Methods for the Detection of Circulating Biomarkers in Cancer Patients

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Abstract

Liquid biopsy has emerged as one of the main pillars for personalized oncology. The term englobes body-fluid samples which contain tumor-derived material such as circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and circulating extracellular vesicles (cEVs). Potential clinical application of liquid biopsy analyses includes cancer screening, detection of minimal residual disease and recurrence, therapy selection, and evaluation of acquired resistance. Despite the great developments of technology focused on circulating biomarkers characterization only cfDNA testing is nowadays implemented for the therapy selection in some advanced tumors. This can be partially explained by the fact that there is still a lack of global standardization of procedures both in the pre-analytical and analytical steps. In the present chapter, we summarize the different strategies for addressing the study of liquid biopsy taking into account their pros and cons to be

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applied in a clinical context and we also discuss the main technical and clinical challenges in the field of circulating biomarkers and personalized oncology.

Keywords

Circulating tumor biomarkers · Liquid biopsy · Personalized oncology · Circulating tumor cells · Extracellular vesicles

21.1 Introduction: Clinical Relevance of Liquid Biopsy for Personalized Oncology

Advances in molecular biology have clearly changed the way to manage cancer, allowing us to coin the term "personalized oncology." This term refers to the individualized diagnosis, treatment and disease monitoring based on the specific molecular characteristics of each tumor [1]. In this context, the analysis of tissue remains the gold standard for making cancer diagnosis and characterization, but this procedure has limitations such as access difficulty, mainly to biopsy metastasis, the lack of representativity of the tumor heterogeneity and the possibility of follow-up the clonal evolution under the therapy pressure [2]. Therefore, the analysis of circulating biomarkers has emerged as a key tool reaching more personalized management of cancer patients [3].

The term "liquid biopsy" was first used by Pantel and Panabiéres in 2010 to refer to the analysis of circulating tumor cells (CTCs) in blood from cancer patients [4]. Currently, the concept is generally employed to talk about the sampling and analysis of tumor-derived material present in different body-fluids, mainly blood, but also other body fluids such as saliva, urine, cerebrospinal fluid, ascites, or pleural effusions [3]. Circulating biomarkers present in fluid biopsies comprise CTCs, circulating tumor DNA (ctDNA), circulating cell-free RNA (cfRNA), circulating extracellular vesicles (cEVs), and other circulating elements such as immune cells or tumor-educated platelets, among others. These circulating biomarkers have shown great potential for cancer screening, molecular diagnosis, predicting the patients' prognosis, assessing minimal residual disease after surgery, the therapy selection/monitoring and for characterizing the mechanisms of resistance in different tumor types [3].

In the clinical setting, ctDNA has been recently implemented to analyze driver mutations that condition the response to targeted therapies and some molecular tests have been approved as a companion diagnostic in the context of advanced breast, non-small-cell lung (NSCLC), prostate and ovarian tumors. NSCLC was the first tumor in which the analysis of ctDNA for tumor phenotyping was included in the guidelines. Thus, genomic alterations in *EGFR, ALK, ROS1, BRAF, MET*, and *RET* must be analyzed in tissue or ctDNA to determine the appropriate treatment [5] (National Comprehensive Cancer Network (NCCN). Non-Small Cell Lung Cancer. Version 4.2020. NCCN Clinical Practice Guidelines in Oncology. Accessed January 6, 2020. nccn.org/professionals/physician_gls/pdf/nscl.pdf). In advanced breast cancer, NCCN guidelines recommend the analysis of *PIK3CA* status using tissue

samples or ctDNA to guide the administration of Alpelisib [6] (National Comprehensive Cancer Network (NCCN). Invasive Breast Cancer. Version 4.2020. NCCN Clinical Practice Guidelines in Oncology. Accessed January 6, 2020. nccn.org/professionals/physician_gls/pdf/breast.pdf). Also, in advanced colorectal cancer, several studies have highlighted the feasibility of interrogating *RAS* and *BRAF* status to guide anti-EGFR therapy [7, 8]. The value of ctDNA analyses for the response assessment has been reported in many tumors such as melanomas or breast cancer, and for different targeted and non-targeted therapies [9–12]. Plasma ctDNA has been also explored as a prognostic biomarker to stratify the risk of recurrence in localized tumors after curative surgery, indicating those patients with a need of more intensive adjuvant therapy [13, 14]. In the same line, cfDNA studies have shown value as diagnostic tools. For this purpose, the identification of methylated patterns has been successfully applied to detect the presence of different tumor types [15]. Actually, the detection of methylation in the promoter region of the *SEPT9* gene in plasma cfDNA (Epi proColon test) represents the first blood-based test approved by the FDA for the screening of CRC [16, 17].

