 2020 [1]. Given the asymptomatic course of the disease, the disease is often diagnosed in the later stages. In cases of radical resection, the five-year survival rate is 20 to 25%. Given the poor response to radiation and chemotherapy, the predicted five-year survival without surgery is 3 to 5%.

Cancer diagnosis is mainly based on data from radiological examinations such as CT, MR, PET in combination with endo US data, oncological markers, as well as the results of examinations of tissue samples obtained during surgical biopsies or Fine Needle Aspiration Cytology (FNAC). Although conventional tissue biopsies are a gold standard in cancer diagnostics and can provide invaluable information, these methods are painful, invasive, and anatomically difficult to perform, often carry risks of additional complications such as bleeding, infection, and dispersal of cancer cells into healthy tissue and therefore, they are not always feasible to perform and/ or repeat. Additionally, the use of all these methods cannot completely exclude the presence of micrometastases, thus, unresectable patients often undergo surgery that delay the initiation of systemic therapy.

Considering the above-mentioned shortcoming of the classic PC diagnostic methods, there is an urgent need to find highly sensitive and high-precision minimally invasive methods for early diagnosis of PC and PC dissemination. Circulating tumors cells in these patients could be used in lieu of tumor tissue samples. Considering that tumors shed various complex elements of cancerous tissue such as Circulating Tumor Cells (CTCs) and cell-free nucleic acids (cfNAs) (i.e., cell-free circulating tumor DNA (ctDNA), cell-free mRNA (cfmRNA) and cell-free miRNA (cfmiRNA)), into the circulatory system, liquid biopsy has emerged as a novel, minimally invasive tool for early stage diagnosis, determining prognosis, monitoring therapeutic responses and tumor recurrence, as well as designing innovative therapies in cancer treatment.

In the past decade, liquid biopsy proved to be highly diagnostically valuable in lung, brain, and breast cancers. Importance of biopsies is evident – application of such method in diagnosis of PC has been highlighted in several studies, where early detection of pancreatic cancer is associated with decreased mortality and increased survival rates (increase from 3% up to 32%) amongst patients. While numerous methods of tumor-derived components enrichment, detection, and analysis are available, there's scarcity of reports evaluating their diagnostic value and provide comparative analysis of different methods that can be utilized in PC.

This review compiles examples of current methods and strategies from different categories employed to capture CTCs and cfNAs. We will provide an overview of the advantages and disadvantages, and overall compare the different strategies and technologies in use.

3. Tumor-Derived Components for Liquid Biopsy

Blood and bodily fluids are used as a means of biomarker detection. Through either exocrine and endocrine glands, pancreatic tumors secrete CTCs and cFNAS into the circulatory system. While blood is extensively in liquid biopsies due to easy acquisition and minimal invasiveness, it contains circulating materials other than tumor-specific components, which in result decreases the concentration of cancer-specific components within the sample. Other body fluids, such as pancreatic juice, bile, stool, saliva, urine, and pleural effusion may be used as a primary or complementary source of biomarkers for the detection of pancreatic cancer.

Enrichment is a crucial step in liquid biopsies, as bodily fluids contain circulating materials besides CTCs and cfNA's. The presence of these materials is particularly prevalent in blood samples. In context of liquid biopsies, "enrichment" implies a purification process of isolating tumor cells from other components (e.g. leukocytes) by running the sample through a platform. Generally, enrichment methods rely on different properties of the target component: (a) biological properties (e.g., specific surface protein expression) and (b) physical properties (e.g., size, density, electric charges, and deformability). Following enrichment, tumor-specific components require subsequent detected.

3.1. CTCs

CTCs, first described by Thomas Ashworth in 1869, represent a heterogeneous group of cells with varying phenotypic and genotypic properties that shed from primary and/or secondary tumor sites, entering into the bloodstream, and circulating throughout the body. Although CTCs are excellent candidates for liquid biopsies, as their presence indicates malignancy, they were not used in clinical applications until the late 1990s.

According to literature, mechanical forces (exerted by internal tumor growth or external surgery) and/or Epithelial to Mesenchymal Transition (EMT), may be causalities of CTC secretion.EMT is a biological process, where cells gain mesenchymal properties, which in return leads to an enhanced migratory capacity, invasiveness, and elevated resistance to apoptosis. This process allows polarized cells to transgress through the basement membrane, stroma and vessel walls, and enter into the circulatory system. At a distant site, a reverse process called the Mesenchymal–Epithelial Transition (MET), as well as interaction with the Extracellular Matrix (ECM) of the site may induce the formation of a new metastasis. This mechanism exists even in the premalignant pancreatic lesions, which are still not recognizable with conventional diagnostics methods, which suggests that the metastatic spread may be an early event in cancer progression and not its late consequence.

With meager quantities of CTCs in peripheral blood samples (i.e., one milliliter of peripheral human blood carries only about 1–10 CTCs), their detection is a challenge. CTCs in peripheral blood are very rare mainly because they are around 25 μm in comparison

to the capillaries ~8μm diameter, meaning they are too large to pass. Studies using animal models have demonstrated that when injecteed into a vein, radiolabeled tumor cells are rarely detected in peripheral blood as they are trapped in the capillary beds of the initial target organ. In case of PC, the portal vein drains periampullary and pancreatic tissues and carries CTCs from the tumor site to the liver, where CTCs are filtered. If CTCs become largely diluted within the peripheral blood system, rather than remaining in the liver. Blood can be sampled from the portal vein prior to CTC sequestration in the liver, in order to increase the likelihood of CTC detection. The procedure was first performed on a sample of 20 people, including a resealable Pancreatic Ductal Adenocarcinoma (PDAC) that could easily and safely take a portal blood sample during surgery.

CTCs were found in 9/20 (45%) portal blood samples and 4/20 (20%) peripheral blood samples in these patients. CTC was detected only in portal blood samples and would not have been detected if only peripheral blood was used in 25% of the 20 patients.

Additionally, there's association between detection of CTCs in portal venous samples and an increase in liver metastases [2]. Likewise, study of 60 patients with periampullary cancer (41 PDAC, 15 ampullary cancers, 1 duodenal cancer, and 3 common bile duct cancers) showed higher CTC detection rate (58.3% v 40%) and substantially larger number (mean: 230.1 vs 71.7; median: 60.0 vs 40.5) in portal blood samples v. peripheral venous blood samples [3]. Probability of metastases being directly proportional to quantity of tumor cells administered has been shown in animal studies. Consequently, patients with high CTC counts may be at an increased risk of metastases.

In the study, Tien et al. 21 (60%) of the 35 patients with CTC found in the portal vein did not develop liver metastases within 6 months of surgery.

This would imply that CTC detection in portal venous blood does not automatically lead to metastases of the liver. Nevertheless, the possibility of developing post-surgical liver metastases was directly proportional to the CTC count in portal venous blood samples collected intraoperatively: 11/13 patients with high CTC count (>112 CTCs in 2ml portal venous blood) suffered from liver metastases and all 11 patients developed liver metastases in a period of 6 months after surgery.

EUS-guided transhepatic portal venous sampling is feasible and effective, according to several reports [4]. Thakrar and Madoff have described the safe collection of blood samples from the intrahepatic portal vein for the assessment of CTC during percutaneous transhepatic portal vein embolization with a large-caliber catheter prior to major hepatectomy.

CTCs vary amongst different cancer types and stages accordingly, therefore presenting additional challenge in generalized approach to CTC identification solely based on morphological aspects. Inter- and intra-patient heterogeneity is possible, meaning that different morphologically distinct CTCs can be found even within one patient, as it has been shown in lung [5] and breast [6] cancer studies. In pancreatic cancer, CTCs are highly heterogenous, expressing plethora of stem cell markers, as well as ECM proteins. Kulemann et al. studies showed heterogenous group of cells with different staining and KRAS mutational properties in blood samples. Circulating Tumor Microemboli (CTMs), representing clusters of tumor cells, can be detected and especially in patients with metastatic disease. CTMs may also form aggregates with parental tumor cells or fibroblasts, leukocytes, endothelial cells, or platelets. Mutations in the primary tumor and metastatic lymph nodes are seldom shown to connected, despite high variability in CTCs.

Considering the extreme rarity and substantial heterogeneity of CTCs, a key challenge is the availability of systems capable of isolating CTCs from patient blood samples in a way that enables downstream processing and analysis of said samples. While numerous systems, which utilize a vast array of strategies have been developed, a great need still exists for low-cost, non-invasive, and efficient devices for CTC capture. Most of the current CTC technologies consist of a two-step process: enrichment and detection. Initially, whole blood samples are collected in vacutainers coated with ethylenediaminetetraacetic acid. Whole blood contains single- or double-digit quantities of CTCs among more than 109 RBCs per ml, therefore the necessity for pre-processing whole blood should be evaluated. Albeit pre-processing may render blood samples useless (e.g., by centrifugation or RBC lysis), resulting in CTC loss. Conversely, RBCs can interfere with CTC isolation and/or detection, and while CTCs can be immediately identified after extracting red blood cells using high-throughput imaging techniques, a successful enrichment phase will vastly improve the chances of CTC detection.

3.1.1. CTC Enrichment Methods: Antibody (Label) Dependent – Immunoaffinity: Since description by Racila et al. in 1998, CTC enrichment by immunoaffinity still remains the most commonly used method of CTC enrichment. This method uses antibodies to target specific surface antigens to capture cells. Method could be subdivided into positive and negative enrichment – while positive enrichment captures CTCs by targeting tumor-associated cell surface antigens, like EpCAM, negative enrichment, on the other hand, is aimed at removing materials other than tumor-specific components by binding to antigens that are not expressed by CTCs (e.g., CD45).

3.1.1.1. Positive Enrichment Techniques: Considering that until recently immunohistologic definition of a CTC included positive expression of EpCAM+/CK+ and negative expression of CD45- molecules in nucleated cell, majority of positive enrichment techniques and available devices have initially specifically targeted EpCAM with subsequent detection with cytokeratin (CK) and 4',6-diamidino-2-phenylindole (DAPI) nuclear staining (7). How-

ever, over the last several years the established definition of CTCs, as well as the strategy of immunoaffinity methods has come under scrutiny as several studies have revealed the existence of CTCs with a) low expression of EpCAM, b) EpCAM−, c) CK– d) CK+/CD45+ ("double positive"), and e) EpCAM+/CK+/CD45− cells in the circulation of patients with benign colon diseases [7], pancreatic lesions [8], and benign breast disease [9, 10].

- Immuno-magnetic cell separation platforms.
    - CellSearch. While technologies that target epithelial markers for positive selection may have several drawbacks, relevance of EpCAM+/CK+/CD45- cells in PBS is supported by prognostic value of platform like CellSearch [11-13]. CellSearch uses EpCAM conjugated ferrofluid nanoparticles to immunomagnetically capture CTCs, which may then be differentiated from contaminating leukocytes with immunostaining for CK+ and CD45−. Although CellSearch retains its so-called gold standard status within the field as the only FDA approved method for CTC detection in breast, colorectal, and prostate cancer; newly introduced technologies are primarily validated and compared against CellSearch, results obtained by CellSearch in PC studies, on the other hand, seem controversial. Study carried by Allard et al. reviewed CTCs using CellSearch system in 2183 blood samples from 946 metastatic patients with 12 different cancer types (from which 21 blood samples were from 16 pancreatic cancer patients). Compared to other neoplasms, pancreatic cancer patient strata showed lower counts of CTCs in blood samples (mean 2 CTCs/7.5 mL). Detection of CTCs above cutoff level of ≥2 CTCs was only possible in 4/21 (19%) samples [14]. Amongst other studies, CellSearch CTC detection capabilities were not consistent amongst multiple studies, ranging between 5% (UICC III cohort) to 42% (mainly stage IV) [15-18]. Dotan et al. recounted only 48% positive cases among metastatic patients [19].
    - AdnaTest. In order to improve CTC capture and enrichment, AdnaTest uses immunomagnetic beads coated with a mixture of antibodies. For CTC validation, the positively enriched cells are screened for different gene panels depending on tumor form using reverse transcription–PCR (RT-PCR). Although there has been limited use of the AdnaTest in PC research to this date, it has been widely used and verified in other cancer types, for example, AdnaTest found CTCs in 34/47 (62%) of patient samples in comparison to just 23/47 (45%) with CellSearch in a clinical trial to determine the prognostic relevance of CTCs in metastatic castration-resistant prostate cancer. The overall sensitivity of the CellSearch findings was found to be unfavourable in this review [20]. Similarly, AdnaTest proved to be superior identifying CTCs in 81% of metastatic CRC patients comparted to result of 21% with CellSearch [21]. This distinction may be explained by the fact that AdnaTest detected CTCs using several gene panels, while CellSearch relies solely on EpCAM for capture. Despite the fact that Adnatest incorporates two methods into its methodology, CellSearch outperforms AdnaTest in some area due to the technological challenges associated with AdnaTest blood sample processing and preservation.
    - Magnetic Cell Sorter (MACS). MACS captures cells labelled with magnetic nanoparticles by using high gradient magnetic separation. conjugated to anti□EpCAM [22, 23] or anti-CD45 [22] antibodies. The blood sample is passed through a plastic-coated steel wool-filled column. In the absence of magnetic field, this wool could be either magnetized or demagnetized allowing for capture and isolation of magnetically labeled cells [24]. In a study by Effenberg et al. [25] CellSearch and MACS enrichment were used to analyse a subset of 20 patients in tandem, and the results show that in the case of PDAC, CellSearch could not be the best tool for CTC detection as the CTC rate was twice as high with MACS.
    - MagSweeper. In microfluidic platforms, a large surface area is coated with ligands or capture molecules, that bind to the CTC surface markers (e.g., anti-EpCAM, EpCAM). High affinity of ligands to CTC surface markers allows for retention and successful capture of cells, while other sample components are carried away by the flow. Precise sample flow control is utterly important in such devices, as it affects capture success rate; therefore, flow control is integrated in these devices.
- Microfluidic-based technologies. In microfluidic platforms, ligands (or capture molecules) with affinity to CTC surface markers are bound to large surface area. Most commonly, well-established antibody-antigen pairs like anti-EpCAM and EpCAM

are used in such instance. During processing of blood sample, CTCs are captured from the flow because of antigen-antibody interaction, retaining only CTCs. Ability to precisely control and alter sample flow is integral feature of these devices, as the flow rate directly influences cell capture efficiency. In addition, microfluidic approaches allow for gentle capturing of live rare cells so that further analysis can be performed using cellular, microscopic, or molecular techniques.

- Micropost arrays. Introduction of microfabrication methods provides an opportunity to utilize structures at or below cellular length scale in cell separation technologies.
  - CTC-Chip. CTC-Chip, the first microfluidic device designed for CTC enrichment, was created by Nagrath et al. in 2007. that consisted of anti-EpCAM antibody chemically functionalized and geometrically arranged 78,000 microposts. In 116 samples from 68 cancer patients, including 15 with pancreatic cancer, Nagrath et al. used the CTC-Chip to enrich for CTCs and found CTCs (CK+/CD45−) in 115 samples (>99%) [27]. The advantage of CTC-Chip and other microfluidic chip technologies discussed hereof, is the ability to process whole blood. The major downside is the slow throughput rate and inability to accommodate big enough sample volumes; in CTC-Chip, for example, a fluid flow of only 1–2 ml/h was used.
  - Geometrically enhanced differential immunocapture (GEDI) device. By combining positive enrichment with hydrodynamic chromatography, GEDI allows for cell separation based on their [28, 29]. Rhim et al. used GEDI chip to capture CTCs across three different subject groups with different diagnoses (PDAC patients of all stages, precancerous cystic lesion patients, and cancer-free (negative) controls) [30]. The detection rates of CTCs were 8/11 (73%), 8/21 (38%) and 0/19 (0%) in PDAC patients, patients with precancerous lesions, and cancer-free group, respectively [30]. No comparison studies were performed on GEDI chip v CellSearch performance in PC; in a study by Kirby et al method of choice for CTC, that were defined as PSMA+/CD45-, isolation was GEDI. In their study, GEDI chip showed a 2-400-fold increase in isolated CTC counts compared to CellSearch [29]. Similar to CTC-Chip, the GEDI device uses a low flow rate of only 1 ml/h.
- Surface-based arrays. Considering that large-scale production of micropost-based devices, as well as detection and characterization of CTCs through high-resolution imaging in the presence of micropost arrays is inherently difficult, development of surface-based microfluidic devices was initiated to overcome these limitations. As these devices use antibody-coated surface for facilitated CTC capture instead of micropost arrays, the overall simplified architecture is more suitable for large-scale production with possible addition of added device transparency amenable to imaging. Additionally, these devices can be run at higher flow rates.
  - Herringbone (HB) Chip. The HB Chip generates microvortices within the translucent wall of the system by using herringbone grooves to guide target cells against the antibody-coated walls. Higher blood volume throughput (4.8 ml/h) and improved CTC capture quality and purity are possible with this "second generation" unit. CTCs from patients with metastatic prostate cancer were successfully captured using the HB-Chip. – CTCs were detected in 14/15 (93%) patients with metastatic disease [31].
  - Geometrically enhanced mixing (GEM) Chip. In case of HB and GEM chip, method published by Stroock et al. of inducing mixing within microchannels with transverse flow is being employed resulting in enhanced cell-antibody [32]. The GEM chip is built around geometrically optimised micromixer constructs that improve transverse flow and flow folding, enabling CTCs and antibody-coated surfaces to interact more effectively.The device runs at a flow rate 3.6 ml/h. The GEM Chip was used to identify CTCs

in the blood of patients with metastatic pancreatic cancer.; and CTCs were found in 17/18 samples (>94%) [33].

- Graphene Oxide (GO) Chip. The GO nanosheet with EpCAM is adsorbed on substrate consisting of 58,957 flower-shaped gold patterns that facilitate CTC capture at a rate of 1-3ml/h. The GO chip was used in a study of 20 cancer patients, including 9 patients with metastatic PC, and compared with cancer-free controls. Overall, CTCs were captured with high sensitivity at low concentration of target cells, and all patients with PC had ≥ 2 CTCs/mL, with a mean of 5 ± 4 CTCs/mL, while no CTCs were measured in healthy controls [34].

- Immunomagnetic arrays. Lack of convenient downstream analysis in surface-capture devices is a major method design drawback, as a result of CTC immobilization on the surface. To overcome the limitation posed by such platforms, a dual-modality platform has been design, that integrate immunomagnetic beads. In this case, immunomagnetic beads serve as mean of CTC retrieval from whole blood sample; then the marked cells are separated based on size or by inertial focusing, giving greater enrichment efficiency (>99%) and higher purity of isolate.

  - Ephesia Chip. In Ephesia chip, magnetic traps (designed as period arrays of 48,000 columns within microfluidic channel) are created by microcontact printing. This design promotes self-assembly of supermagnetic beads coated with EpCAM antibodies in those channels [35, 36]. Ephesia is not currently commercialized, but is seen to be promising for diagnostic purposes, particularly with its high capturing precision [37]. CTCs were found in clinical samples taken from metastatic breast cancer (4/5) or metastatic prostate cancer (6/8) patients in a study comparing Ephesia cell capture technology with CellSearch. In comparison to CellSearch, the Ephesia process revealed a comparable or higher quantity of caught CTC. in 10/13 samples [37]. Compared to micro-post arrays, the main benefits of this platform are reduced production costs and a more amenable design to commercialization, lesser obscuration of cell imaging, and higher throughput of >3 ml/h.

  - CTC-iChip. In the CTC-iChip, whole blood is processed and cells are magnetically labeled and then passed in 3 microfluidic technologies within the device. First, whole blood samples are processed using a micropillar array deterministic lateral displacement that separates nucleated cells from smaller blood components (i.e., red blood cells, platelets) based on cell size. Second, using inertial focusing, larger nucleated cells are arranged into a single line. Third, magnetically-labeled cells are deflected from unlabeled cells from separate cells via magnetophoresis – in CTC-iChippos using positive enrichment; or in CTC-iChipneg with negative enrichment utilizing CD44- and CD66b- labeled magnetic beads [38]. A study comparing CellSearch v CTC-iChippos was performed on 42 cancer patients (6 of which were PC patients); while both assays had good performance with high CTC loads (>30 CTCs per 7.5 mL), in case of lower CTC counts in the sample, there was a drastic difference in efficiency. The number of CK+ CTCs isolated with the CTC-iChippos was substantially higher in 22 cases among 36/42 (86%) of metastatic cancer patients. This draws the conclusion, that higher CTC-sensitivity of iChip is of particular importance in patients with low CTC burden [38]. In regard to iChip, it is also worth mentioning the faster sample processing speed of 8 ml/h, compatibility with normal post-capture CTC research protocols, as well as its development for diagnostic purposes [39].

- In vivo technologies. Saucedo-Zeni et al. developed and presented a new diagnostic tool for analysing large blood samples vivo called The GILUPI CellCollector. The CellCollector uses a functionalized medical wire (FSMW), which based on the

Seldinger guidewire. Phenotyping CTC collected by the FSMW with antibodies guided to cytokeratins and/or epithelial cell markers allows CTC to be identified. . Antibodies to the epithelial cell surface antigens are attached to a polycarboxylate hydrogel which is coated on a gold-plated Seldinger guidewire. Then the hydrogel is functionalized with antibodies to the target molecule. This FSMW binds to target cells that have a particular antigen on their surface. In breast and non-small cell lung cancer patients, the wire successfully caught CTCs in 22/24 (92 percent) of cases, with a median of 5.5 CTCs (range 0–50) and 16 CTCs (range 2–515) observed, respectively [40].

3.1.1.2. Negative Enrichment Techniques: These techniques are based on indirect isolation of CTCs: background cells like leukocytes are targeted for removal to acquire a CTC-enriched sample. While purity level is incomparable with superior levels of positive enrichment techniques, in some studies depletion methods may be techniques of choice, avoiding sample bias depending on selected markers or application of difficult to-remove labels [41]. EasySep and Quadruople Magnetic Seperator (QMS) are two commercialized systems used in studies to isolate CTCs from clinical samples.