CTC research is considered the start-point of the liquid biopsy field. Early in the formation and growth of a primary tumor, cells are released into the bloodstream. Several groups are studying the clinical benefit of CTC monitoring [18]. CTCs have been validated as a prognostic marker in metastatic breast cancer and other solid tumors such as prostate, colorectal, and lung cancer, showing even more accuracy than conventional imaging methods for response evaluation [19]. However, there are still technical challenges to using CTC monitoring to detect minimal residual disease in patients at early stages. On the other hand, the molecular characterization of CTCs is of great interest to guide the selection of targeted therapies since it allows clinicians to have a dynamic view of different molecular targets such as ERBB2, EGFR, AR or PD-L1, among others [19, 20].

On the other hand, the field of cEVs and miRNAs is continuously increasing due to their relevant function during the process of carcinogenesis and tumor spread. They can be detected in different body fluids and have shown great potential as cancer biomarkers for diagnostic and prognostic purposes. In particular, EVs contain both proteins and nucleic acids that can serve to increase tumor detection sensitivity [21, 22]. However, one of the main limitations for cEVs based approaches is the absence of tumor-specific markers to identify the tumor-derived EVs. Only hot shot protein 60 (HSP60) and Glypican-1 (GPC1) have been identified as potential identifiers for detecting EVs from colorectal, pancreatic, and breast cancer detection [23]. Thus, studies based on cEVs and in fluid samples are still in an infancy stage and further validation in clinical studies is required to clarify their impact on precision oncology.

Finally, other blood elements such as tumor-educated platelets (TEPs) or the different circulating immune cells have been evaluated as liquid biopsy biomarkers for prediction or monitoring of therapy responses, but their application is still far from the clinical routine [24, 25].

In the present chapter, we summarize the analytical strategies developed to interrogate the presence of CTCs, ctDNA, and cEVs as the main type of circulating markers with clinical interest for personalized oncology.

21.2 Strategies for CTCs Isolation and Characterization

Circulating tumor cells are present in the bloodstream at a low proportion, about 1 CTC per 10^6–10^7 leukocytes [26] and with a very short half-life (1–2.4 h) [27, 28]. Due to the low concentration in blood, CTCs identification and characterization require methods with high analytical sensitivity and specificity [28], being their isolation technically challenging. In the last years, a high number of promising CTC-detection technologies have been developed, focused on the differential features between CTCs and the surrounding normal blood cells, including physical properties (size, density, electric charges, deformability) and biological properties (cell surface protein expression, viability) [29]. Here, we divide the different methods to enrich CTCs into two main principles: antigen-dependent methods and antigen-independent methods (Fig. 21.1).

21.2.1 CTCs Isolation Strategies

21.2.1.1 Antigen-Dependent

Antigen-dependent isolation approaches are the most common methods employed and they are based on the presence of specific surface markers by CTCs (called positive enrichment) or by blood cells (negative enrichment).

Positive enrichment, the most employed strategy is usually carried out using antibodies that recognize epithelial cell adhesion molecule (EpCAM) [29] conjugated with magnetic nanoparticles**.** Among the current EpCAM-based technologies, CellSearch® system (Menarini, Silicon Biosystem, Bologna, Italy) [30] has become the "gold standard" for the CTC-detection methods. CellSearch® system employs anti-EpCAM-coated ferrofluid nanoparticles for the selection of EpCAM positive cells. Next, an immunostaining step discriminates CTCs from leukocytes based on the positive expression of cytokeratins and the absence of CD45 staining together with morphologic criteria. Although a high number of alternatives that employ magnetic nanoparticles conjugated with anti-EpCAM antibodies are also available [31], until now CellSearch® system is the unique method approved by the Food and Drug Administration (FDA) for clinical use in metastatic breast, prostate and colorectal cancer [32–34].

Recently, new positive enrichment methods are being developed, in which the specific surface markers are immobilized on the surface of microfluidic chips [31] to increase the contact between the cells and, therefore, to enhance capture efficiency. However, the isolation in all these approaches is based on the EpCAM expression, therefore they are not able to detect CTCs that show no EpCAM expression, for example, CTCs of non-epithelial tumors such as sarcomas or CTC that have undergone epithelial-to-mesenchymal (EMT) transition [35, 36].