- EasySep. EasySep is an immunomagnetic cell isolation platform suitable for the enrichment of CTCs by targeting unwanted cells for depletion by, firstly, incubating samples using the EasySep Human CD45 Depletion pack, which includes magnetic nanoparticles linked to tetrameric antibody complexes for CD45 targeting. Furthermore, by inserting the sample-containing tube into the EasySep magnet, magnetically labelled cells are isolated from unlabeled cells. Unwanted cells are pushed to the tube's edges, and the enriched cells are pipetted or pumped into a new tube [42].

- QMS. The QMS is a flow-through, high-throughput magnetic cell sorter, which employs a quadrupole magnetic field and annular channel geometry. A separate inlet stream forms a sheath flow between the feed stream and the cylinder wall (stream b), while the sample is fed into the cylindrical separation mechanism around a centre (stream a). The separation mechanism is surrounded by four magnets, which provide a magnetic gradient that deflects immunomagnetically labelled cells and allows them to shift from stream a to stream b. The two streams will stay distinct in a laminar flow regime with no merging, and each stream will leave the column separately through a flow splitter at the outlet [43, 44].

Any of the aforementioned positive immunoaffinity methods may be utilized together with negative enrichment techniques by employing antibodies targeting leukocyte-associated antigens, usually, CD45 [42]. Additionally, many positive immunoaffinity methods, especially those using immunomagnetic separation with antibody-functionalized beads or particles, can function as negative enrichment technologies by applying different antibodies (e.g., replacing anti-EpCAM with anti-CD45) [22, 38, 39, 45].

3.1.2. Ctc Enrichment Methods: Antibody (Label) Independent: Another major type of CTC enrichment strategy, that has been gaining in popularity in the field, known as label-independent (label-free) or physical property-based enrichment, relies on biophysical properties (e.g., size, including inertial focusing, electrical charge, and density) of CTC's. Compared to affinity-based methods, label-free methods are not restricted by the need to target a specific antigen for enrichment, and additionally CTC are not labeled with an antibody that can hinder downstream analysis.

3.1.2.1. Density-Based Gradient Centrifugation Techniques: One of the first methods reported for CTC isolation is centrifugation. In 1959, Seal documented different specific gravity for red blood cells, leukocytes, and cancer cells; using silicone flotation and subsequent filtration he isolated CTCs from 39/86 (45%) of samples from patients with cancers of varying origin [46].

In density-based gradient centrifugation, separation of CTC-containing peripheral blood mononuclear cell (PBMC) layer is done based on density properties, after subjecting the sample to centrifugation on a resolving medium.

- Ficoll-Paque. Although it was designed for the isolation of mononuclear cells from blood and bone marrow aspirates using gradient centrifugation, researchers have used Ficoll-Paque in CTC isolation. Ficoll-Paque media products are aqueous solutions containing Ficoll PM400, a neutral, highly branched, high-mass, hydrophilic polysaccharide, and sodium diatrizoate with calcium disodium ethylenediamine-tetraacetic acid. In a study by Soeth et al. bone marrow samples and venous blood samples of 172 patients with PDAC were enriched by Ficoll-Paque and centrifugation with subsequent detected using CK20 RT-PCR. In 117/172 (68.0%) patients a positive result in bone marrow and venous blood was obtained, while in 55/172 (32.0%) patients result from only one compartment could be introduced (18/172 (10.5%) bone marrow samples and 37/172 (21.5%) blood samples) [47].

- Nycoprep. Nycoprep is a ready-made, sterile and endotoxin tested solution of Nycodenz, NaCl, and Tricine-NaOH. Using NycoPrep and immunohistochemistry for pan-cytokeratin antibodies, Z'graggen et al. isolated CTCs from blood samples of 27/105 patients (26%) with resectable and advanced PDACs. AE1/AE3 [48].

- OncoQuick. OncoQuick combines density gradient centrifugation in conjunction with filtration by adding a porous barrier above the separation media that enables RBCs and some leukocytes to move through while keeping CTCs. In a comparative study of Ficoll-Paque and OncoQuick by Rosenber et al., spiking experiments with the colorectal carcinoma cell line HT-29 were performed. While the tumor cell recovery rates for each system following centrifugation were similar, OncoQuick resulted in a 632-fold enrichment ratio against leukocytes compared to 3.8 with Ficoll-Paque [49]. Balic et al. conducted a comparison analysis

between OncoQuick and CellSearch., and In addition to identifying CTCs in a greater number of patients, (33/61 (54%) with CellSearch vs 14/61 (23%) with OncoQuick), CellSearch also found a higher number of CTCs per sample. (mean 20/7.5 ml blood) than OncoQuick (mean 3 CTCs/7.5 ml blood) [50].

- RosetteSep. RosetteSep. RosetteSep design encompasses isolation of monocytes from whole blood samples by means of negative selection. Unwanted cells (monocytes, RBCs) are targeted with Tetrameric Antibody Complexes (TAC) for removal. Further centrifuged with Ficoll-Paque or Lymphoprep (buoyant density media), antibody-label cells sink to the bottom. Highly enriched population of monocytes is present at the interface between plasma and buoyant density media. In a comperative study of RosetteSep and OncoQuick by Buscail et al., CTC recovery capacities from total blood samples were tested using CAPAN-2 cell line spiking experiments. Both enrichment methods were compared head-to-head, followed by KRAS mutant DNA detection by droplet digital PCR (ddPCR). While recovery was higher in OncoQuick as compared to RosetteSep (67.5% ± 3.5%, n = 59 and 50.7% ± 3.5%, n = 65, respectively), cell enrichment was 10 times lower, leading to high levels of contamination. In addition, a 3- to 4-fold more sensitive molecular detection of mutant KRAS alleles by ddPCR was seen after RosetteSep vs OncoQuick. Thus, OncoQuick was superior to RosetteSep in recovering tumor cells, but RosetteSep was more sensitive in detecting tumor DNA [51].

The advantage of this method is that it offers a quick, inexpensive, and simple way to isolate CTCs. But the drawback of this technique is its poor sensitivity, due to the loss of some CTCs migrating to the plasma layer (presumably, due to cytotoxicity of density medium), or the formation of CTC aggregates settling to the bottom of the gradient. Discrepancies regarding use of these media are seen in the literature on the basis that different studies report polarized results, e.g., showing either high purity rate vs low isolation efficiencies. Another major fallback of even the most advanced centrifugation systems is inability to easily eliminate leukocyte contamination from the samples, resulting in in purities of less than 1% [52].

3.1.2.2. Microfiltration Techniques: In most cases, CTCs are assumed to be significantly larger than the surrounding cells in blood samples (12–25 μm vs. 8–14 μm). In this method circulating CTCs are processed through an array of microscale constrictions to capture target cells based on their size. Isolation of CTCs from samples based on cell size has been demonstrated to be an efficient, inexpensive, and quick method of CTC enrichment.

- Two-dimensional microfiltration systems. Since the first microfiltration setup for CTC enrichment constructed by Seal in 1964, more advanced techniques, e.g., track-etching and photo-/soft-lithography, for generating microfilters have been developed.
- Through a combination of surface bombardment with charged particles (or irradiation) and chemical etching, tracketching creates nano to micron-sized pores in thin polycarbonate films, allowing decoupled regulation of pore size and density [53].
  - ISET. ISET consists of a module of filtration and a polycarbonate track-etched membrane with calibrated, 8-μm-diameter, cylindrical pores. The module of filtration has 10-12 wells, and each sample is filtered through a 0.6-cm-diameter surface area in the membrane [54]. In a study of patients with metastatic or inoperable PC, Khoja et al. demonstrated that ISET identified CTCs in more patients than CellSearch (93% vs 40%) and in larger numbers (median CTCs/7.5 ml, 9 (range 0–240) vs 0 (range 0–144)), respectively.
  - ScreenCell. ScreenCell is a filtration-driven, single-use, marker-independent approach based on cell size and morphologic parameters. It employs a circular track engraved polycarbonate filter with randomly spaced cylindrical micropores (diameter 7.5 0.36 m; 1 X 105 pores/cm2) that enable regular blood components to flow through while retaining the much larger CTCs [55]. ScreenCell offers microfiltration setups for cytological studies (ScreenCell Cyto), live cell culture (ScreenCell CC), and molecular biology assays (ScreenCell MB). Using ScreenCell Cyto in combination with ScreenCel, CTCs were found in the majority (73%) of 11 pancreatic cancer patients studied by MB Kulemann et al. In this analysis, cytology alone was able to detect CTC in 18% of cases. The simplicity of the ScreenCell filtration devices' filtration technique, which does not require costly machines or facilities, is one of their main advantages [56].
  - MetaCell. MetaCell. Filtration of PBS through porous (8 μm diameter) polycarbonate membrane is applied in this method. Bobek et al. used this technique in their study with 24 PC patients, candidates for surgical treatment. In 16/24 patients (66.7%), CTCs were detected. Positive findings did not reflect disease stage, tumor size, or lymph node involvement. Same results were acquired in metastatic and non-metastatic patients.
    - Photolithographic fabrication has the capability of producing uniform patterned microfilters for use in CTC isolation. This method has been accomplished in various academic settings using

parylene, silicon, silicon nitride and nickel substrates [54, 54-59].

- CellSieve. CellSieve microfilters are generated through photolithography from a 10-μm thick layer of photoresistor patterned with ~160,000 7-μm pores per 9-mm diameter filter. In a study by Adams et al. CellSearch and CellSieve assays were compared using blood from 29 patients with cancer. Comparing identification systems, at least one CTC in blood samples showed 72% for CellSieve, 58% for CellSearch [60]. Photolithography also has significant disadvantages, such as the need for high-energy radiation, as well as the high cost of high-precision instruments. It can also be applied only to flat surfaces. Soft lithography is an improved version of photolithography that is simple and inexpensive.

• Three-dimensional microfiltration systems. Prior fixation of blood samples is vital for prevention of cell lysis during filtration in two-dimensional filtration systems yielding whole process unviable for effective CTC enrichment. To overcome shortcoming of this method, a three-dimensional system has been developed, where blood samples are processed without prior fixation. These systems, e.g. FaCTChecker, Parsortix system, and cluster chip, utilize larger size of tumor cells in multi-layer filtration for capture.

- FaCTChecker. The FaCTChecker is composed of two porous, vertical parylene-C membrane layers – bottom layer consisting of hexagonally arranged 8-μm pores, and top layer being made of 40-μm pores in hexagonal arrangement; hexagonal patterns on top layer are aligned with the bottom layer [61]. Size difference in membrane pore openings allows for easy CTCs 10-μm distance passage through top layer and subsequent fixation on bottom layers, allowing to them to be removed. Although, no data substantiates its use in clinical practice, Zhou et al. analyzed whole blood samples from breast cancer mouse model system in vivo to assess system's effectiveness; results proved successful collection and culture of viable CTCs from mice previously injected with tumor cells with variable metastatic potential.
- Parsortix. Unlike the FaCTChecker system, which uses a vertical configuration, The Parsortix has devised a horizontal stair-type scheme that decreases the channel width stepwise to ≤10 μm. CTCs that are larger than the filtration channel width is trapped in the gap, and can be harvested post-filtration via opposite direction flow and further molecular analysis [62]. Similarly, to the FaCTCheck system, Parsortix has not been extensively utilized in PC research, but a study by Xu et al. demonstrated that in seven prostate cancer patient samples where both systems were used, the Parsortix system not only processed blood samples with at least equal speed and blood volume capability to the CellSearch system, but it also harvested slightly more CK positive CTCs than the CellSearch system (an average of 32.1 and 10.1 respectively).
- Cluster Chip. The Cluster chip positions itself in a unique spot amongst size selection technologies being specifically designed for CTM capture. By design it is projected in multiple staggered rows of triangular pillars, where a repeating unit is the "cluster trap"; it consists of two adjacent pillars with third bifurcating beneath them. In practice, such device allows capture of CTMs as small as two cells, whereas other strategies are more likely to fail or damage CTMs. Results from study by Sarioglu et al. show the efficacy of this model – clusters were identified in 11/27 breast cancer patients (41%), 6/20 melanoma patients (30%), and 4/13 (31%) prostate cancer patients [63].

While microfiltration allows rapid processing of blood for CTC enrichment and allows capture of viable cells without difficult to remove labels, some design flaws exist, however. Major caveats include clogging issue and requirement of parallel processing for large sample volumes (>1.5 mL). Additionally, it typically difficult to achieve purity levels higher than 10% because of overlap in size distributions between leukocytes and CTCs.

3.1.2.3. Inertial Focusing: Fluid inertia properties at high flow rates in specific microchannels varying in shape are used in inertial focusing for microparticle and cell alignment. Randomly dispersed particles with Reynold's number of 1 or higher are subjected to two opposing inertial lift forces (shear gradient lift force and a wall effect lift force) within the microchannel. Channel dimensions, channel aspect ratio, flow rate, and particle diameter all influence the magnitude and orientation of these lift powers. Particles migrate 2 to 4 complex equilibrium positions between the rectangular or square channel's centerline and the wall. Cells are harvested in smaller volume due to concentration of collection

in size-dependent manner [64, 65]. Vortex Chip. Vortex chip uses multiple expansion-contraction reservoirs placed in series and parallel, which generate multiple vortices when a laminar flow of a sample occurs at a high rate. By further lowering the flow rate in these reservoirs, sample can be purified from remaining small particles, releasing the CTCs. Such chip has a processing capability of 7.5ml/20min, either with or without RBC lysis. Sollier et al. trialed this system and isolated CTCs from 12 clinical samples, achieving yield of ≥5 CTCs from 9/12 samples and a 57–94% purity [66].

• ClearCell FX Chip. The ClearCell FX Chip is a spiral microfluidic system that traps CTCs from a blood sample by combining inertial focusing and secondary Dean's flow caused by curved channels. The ClearCell FX chip can process a 7.5 ml sample in less than 10 min but requires RBC lysis prior to enrichment (67). Khoo et al. validated this system by isolating CTCs in samples from patients with advanced metastatic breast cancer or NSCLC. CTCs were detected in 10/10 (100%) of patients, with a varied range of CTCs median: 55 CTCs/mL in breast cancer samples, and 82 CTCs/mL in NSCLC samples (68).

Inertial focusing induces mild agitation to trapped cells and allows for the retrieval of viable cells for downstream study. It also eliminates the need for complicated high-resolution imaging methods and the use of costly antibodies.

3.1.2.4. Dielectrophoresis: Dielectrophoresis (DEP) is a phenomenon by which a subject (e.g., cell) is forced to move by the application of divergent electric field gradients due to polarization forces. DEP forces depend on different factors, such as cell-membrane and cytoplasm-charge properties, and size. The electric field induces charges within the cell, forming dipoles. If the cell is more polarizable than the suspending medium, it is attracted towards the regions of higher electric fields, and the motion is called positive DEP (pDEP). Conversely, if the cell is less polarizable than the suspending medium, it is repelled from the regions of higher electric field, and the motion is called negative DEP (nDEP) [69-71]. DEP can be applied to cell separation via two distinct strategies: DEP migration and retention.

• ApoStream. ApoStream applies DEP migration, when the electrical field pushes cells in opposite directions by applying opposing forces on them, through dielectrophoretic field-flow fractionation (DEP-FFF) [72]. Cell separation occurs on particle conductivity property basis. This method has shown 70% recovery rate with captured cell viability >97%. Sample processing takes 1h for 10ml blood sample, although initial enrichment step is required, and purity of resulting product is <1%.

• DEPArray. DEPArray applies DEP retention by trapping single cells in DEP cages generated via an array of individually controllable electrodes [73]. This platform is designed for single CTC capture, allowing downstream gene analysis and sequencing [74].

In all described DEP methods, the isolation is performed independently from cell surface markers, preserving isolated cells and allowing them to be maintained in culture. The major limitations of this method are the low sample volumes, varying dielectric characteristics of cells due to ion leakage, and the need for low electric resistance of the used running medium, which is not always achievable, for example, in samples from diabetic patients [52].

3.1.3. Ctc Detachment/Release from Surfaces: Although acquiring viable cells presents a tempting opportunity for further analysis, culturing, metastasis and treatment response monitoring, and designing innovative therapies in cancer treatment, effective release of CTCs is a challenge that, to date, has not been overcome for many enrichment techniques. Detachment from filters, immunoaffinity chips, and other substrates require the removal of receptorligand interactions and/or focal adhesions, while having little to no impact on cell viability and function. Therefore, a variety of methods have been developed to permit the efficient recovery of cancer cells after capture by using chemical, enzymatic, self-assembly, mechanosensitive, and thermal release mechanisms.

• Chemical release of CTCs. Layer-by-layer deposition of alginate in the presence of Ca+2 coats magnetic beads, and biotin/streptavidin binding attaches EpCAM unique antibodies to the alginate coat. The addition of ethylenediaminetetraacetic acid (EDTA) causes the Ca+2 ions to be sequestered, disturbing the cells' contact with the coated beads and triggering cell release [75, 76].

• Enzymatic- and self-assembly based release of CTCs After capture with aptamer-tagged magnetic beads, cells may be freed up with nucleases, that digest the aptamer, or a sequence complementary to the aptamer, releasing CTCs from cell-aptamer complex [76-79].

• Mechanosensitive and thermal release of CTCs. CTCs captured on antibody-functionalized gelatin base can be released mechanically or by denaturing gelatin nanocoating at temperature above 30C [76, 80].

3.1.4. Ctc Detection: While some physical property-based technologies, such as ScreenCell, ISET, ClearCell FX, and density or gradient centrifugation-based methods can be used to simultaneously enrich and detect CTCs, and other technologies, such as CellSearch and DEPArray, have incorporated automated high-resolution fluorescence imaging into their systems for CTC detection, many of the enrichment methods mentioned above require subsequent verification of the captured cells.

Table1

Principle				Advantages	Limitations	Assay/platform
Label dependent	Positive enrichment	Immuno-magnetic cell separation platforms		• Fast • Multiplexed processing • High capture and enrichment efficiency • Magnetic field not harmful to CTCs • Most widely studied and used technique	• High cost • Influanced cell viability • Dependence on expression of CTC markers • Difficult to be automat	• CellSearch • AdnaTest • MACS • MagSweeper
		Microfluidic-based technologies	Micropost arrays	• Minimal sample preparation • Less sample and reagent demand • Low cost • High sensitivity and efficiency • High cell viability	• Limited sample volume • Slow flow rate • Shear force must be sufficiently low to ensure maximum cell-substrate attachment • Difficult large-scale production • Difficult CTC detection and characterization through high-resolution imaging • Dependence on expression of CTC markers	• CTC-Chip • GEDI
			Surface-based arrays	• Minimal sample preparation • Less sample and reagent demand • Low cost • High sensitivity and efficiency • High cell viability • Easier large-scale production • Easier CTC detection and characterization through high-resolution imaging	• Limited sample volume • Slow flow rate • Shear force must be sufficiently low to ensure maximum cell-substrate attachment • Dependence on expression of CTC markers	• HB Chip • GEM Chip • GO Chip
			Immunomagnetic arrays	• Very high enrichment efficiency Very high purity	• High cost • Poorly investigated • Moderate sensitivity • Dependence on expression of CTC markers	• Ephesia Chip • CTC-iChip
		In vivo technologies		• Higher capture capacity of CTCs • Relatively low requirement of sensitivity • Low false negative rate	• Very long enrichment time • Technology is immature and no data of CTC purity has been reported • High cost • Dependence on expression of CTC markers	• GILUPI • CellCollector
	Negative enrichment			• Do not rely on expression of CTC markers • Can collect the CTCs in an intact form • Easy to be automated	• Typically low purity • Identification of CTCs needs further analysis • Large amount of antibodies needed • High cost	• EasySep • QMS
Label independent	Density-based gradient centrifugation techniques			• Do not rely on expression of CTC markers • High cell viability and intactness • Fast sample processing • Low cost • Reliable	• Challanging commercialization • Low purity • Identification of CTCs needs further analysis • Loss of large CTC and cell aggregates	• Ficoll-Paque • Nycoprep • OncoQuick • RosetteSep
	Microfiltration techniques		Two-dimensional microfiltration systems	• Do not rely on expression of CTC markers • High cell viability and intactness • Rapid processing of large sample volumes • Low cost • High efficiency	• Challanging commercialization • Low purity • Identification of CTCs needs further analysis • Membrane clogging • Different size of CTCs • Difficult to detach CTCs from the filter	• ISET • ScreenCell • MetaCell • CellSieve
			Three-dimensional microfiltration systems	• Do not rely on expression of CTC markers • High cell viability and intactness • Rapid processing of large sample volumes • Low cost • High efficiency	• Challanging commercialization • Low purity • Identification of CTCs needs further analysis • Membrane clogging • Different size of CTCs • Difficult to detach CTCs from the filter	• FaCTChecker • Parsortix • Cluster Chip
	Inertial focusing			• Do not rely on expression of CTC markers • High cell viability and intactness • Fast sample processing • Low cost • Precise • Simple structure • High throughput • Freedom of external field	• Challanging commercialization • Low purity • Identification of CTCs needs further analysis • Complicated principle • Morphological deformation of captured cells	• Vortex Chip • ClearCell FX Chip
	Dielectrophoresis			• Do not rely on expression of CTC markers • Single-cell isolation • High cell viability • High efficiency	• Limited volume • Low purity in some devices • Cell electrical properties can be affected during the procedure • Large number of parameters must be controlled simultaneously	• ApoStream • DEPArray

Table 2

Principle		Advantages	Limitations	Assay/platform
qRT-PCR-based		• Established method • High sensitivity, high specificity, and high dynamic range • Cost-effective • Fast • Can be used for absolute quantification	• Cannot identify novel miRNAs • Medium throughput	• TaqMan individual assays • TaqMan OpenArray • TaqMan TLDA microfluidic cards • miRCURY LNA qPCR • Biomark HD system • SmartChip human microRNA • miScript miRNA PCR Array
Hybridization-based		• Established method • Cost-effective • High throughput	• Typically lower sensitivity, lower specificity, and lower dynamic range than qRT-PCR or RNA sequencing • Difficult to use for absolute quantification • Typically cannot identify novel miRNAs • Require higher amounts of starting material for analysis • Require a pre-amplification step	• Geniom Biochip miRNA • GeneChip miRNA array • GenoExplorer • MicroRNA microarray • miRCURY LNA microRNA array • NCode miRNA array • nCounter • OneArray • Sentrix array matrix and BeadChips • µParaFlo biochip array
RNA sequencing	**High-throughput NGS**	• Detection of both novel and known miRNAs • High accuracy in distinguishing miRNAs that are very similar in sequence, as well as isomiRs • Requires lower amounts of starting material	• High cost, although this is dropping with the introduction of newer versions of the instruments • Use of DNA "barcoding" • Substantial computational support needed for data analysis and interpretation • Sequence-specific biases due to enzymatic ligation • Cannot be used for absolute quantification	• HiSeq 2000 (or Genome Analyzer IIX) • SOLiD • GS FLX+ (454 sequencing) • Ion Torrent • MiSeq • GS Junior (454)
	Smaller-scale NGS			
	Single-molecule sequencing	• Amplification not required • Potential to determine absolute quantification • Higher, less biased throughput	• High cost • Not widely accessible • Higher error rates • Single-molecule real-time approach not yet demonstrated for miRNAs	• tSMS • SMRT

• ICC. Analyte is fixated to a solid support and immunochemically stained – DNA staining with DAPI, Cytokeratin (CK), and epithelial marker staining; CTCs are usually identified as DAPI+/CK+/CD45-, allowing for discrimination between CTCs and WBCs. Deregulated relative expression of proteins may also serve as a target for staining, like in case of pancreatic cancer SMAD4, TP53, and CDKN2A could be evaluated as well. In this case, different staining methods can be employed – using primary antibodies with detectable tags (fluorescent molecules, gold particles), or secondary antibodies that bind the primary antibody and allow cells to be visualized through enzymatic color reactions or by fluorescence. After that the CTCs are detected and counted with a light or fluorescence microscope.

• Fluorescence in Situ Hybridization (FISH). As a result of EMT, expression of certain CKs could be decreased in CTCs. As such, FISH can be employed for identification of additional CTC markers. Aneuploidy, a common manifestation of chromosome instability, is a hallmark of malignant solid tumors [81]. As chromosome numbers are reflected by chromosome 8 centromere (CEP8), FISH aneuploidy detection in peripheral blood provides a fresh perspective for CTC detection [82]. Previous studies have shown centrosome abnormalities in 85 % of pancreatic cancers and FISH has shown CEP8 abnormalities in all of 16 pancreatic cell analytes [83, 84].

• Enrichment-free imaging methods. Enrichment steps has been foregone in some systems in favor of advanced high-speed multi-parameter fluorescence imaging.

  - FASTcell. Somlo G. et al. developed a fiber optic array scanning technology (FASTcell), which uses an array of optical fibers to form a wide collection aperture that allows a wider field of view. The FASTcell systems can scan a sample-containing glass slide at a rate of 25 million cells/min and enables localization and potential detection of CTCs identified by DAPI, CK and CD45 staining without the need for enrichment (85). Limitations of the system include a decrease in image resolution because of the in-

creased field of view, and a need for subsequent verification of potential CTCs by high resolution imaging with an automated digital microscope after the initial screening.

- Cytotrack. In CytoTrack, laser scanner system scans special glass disc (CytoDisc) with spread blood sample, that spins at high velocities, for fluorescently labelled cells against EpCAM at a rate of 100 million cells/min. Incorporated pipette system (CytoPicker) compliments the technology by allowing for retrieval of single cell for downstream analysis in the scanner [86].

• Functional assays. Functional assays exploit aspects of live cellular activity for CTC enrichment and isolation. These include Epithelial Immunospotting (EPISPOT), which analyzes CD45 protein levels, and CAM assays (i.e., Vita-Assay), which analyzes Collagen Adhesion Matrix (CAM) removal and uptake.

- EPISPOT. In EPISPOT, the seeded cells are cultured on plates coated with specific antibodies for 24-48 hours. Proteins secreted by those cells are captured on the antibody-coated membrane. Cells are washed off and the specific protein marker is detected by a second antibody conjugated with a fluorochrome. Immunospots are counted by video camera imaging and computer-assisted analysis: one immunospot corresponds to the fingerprint of one viable marker protein-secreting cell [87]. Though a very promising technique, problems in EPISPOT detection arise when antigen levels are lower or binding efficiency is reduced [88].
- Vita-Assay. This system employs functional cell separation using density gradient centrifugation followed by preferential adhesion of CTC to collagen adhesion matrix (CAM-enrichment) [89]. Vita-Assay produces results with high sensitivity and specificity, but requires over 12 hours for isolation, may fail to isolate more heterogeneous cells due to its biomarker dependence [90], and in a study by Friedlander et al. achieved low purity of (0.5–35%) [91].

• PCR-based detection. Quantitative reverse transcription-PCR (RT-qPCR) targets and enables the quantitative detection of tumor- or epithelial-specific mRNAs for CTC detection. This offers a highly specific detection method that can be combined with various isolation techniques [92], allows multiple markers to be analyzed simultaneously in multimarker assays [93] and compared to immunocytochemistry, is less subjective, and automation is easily achieved. However, challenges are faced when quantifying gene expression in unpurified samples, leading to false-negatives and false-positives [94, 95]. Several pancreatic cancer studies have utilized RT-qPCR for the detection of CTCs [96-98].

2.2. CCFNAS

Cell-free nucleic acids (ccfNAs) are a mixture of single- or double-stranded nucleic acids, released into the blood plasma/serum by different tissues via apoptosis, necrosis, and secretions.

2.2.1. Cell-Free DNA: In 1948, Mandel and Metais [99] were first to report that cell-free DNA (cfDNA) was detectable in human plasma. In 1977, Leon et al. detected increased concentrations of cfDNA in patients with PC, which decreased after therapy in some cases [100]. In 1989, Vasioukhin et al. successfully detected cfDNA with neoplastic characteristics, providing the first evidence suggesting that tumors can shed DNA into the circulation [101]. The clinical potential of cfDNA was recognized in 1994, when Sorenson et al. detected a mutation of the KRAS gene in a sample of plasma from patients with PC [102].

In healthy individuals, cfDNA originates mainly from myeloid and lymphoid apoptotic cells due to the physiological turnover of hematopoietic cells [013, 104] and plasma concentration ranges from less than 10 ng/ml to more than 100 ng/ml [105], while high levels of cfDNA are most often associated with inflammation, trauma, or exhaustive exercise [106]. A subpopulation of cfDNA, which is specifically released from cancer cells, is called circulating tumor DNA (ctDNA). ctDNA represents a small fraction of total cfDNA, varying from 0.01% to 60% [107-110] depending on tumor volume, stage, vascularization, cellular turnover, and response to therapy [107, 111]. Although different mechanisms for ctDNA release in the bloodstream have been postulated, it is agreed upon that ctDNA may originate from apoptotic and necrotic tumor cells, from living tumor cells, or even from CTCs.

Because ctDNA harbors tumor-specific genetic alteration that reflects the genomic status of the malignant cell of origin [111-113], quantification and the detection of tumor-derived genetic aberrations (point mutations, allelic imbalances, microsatellite instability, genetic polymorphisms, loss of heterozygosity, and methylation) both have a direct impact on the clinical utility of ctDNA [114].

3.2.1.1. Collection and Storage: CfDNA is typically obtained from blood plasma or serum but other biological source, such as urine [115-117], cerebrospinal fluid [118-120], and pleural effusion fluid [121-123], have also been used. Although serum contains 2–24 times higher amounts of cfDNA than plasma, serum is not the preferable biological source as it is prone to contamination by genomic DNA (gDNA) from cellular component (typically healthy cells) during the clotting process [124-127].

Wide spectrum of preservative tubes for blood collection are available for preservation of cfDNA quality and quantity. In case, if specimen analysis can be performed within 6h after collection,

standard Ethylenediaminetetraacetic Acid (EDTA) collection tubes are most commonly used and also the most cost-effective; in this case, EDTA preserves cfDNA by inactivating DNase activity [128]. Analysis delay up to 48h demands other media - CellSave Tubes, Streck's Cell-Free DNA BCT or Roche Cell-Free DNA Collection Tubes, which can preserve the cfDNA quality up to 48–96 h after the blood collection, should be used [131-134]. Studies have shown that samples drawn into Roche tubes and Streck tubes are stable for up to 7 days and 14 days, respectively [135-137], and can be conserved at room temperature without affecting DNA quality [130, 137-139].

After collection, a mandatory preparation step for cfDNA analysis is blood sample centrifugation, which is performed at 2000–3000 X g for 10 min [140-146] at 4OC or room temperature [147-154] and allows obtaining cell-free plasma fraction by removing cellular components from whole blood [129, 155-157]. A subsequent second centrifugation of the collected plasma may be used to remove residues of cells and cell debris that may have not been removed during the first centrifugation (158). The double centrifugation procedure consists of a first centrifugation at 1600–3000 X g for 10 min followed by a second centrifugation at 10,000–14,000 X g for 10 min [147-154, 159].

After centrifugation, isolated plasma, not whole blood, can be stored up to a few years [160, 161] at –80OC [162, 163]. Current evidence shows that plasma cfDNA levels are not affected by 2 weeks of storage at –80OC [162], while a storage of 1 year can increase the degradation level of cfDNA by 30% [164].

3.2.1.2. Cfdna Isolation: The extraction of cfDNAs from the sample may be performed using a multitude of extraction kits, which mainly apply two techniques: spin columns and magnetic beads.

- Spin column-based kits. This technique is using spin columns primarily with silica matrix, which can bind DNA fragments from preprocessed plasma sample passing through it. Next following step is removal of contaminants, which is done by a vacuum pump or minicentrifuge [165]. These kits include, but are not restricted to, QIAamp circulating nucleic acid kit (QIA), FitAmp Plasma/Serum DNA Isolation kit (FA), Plasma/Serum Circulating DNA Purification Mini Kit (PSN), Plasma/Serum Cell-Free Circulating DNA Purification Mini Kit (PScfN), and NucleoSpin Plasma XS Kit (NS) [158]. While the spin column technique remains the gold standard of DNA extraction because it is fast, reproducible and can provide a higher yield and purification than other extraction methods, but with the main drawback being necessity of additional equipment – small centrifuge.

- Magnetic bead-based kits. This method employs magnetic beads that bind nucleic acids with elimination of centrifugation step, which reduces the risk of damaging DNA due to sheer forces acting on the sample during centrifugation (165). These kits include, but are not restricted to, QIAsymphony PAXgene Blood ccfDNA Kit (QS), EpiQuick Circulating Cell-Free DNA Isolation Kit (EQ), Maxwell RSC ccfDNA Plasma Kit (RSC), MagMAX Cell-Free DNA Isolation Kit (MM), MagNA Pure Compact Nucleic Acid Isolation Kit I (MPC), and NEXTprep-Mag cfDNA Isolation Kit (NPM) [129, 143, 158, 162, 163, 166-169].

Sorber et al. compared cfDNA isolation efficiency of 5 extraction kits (QIA, PME free-circulating DNA Extraction Kit (PME), RSC, EQ, and 2 consecutive versions of NPM (NPMv1 and NPMv2)) with a digital droplet PCR-based method from 10 plasma samples, including five with a KRAS mutation, from 9 patients (2 with benign pancreatic cysts, 3 with PDAC who underwent surgery, and 4 with metastatic PDAC, including one of whom a second blood sample was taken during follow-up). They found that QIA and RSC performed similarly, with yields remarkably higher compared to PME, EQ, and NPMv1/NPMv2. They found that QIA and RSC performed similarly, with yields remarkably higher compared to PME, EQ, and NPMv1/NPMv2. They were able to detect KRAS mutation in 5/5 patients with cfDNA extracted with QIA and RSC, in 3/5 patients with NPMv2, and in 0/5 patients using the other kits [169].

2.2.1.3. Cfdna Amplification/Quantification: After washing and elution, detection of ctDNAs from the pool of extracted cfDNAs is based on identification of known oncogenic mutations. For PC, global genomic sequencing of 24 patients revealed an average of 63 genetic alterations, the majority of which were point mutations, which defined 12 core cellular signaling pathways [170] and the average number of mutated genes in PC ranged from 26 to 42 [171, 172]. The most prevalent mutated genes in PC, according to literature, KRAS, TP53, SMAD4, CDKN2A and ARID1A [171, 172]. Over 90% of mutation in PC contains mutated KRAS gene and is an early event during carcinogenesis [173-177]. Of KRAS-mutated cancers, 90% will have G12D, G12V, or G12R [175, 178, 179] (40, 36, and 12 percent, respectively, for tissue samples) polymorphism [17, 180]. Interestingly, chronic pancreatitis also shows the KRAS mutation in 10 to 15% of the cases in cfDNA [181].

Two major methods exist for the detection and analysis of ctDNA: a) targeted approaches, which are used to analyze single nucleotide mutations or structural chromosomal rearrangements in specified genomic regions of plasma DNA and to estimate the allelic frequency of a particular mutation within a sample; b) untargeted approaches for the detection of de novo mutations and somatic Copy Number Variations (CNVs) that do not require any prior knowledge of molecular alteration, such as WGS (whole genome sequencing) or WES (whole exome sequencing).

- Targeted approaches.
    - PCR-based technologies. PCR is an in vitro DNA amplification technique. This method allows for

synthesis of specific DNA fragments from trace amounts of target sample using dNTPs, primers, and DNA-polymerase enzyme, which synthesizes complimentary DNA after initial denaturation of sample DNA. PCR typically consists of 20 to 40 temperature cycles yielding double the amount of target DNA each cycle (theoretically reaching 2n copies in n cycles, representing exponential amplification). Amplification products are analyzed at the end of the reaction by gel electrophoresis and detected after fluorescent staining. Currently among the most promising of PCR-based techniques, digital PCR (dPCR), which uses droplets to compartmentalize individual DNA strands, demonstrated detection of highly recurrent hotspot mutations with high specificity and a high sensitivity. dPCR variants include BEAMing (beads, emulsion, amplification and magnetics) and the droplet dPCR (ddPCR) platform.

- BEAMing. In BEAMing magnetic beads in water-in-oil emulsions are used to perform a single molecule amplification by PCR, followed by a flow cytometry to quantify the genetic variants. BEAMing quantifies independently the fluorescently labeled particles, which can detect the rare variants with allele frequency < 0.01%.
- ddPCR. In ddPCR the sample is divided into thousands of droplets representing a partition of single molecules, each of them undergoing a PCR analysis by selected primers against known regions of ctDNA. The molecular alterations are then measured by fluorescent probes, which bind to the amplified region.

While dPCR-based approaches have been shown to detect somatic point mutations with a sensitivity of 1% to 0.001% [182], these technologies need advanced knowledge of the area of interest in order to detect identified mutations and are constrained by scalability for larger studies.

Other promising PCR-based technologies include, but are not restricted to, real-time quantitative PCR (qPCR), Amplification-Refractory Mutation System (ARMS)-based qPCR, competitive allele-specific TaqMan PCR (cast-PCR), and coamplification at lower denaturation temperature PCR (COLD-PCR).

- Targeted next-generation sequencing (NGS)-based technologies. Several NGS-based strategies have been developed for targeting selected, predefined regions of the genome by employing gene panels. NGS detects a wider range of mutation with higher coverage, but with lower sensitivity (approximately 1%) than dPCR.
  - Safe-SeqS (Safe-Sequencing System). First described by Kinde et al., Safe-SeqS is based on a two-step process: a) the assignment of a unique identifier (UID) to each DNA template molecule; b) amplification of each uniquely tagged template to generate many daughter molecules with the identical sequence (defined as a UID family). In this manner, all molecules with the same UID should have the same DNA sequence, and it is possible to identify the "supermutants", namely a UID family in which almost 95% of members show the same mutation [183]. Safe-SeqS reduces the NGS error rate to 1%, increases the sensitivity to rare mutants [183], and yields an error frequency of $1.4 \times 10{-}5$ [184].
  - TAm-Seq (tagged amplicon deep sequencing). First described by Forshew et al. in 2012, Tam-Seq works by amplifying multiple regions in parallel using a two-step amplification process. An initial preamplification step is performed using a pool of the target-specific primer pairs to obtain a representation of all alleles in the template material. After that regions of interest in the preamplified material are selectively amplified in individual (single-plex) PCR, thus excluding nonspecific products. Finally, sequencing adaptors and sample-specific barcodes were attached to the harvested amplicons in a further PCR [185]. This methodology has been used to recognize cancer-specific mutations with allele frequencies as low as 2% and known hotspot mutations in EGFR and TP53 with allele frequencies as low as 0.2 percent [186, 187].
  - CAPP-Seq (cancer personalized profiling by deep sequencing). CAPP-Seq

is a capture-based NGS method for detecting ctDNA that utilizes biotinylated oligonucleotide selector probes to target various DNA sequences. It was developed by Newman and Bratman [187]. The procedure found ctDNA in 50 percent of stage I NSCLC patients and 100 percent of stage II–IV NSCLC patients [187]. Newman et al. created an advanced digital error suppression (iDES)enhanced CAPPSeq in 2016, by applying a molecular barcode solution to their original CAPP-Seq system and integrating an in silico bioinformatics strategy to minimise background noise. When profiling EGFR kinase domain mutations in cfDNA of NSCLC samples, this technique improved the sensitivity of the original CAPP-Seq procedure by 15-fold and registered a sensitivity and specificity of 92 percent and 96 percent, respectively [188].

- PARE (personalized analysis of rearranged ends). PARE starts with identifying unique somatic rearrangements, or breakpoints, in the tumor using next-generation mate-paired sequence analysis. Then it uses PCR to monitor the observed cfDNA rearrangements quantitatively [189]. It has high sensitivity, detecting ctDNA levels of less than 0.001% in patient plasma samples [189]. According to some studies, ctDNA values greater than 0.75 percent could be found in cancer patients with a sensitivity of 90 percent and an accuracy of 99 percent [189]. One disadvantage to this approach is that such rearranged sequences must not be driver events, since they can be lost over the course of the disease and thus may not represent the tumor genome's evolution [189, 190].
- Guardant360 CDx. The Guardant360 CDx is an NGS panel of 54 clinically actionable genes utilizing digital sequencing of ctDNA [191]. The test detects single nucleotide variants in all 54 genes and copy number amplifications in EGFR, ERBB2 (codes for HER2) and MET [192]. Zill et al. used Guardant360 assay to sequence cfDNA in 21867 advanced cancer patients including 867 PDAC samples and reported the genomic findings and the response outcomes. The FDA has approved Guardant360 CDx for comprehensive genomic profiling in patients with any solid malignant neoplasm, as well as a companion diagnostic to identify patients with non-small cell lung cancer (NSCLC) with EGFR alterations who may benefit from treatment with osimertinib [193].
- FoundationOne Liquid CDx. The FoundationOne Liquid CDx is a qualitative NGS-based in vitro diagnostic test that examines 324 genes using cfDNAs obtained from plasma obtained from anti-coagulated peripheral whole blood of advanced cancer patients using optimised high throughput hybridization-based capture technology. studies included >7,500 tests and >30,000 unique variants across >300 genes and >30 cancer types, including PC [194]. The test has been approved by the FDA for general tumor profiling, as well as a companion diagnostic for three lung cancer therapies and a prostate cancer therapy [195].

In a study by Pécuchet et al., comparisons between a targeted NGS approach (Ion Proton) and a microfluidic dPCR (RainDrop) were blindly performed for EGFR and KRAS mutations in 77 plasma samples from patients with pancreatic cancer and 154 with lung cancer. The EGFR/KRAS mutation status was concordant in 95% of samples. They found 11 discordant samples: 2 pancreatic cancer samples (2/231, 0.9%) had a KRAS mutation detected by dPCR only, and 9 lung cancer samples (9/231, 3.9%) had a mutation detected by NGS only [196]. In a similar study, Pietrasz et al. compared the ability of Ion Proton and RainDrop in detecting KRAS mutant ctDNA in samples from 135 patients with PDAC. Likewise, they reported high concordance between the two methods [197]. Interestingly, a combined strategy of prescreening with NGS with subsequent validation with ddPCR (Bio-Rad), has been suggested as cost-effective and efficient method for analyzing ctDNA in PDAC patients [198].

- Untargeted NGS-based technologies. While targeted panels have a high sensitivity and are inexpensive, they can only detect point mutations and indels. Untargeted approaches do not

depend on prior knowledge of molecular alteration and aim at a comprehensive analysis of the tumor genome.

- WGS (whole genome sequencing). WGS allows for the complete genomic profile of tumor DNA to be obtained, including point mutations, indels, rearrangements, and somatic copy number alteration (SCNAs) [199-203]. Although WGS provides abundant information, it is expensive and less sensitive.
- WES (whole exome sequencing). As a popular alternative to WGS, WES can be adopted for sequencing of cfDNA for the identification of clinically actionable mutations [204]. WES is less expensive than WGS by only sequencing exons.

Although untargeted approaches have several advantages over targeted approaches, both WGS and WES are restricted by an overall lower sensitivity, are expensive and require high input sample volume, hindering their application in screening and early diagnosis when the concentration of ctDNA is considerably low.

3.2.1.4. Cell-Free RNA: Since the detection of tyrosinase mRNA in the serum of patients with malignant melanoma [205] and Epstein-Barr virus-associated RNA in nasopharyngeal carcinoma [206] in 1999, many studies have demonstrated the stable presence of cell-free mRNAs (cfmRNAs) in the blood of patients with cancer, even though RNA is easily degraded by endogenous ribonuclease (RNase), the concentration of which is high in these patients.

Although there are numerous studies investigating the correlation between cfmRNA in the bloodstream and several solid cancers [207-209], there exists a limited number of studies that focus on the use of mRNAs in liquid biopsies for PC. Regarding PC, there are several studies investigating cfmRNA as a marker for the detection of CTCs [210-213]. For example, Clarke et al. used an RT-PCR assay to detect EGFR mRNA in the blood of 43 patients with pancreatic, lung (NSCLC), colon, and renal carcinomas. EGFR-positive cells were detected in 2/11 (18%) PC patients, and it lends further support for the use of EGFR mRNA as a marker of CTCs in the blood of patients with certain types of solid tumors [214].

Most RNA-based PC studies have focused on noncoding RNAs (ncRNAs), particularly miRNAs, due to their putative capability to be more informative than mRNA, and high stability in body fluids [215].

Victor Ambros and Gary Ruvkun's laboratories collaborated to discover lin-4 in the nematode Caenorhabditis elegans in 1993, making it the first miRNA found in the nematode [216, 217]. Chim et al. published the first paper on miRNAs as biomarkers in biological fluids in 2008, in an analysis that found placental miRNAs in maternal plasma [218]. In the same year, Lawrie et al. discovered that the levels of miR-155, miR-210, and miR-21 in the serum of patients with diffuse large B-cell lymphoma were dramatically higher than in healthy controls [219]. To date, deregulation of miRNAs in cancer patients' serum has been reported in leukaemia, lymphoma, gastric, colorectal, lung, dental, and squamous cell cancers, as well as breast, ovarian, prostate, pancreatic, and hepatocellular tumors [220].

miRNAs are short (~22 nucleotides (nt) in length) nRNAs that control gene expression by binding to particular mRNA targets and encouraging degradation and/or translational inhibition. As compared to the number of mRNAs and proteins, most organisms have a small number of miRNAs; for example, the human genome is thought to encode 1,000 miRNAs, while the number of mRNAs is usually measured at 30,000. One miRNA, on the other hand, can control hundreds of mRNAs and thus have a significant impact on gene expression networks [221]. Tumor suppressor miRNAs and oncogenic miRNAs (also known as oncomiRs) are two types of miRNAs that play a role in carcinogenesis [222]. The role of the target mRNAs in the tumor initiation process determines the classification. Oncogenes and tumor suppressor genes are normally controlled to achieve an optimum activation/inhibition equilibrium. A tumor suppressor miRNA is identified when downregulation of a particular miRNA increases the activity of a related oncogene. On the other hand, upregulation of an oncomiRwould result in the target tumor suppressor gene being continuously inhibited. The loss of control over specific tumor formation pathways would result from this uncontrolled inhibition. The growth of tumors is aided by deregulation of any of the miRNA forms [223].

Expression patterns of miRNAs are unique to individual tissues and differ between cancer and normal tissues [224, 225]. Some miRNAs are overexpressed or downregulated exclusively or preferentially in certain cancer types. This specificity together with the remarkable stability in a range of specimen types make miRNAs useful biomarkers in cancer diagnosis [226].

Poy et al. first identified specific miRNA signature for the normal pancreas in 2004 [227]. Thereafter, numerous studies on circulating miRNAs have been conducted to identify differentially expressed miRNAs in PC. In a study by Lee et al. 100 miRNAs aberrantly expressed in PC were identified, including microRNAs previously reported as differentially expressed in other human cancers (miR-155, miR-21, miR-221 and miR-222) as well as those not previously reported in cancer (miR-376a and miR-301) [228] (228). MiRNA profiling performed by Zhang et al. identified eight miRNAs that were significantly upregulated in most PC tissues and cell lines, including miR-196a, miR-190, miR-186, miR-221, miR-222, miR-200b, miR-15b and miR-95 [229]. Wang et al. reported that plasma levels of four miRNAs (miR-21, miR-155, miR-196a, and miR-210) can discriminate patients with PC from normal healthy individuals with a sensitivity of 64% and a specificity of 89% [230]. A study by Ali et al. identified miR-2 to be

overexpressed in PC [231]. Morimura et al. identified significantly higher levels of miR-20a, miR-18a, miR-155, miR-22, miR-21, miR-99a, miR-24, miR-185, miR-25, miR-885-5p, miR-191, miR-642b, and miR-196a in the blood of PC patients' as compared to healthy donors [232]. In a study by Li et al., elevated serum miR-1290 expression was demonstrated to exhibit the best diagnostic performance among other upregulated circulating miRNAs in distinguishing patients with low-stage PC from controls [233]. More recently, analysis of panels consisting of multiple miRNAs has been performed for diagnosing PC with relatively high sensitivity and specificity. Schultz et al., for example, recorded that four (miR-145, miR-150, miR-223, and miR-636) and ten (miR-26b, miR-34a, miR-122, miR-126, miR-145, miR-150, miR-223, miR-505, miR-636, and miR-885.5p) diagnostic panels based on whole blood expression could be used to diagnose PC with high sensitivity [234]. In a related study, Kojima et al. observed that a panel of eight miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, and miR-4476) could detect PC with high sensitivity, precision, and accuracy [235]. Although CA19-9 has been widely used as a standard PC serum marker, it is limited to monitoring response to therapy and it is not a sensitive or specific marker for diagnosis [236, 237]. Thus, interestingly, results from a study by Liu et al. showed that a combination of miR-16, miR-196a plasma levels, and CA19-9 was more effective for pancreatic cancer diagnosis, especially in early tumor screening, compared to CA19-9 alone. Additionally, this study demonstrated that increased serum expressions of miR-20a, miR-21, miR-24, miR-99a, miR-185, and miR-191 show high sensitivity and specificity in different stages of pancreatic cancer compared to controls and pancreatitis patients [238]. Deng et al. revealed that serum miR-25 overexpression has strong potential as a novel biomarker for early detection pf PC [239]. Multiple research groups have used miR-18a, miR-21, miR-155, miR-185, miR-196a, miR-210, and miR-212 in pancreas tissue, serum, or plasma to differentiate PDAC from non-cancerous lesions of the pancreas [230, 240-245]. Kong et al. demonstrated that the expression of serum miR-196a was up-regulated in patients with unresectable PC and had potential for predicting cancer progression and prognosis [246]. Additional studies have emphasized an increased level of plasma miR-21 as a biomarker for disease progression and poorer survival [247]. Kawaguchi et al. reported that higher plasma concentration of miR-221 exhibited significant correlation with distant metastasis in PC patients [248], and on top of that several studies have shown that elevated plasma miR-221 and miR-18a levels significantly decreases after tumor resection [232, 248, 249].

Overall, the spectrum of possible miRNA markers for diagnostic and prognostic purposes is very large owing to the continuous discovery of new microRNAs by deep-sequencing technology. Therefore, combinational use of multiple miRNAs is available to enhance the sensitivity and specificity of biomarker panels [226].

Other ncRNAs, such as small nucleolar RNA (snoRNA), small nuclear RNA (snRNA), piwi-interacting RNA (piRNA), and long noncoding RNA (lncRNA) have also been identified as having biological roles and may have considerable potential as novel blood biomarkers, despite the fact that they are mostly unexplored to this day [114].

2.2.1.5. Collection and Storage: High-quality miRNA can be extracted from a variety of cell and tissue types, including cell lines, fresh tissues, Formalin-Fixed Paraffin-Embedded (FFPE) tissues, plasma, serum, urine, and other bodily fluids [221, 250, 251].

Since blood cells (red and white blood cells, as well as platelets) are significant contributors to the presence of miRNAs in the bloodstream and can significantly change specific miRNA levels, whole blood cannot be considered a preferential biological fluid for circulating miRNA identification [252].

Since RNA molecules are released and may alter the true profile of circulating miRNAs during the coagulation process, plasma is usually preferred over serum. On the other hand, plasma includes cellular components that could contribute miRNAs from apoptotic or lysed cells, as well as anticoagulants that could prevent downstream methodologies from functioning [253, 254].

Studies comparing blood plasma and serum in terms of circulating miRNA amount in healthy individuals have obtained controversial results. The average miRNA content in serum was higher than in plasma, according to Wang et al., who hypothesised that this was due to miRNA release from blood cells (such as platelets) during the coagulation phase [255]. Contrastingly, McDonald et al.found that the overall miRNA concentration in plasma was higher than in serum [256], while Mitchell et al. observed that the concentration of the test miRNAs in plasma and serum is comparable [257].

An important factor to consider during plasma isolation in the choice of anticoagulant. Typically, blood collection tubes containing EDTA are recommended over sodium citrate [258, 259] and heparin [260] for PCR-based assays [261]. The use of heparin in blood collection tubes dedicated to RNA analysis has generally been avoided since heparin inhibits the reverse transcriptase and polymerase enzymes used in qRT-PCR [253, 259, 260, 262, 263]. If heparin is used regardless, adequate sample treatment with heparinase prior to analysis is required to increase miRNA detection [264]. Although both EDTA and citrate are appropriate for miRNA detection without any additional treatment [253], EDTA collection tubes should be used instead of citrate collection tubes since citrate can induce hemolysis, and EDTA has also been reported to improve the quality of miRNA expression profiling [265].

The contamination of erythrocyte-specific miRNAs will inhibit the quantification of plasma/serum miRNAs, according to several studies [265-267]. According to a recent study, 58 percent of tumor-associated circulating miRNAs identified in the literature

were highly expressed in blood cells, and hemolysis during blood collection and processing has a substantial impact on the miRNA content in plasma/serum [266], altering circulating miRNA levels by up to 50-fold [252]. Thus, identification of hemolyzed samples remains a crucial issue in miRNA research [268]. Several methods, including spectrophotometry, which measures the main oxyhemoglobin peak absorbance at wavelength (λ) = 414 nm [267], and detection of erythrocyte-specific miRNAs, such as miR-451, have been recently proposed [269, 270]. After the identification of hemolyzed samples, removal of these samples from miRNA analysis should be considered.

Other than sample collection and transportation, one of the most important stages in the pre-analytical phase is serum and plasma recovery by centrifugation. Different centrifugation variables, such as applied force, centrifugation time, temperature, acceleration, and so on, are commonly used to isolate plasma and serum samples [271]. Different centrifugation protocols can produce platelet-rich or platelet-poor plasma/serum, thus influencing the levels of circulating mRNA [262, 272]. For example, prolonged, high-speed centrifugation may cause hemolysis and therefore release of miRNAs from platelets, while brief, low-speed centrifugations may lead to poor separation of serum or plasma from cellular components [273]. Furthermore, several research groups have proposed a double phase of centrifugation for plasma separation to reduce platelet contamination (despite minor discrepancies in centrifugation speed and time between protocols). [269, 272, 274]. Differential centrifugation and size-exclusion chromatography has identified two populations of miRNAs in blood [257]. Most miRNAs are incorporated into the plasma with various proteins (such as HDL and AGO family proteins), whereas a minority of specific miRNAs were predominantly associated with membranous extracellular vesicles (such as apoptotic bodies, shedding vesicles and exosomes), each of which requires different centrifugal separation protocols [275].

Minimal to no differences have been found between fresh specimens and samples stored long-term at −80 °C, even after up to 8 freeze/thaw cycles [129, 257, 272, 276, 277]. After repeated freeze/thaw processes, miRNAs are less stable in serum than in plasma (278–280), and the levels of each c-miRNA are changed in a different manner [280].

3.2.1.6. Mirna Extraction

3.2.1.7. As mentioned before, miRNAs are relatively stable and can be reliably measured in plasma and serum, however, their isolation is challenging, because of their low concentration and potential contaminants from blood [260]. Moreover, the two stable miRNA populations discussed above can possess different levels of resistance and vulnerability to particular isolation methods [255].

3.2.1.8. The principles for isolating miRNA are, normally, the same as for isolation of total RNA, except that miRNA isolation protocols are often slightly modified to retain (and sometimes to enrich) the small RNA fraction [250].

3.2.1.9. In general, there are three major types of circulating miRNA extraction methods: 1) phenol-based approaches that use organic solvents, phase isolation, and precipitation to recover RNA; 2) methods that use phenol/chloroform to separate RNA from other biomolecules and then a column for RNA adsorption; and 3) phenol-free methods that use a lysis buffer to release RNA in the solution and then a column for RNA recovery [215, 281]. Several commercially available kits for miRNA recovery have been developed and widely used.

3.2.1.10. Although no consensus exists regarding the best extraction method, despite several researcher groups having performed head-to-head comparisons, the general agreement is that different isolation methods provide different quality of miRNAs in terms of purity, composition, and yield [215, 256, 282-284].

3.2.1.11. McDonald et al. measured four plasma/serum miRNAs with four different extraction methods and found that mirVana PARIS kit and miRNeasy Mini Kit had the highest mean yield for miR-15b and miR-16 and for mi-24 and cel-miR-39, respectively (256Moret et al. utilized fresh and frozen plasma samples to equate TRIzol-LS, mirVana PARIS kit, miRNeasy Serum/Plasma kit, and a mixture of TRIzol-LS and mirVana. Due to the existence of organic/phenolic pollutants in TRIzol-extracted RNA, column-based methods should be favoured over TRIzol-based methods, according to the report. In this context, miRNeasy Serum/Plasma Kit obtained the highest RNA concentration among the compared commercial column-based kits [274]. Although considering recent studies, where column-based extraction methods performed superiorly to TRIzol-based methods, several publications have created discrepancy regarding the efficiency of different commercially available miRNA isolation kits. For example, in a study by Sourvinou et al., the mirVana PARIS kit and miRNeasy Mini Kit generated the largest recovery yield for a spike-in miRNA as compared to TRIzol extraction, with the first kit also outperforming miRNeasy [274], which was in contrast to a study by Kroh et al., which demonstrated that miRNeasy kit provides 2-to 3-times higher RNA yield compared to mirVana. The possible explanation of difference in results may stem from the fact that in Khor's work, both mirVana PARIS and miRNeasy kit recommended protocol were modified [262]. In another study, Li et al. compared RNA yield and amplification efficiency of plasma RNA using seven different commercially available kits (i.e., RNAdvance miRCURY, MagMAX, Quick-RNA, DirectZol, miRNeasy, and mirVana kits). All of the other kits recovered synthetic RNAs (≥50% recovery). The recovery of MagMAX, Quick-RNA, DirectZol, miRNeasy, and mirVana kits was comparable regardless of RNA length, while RNAdvance and miRCURY kits had

biases in terms of isolated RNA length. Overall, the miRNeasy package outperformed the competition in terms of miRNA purity and recovery [285]. Vigneron et al. compared three commercially available isolation kits (miRNeasy Serum/Plasma, mirVana, and NucleoSpin miRNA Plasma) and found that the NucleoSpin kit produced higher concentrations of miR-16-5p than the miRNeasy and mirVana kits [286], while in a study by Tan et al. the NucleoSpin kit demonstrated the lowest obtained concentration of spike-in miRNAs controls from plasma samples among a total of five commercially available miRNA extraction kits (i.e., miRCURY, miRNeasy, NucleoSpin, mirVana, and Norgen) [284].

3.2.1.12. Mirna Detection: Three major approaches are currently well-established to identify miRNAs and quantify the expression levels of c-miRNAs in plasma/serum samples: quantitative reverse transcription PCR (qRT-PCR), hybridization-based methods (for example, DNA microarrays) and high-throughput sequencing (that is, RNA-seq) (287,288). Each method has its own advantages, disadvantages, and limitations.

- qRT-PCR-based methods. One major approach relies on reverse transcription of miRNA to cDNA, followed by qPCR with real-time monitoring of reaction product accumulation (known as 'realtime PCR') [221]. qRT-PCR is the "gold standard" to quantify cfmiRNAs and cellular miRNAs and is often intended as a single miRNA assay. However, commercially available customizable plates and microfluidic cards (e.g., TaqMan individual assays, TaqMan OpenArray and TaqMan TLDA microfluidic cards, miRCURY LNA qPCR, Biomark HD system, SmartChip human microRNA, miScript miRNA PCR Array) have been developed either to examine a small set of miRNAs or to provide more comprehensive coverage [221]. Advantages: 1) established methods; 2) high sensitivity, high specificity, and high dynamic range; 3) cost-effective; 4) speed; 5) can be used for absolute quantification. Limitations: 1) cannot identify novel miRNAs; 2) limited by medium throughput (i.e., can only analyze a small number of samples) [226, 252, 289, 290].

- Hybridization-based methods. Microarrays were among the first methods to be used for parallel analysis of large numbers of miRNAs [221]. The general detection process begins with enzymatic or chemical fluorescent labelling of targets followed by subsequent hybridization to capture probes on the microarray plate, and the signals are detected using a scanner [291]. Several commercial miRNA microarray platforms (e.g., Geniom Biochip miRNA, GeneChip miRNA array, GenoExplorer, MicroRNA microarray, miRCURY LNA microRNA array, NCode miRNA array, nCounter, OneArray, Sentrix array matrix and BeadChips, µParaFlo biochip array) are available. Advantages: 1) established methods; 2) cost-effective; 3) high throughput. Limitations: 1) typically lower sensitivity, lower specificity, and lower dynamic range than qRT-PCR or RNA sequencing; 2) difficult to use for absolute quantification; 3) typically cannot identify novel miRNAs; 4) require higher amounts of starting material for analysis; 5) require a pre-amplification step [226, 252, 289, 290].

- RNA-seq. As a starting material for next-generation sequencing platforms, a small RNA–cDNA library is developed by reverse transcription of small RNA fractions after enzymatic ligation of adaptors to both ends of mature miRNAs. Following amplification, the samples are subjected to massive parallel sequencing analysis [292]. The abundance of identified miRNAs and candidate novel transcripts is determined by mapping the observed short reads to the reference genome. Read density or cumulative reads numbers, which represent how many reads are mapped to each genomic region, are often used to quantify expression levels. [292]. RNA-seq platforms include high-throughput NGS platforms (e.g., HiSeq 2000 (or Genome Analyzer IIX), SOLiD, GS FLX+ (454 sequencing)) and smaller-scale NGS platforms (e.g., Ion Torrent, MiSeq, GS Junior (454)). Advantages: 1) detection of both novel and known miRNAs; 2) high accuracy in distinguishing miRNAs that are very similar in sequence, as well as isomiRs; 3) requires lower amounts of starting material. Limitations: 1) high cost, although this is dropping with the introduction of newer versions of the instruments; 2) use of DNA "barcoding"; 3) substantial computational support needed for data analysis and interpretation; 4) sequence-specific biases due to enzymatic ligation; 5) cannot be used for absolute quantification. Single-Molecule Real-Time (SMRT) or single-molecule sequencing (SMS) methods offer quicker and less skewed results than the methods described above, but they are currently plagued with higher error rates and costs, and are not readily available [293].

4. Conclusions

Over the last couple of decades' extensive progress has been made in understanding the complexity of molecular and genetic mechanisms of PC and cancer overall. However, several problems, including early detection, real-time disease monitoring, improved management, and possible targeted therapy, remain and are as relevant as ever. This has prompted the search for reliable and non-invasive diagnostic platforms.

Blood-based biomarker evaluation using liquid biopsy is a novel, attractive, and potentially valuable tool in this regard. Similar to traditional biopsies, liquid biopsy of CTCs and cfNAs offers the entire range of information regarding a tumor in a single point in time. Yet, unlike traditional biopsies, with liquid biopsies it's possible to acquire additional samples repeatedly, and track track status of a tumor. However, several issues remain to be tackled before applying liquid biopsies in a clinical setting.

One of the most important hurdles to overcome is the lack of standardized and institutionalized methodologies for sample collection, and biomarker detection and assessment. As demonstrated above, sample types and volume, storage conditions, targeted

biomarkers, detection approaches and assessment techniques have varied greatly among countless research groups.

CTCs have shown enormous potential for different types of cancer, including PC. CTCs can be used to understand the metastatic spread of cancer and have shown promise as a biomarker for early detection, identification, and prognosis. Moreover, CTCs can be used to understand tumor heterogeneity and mutational landscape and help guide treatment and monitor responses to therapy. Although, CellSearch remains as the only FDA approved method for CTC detection in breast, colorectal, and prostate cancer to date, countless platforms employing different enrichment/detection methodologies have been developed, which have shown comparable or superior performances to CellSearch. Overall, there is a broad heterogeneity in the available platforms, none of which are perfect. Moreover, the wide variety of platforms and reported results in the literature makes a comparison difficult.

Similarly, to CTCs, ctDNA captures the heterogeneity across tumor sites and can be utilized to understand tumor cells and mutations, however, ctDNA provides an additional opportunity for real-time treatment response and relapse monitoring and shows great potential in drug-resistance research. Although several ctDNA amplification/quantification methods exist, all of them have advantages and limitations. An interesting concept suggested in several studies would be a combined strategy of prescreening with NGS-based technologies with subsequent validation with PCR-based technologies, which would even out the pros of cons of separate methodologies and provide a cost□effective and efficient method for analyzing ctDNA.

Recent studies have provided considerable evidence showing the use of miRNA expression profiles in the diagnosis and prognosis of pancreatic cancer; moreover, miRNAs are also emerging as promising targets for cancer therapy. In the future, profiling methods such as RNA-seq that have the potential to detect all classes of RNA are likely to shed light on the entirety of the transcriptome, while becoming more available and cost-effective by introducing newer versions of the instruments. Although, miRNAs remain as the focal point of most RNA-based PC studies, other ncRNAs may provide essential benefits in cancer studies and their role as novel biomarkers should be explored further.

Standardization of different methodologies and accumulation of results under equal conditions in large-scale studies should be emphasized in future research. Furthermore, development of sensitive, specific, effective, and cost-efficient strategies that combine immunological, biophysical and genetic methodologies in a single device is an appealing aim in the field of liquid-biopsy that would potentially change the face of cancer diagnostics and treatment.

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