Negative enrichment methods employ magnetic nanoparticles conjugated with antibodies against the common leukocyte antigen CD45 [37] or other antigens expressed in blood cells and represent a good alternative to avoid the limitations of the EpCAM-dependent isolation. They allow isolating CTCs independently of any CTC surface marker expression however due to the low proportion of CTCs and

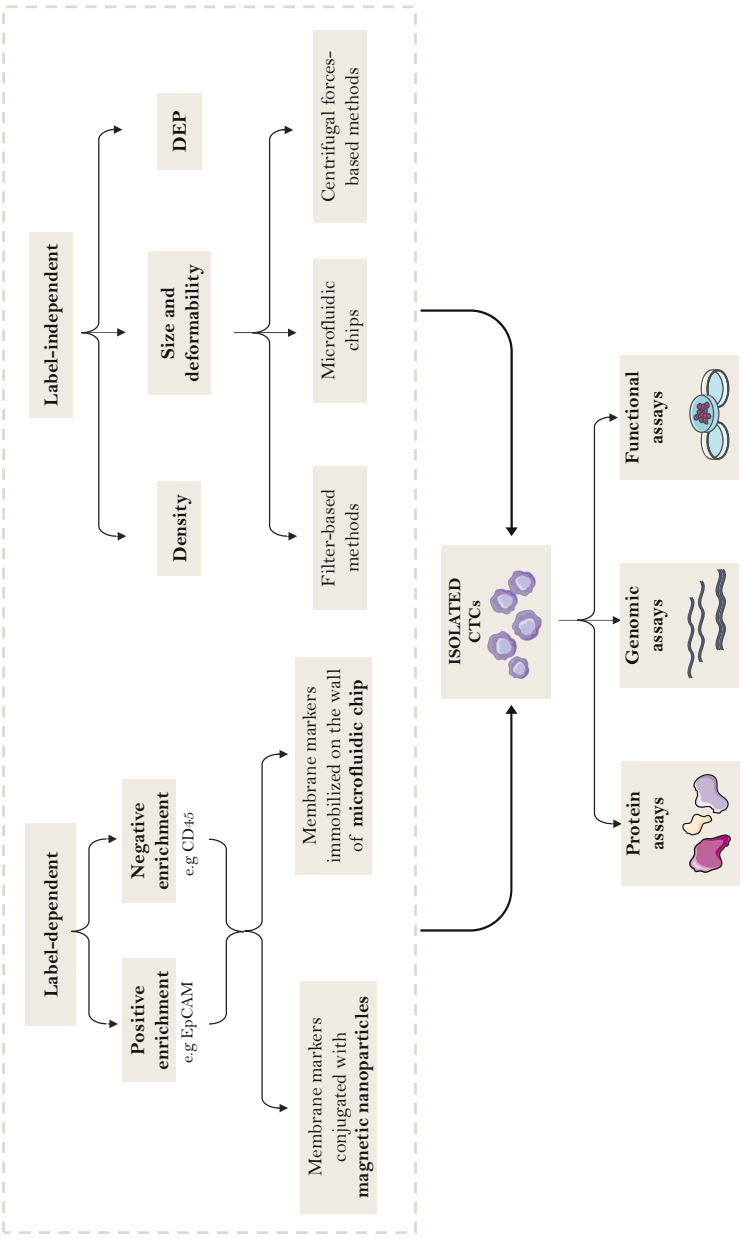

Fig. 21.1 Different strategies for CTCs isolation and characterization. DEP, dielectrophoresis

the recent observation that CTCs travel into the bloodstream coated with blood cells [38], the resulted recovery rate is often relatively low [31].

21.2.1.2 Antigen-Independent

Antigen-independent methods are based on physical properties of CTCs such as density, electric charges (DEP, dielectrophoresis), size, and deformability, among others. The principal advantage and difference with the antigen-dependent methods are that they do not require specific surface markers on CTCs, so they also allow the isolation of CTCs with a low epithelial phenotype. Density-based methods were the first techniques developed. These methods allow to processing of high volumes of blood (about 25 mL) with a quick processing time; however, they generally show a low efficiency and purity of the sample obtained [31]. The size-based methods are the most common. They are based on the fact that tumor cells are larger than blood cells [39, 40] and, therefore, they can be isolated using filter-based strategies (such as ISET assay (Rarecells Diagnostics, Paris, France) [41]), microfluidic chips (such as Parsortix system (Angle, UK) [42]) and methods based on centrifugal forces [43]. The different charges between blood cells and CTCs can also be employed in their isolation. DEP field forces are employed to move CTCs independently to other blood cells, being a highly specific method [44].

Antigen-independent methods are generally easy to implement, however they depend on the availability of advanced materials or assistive engineering technologies for better clinical application [20]. Interestingly, new methods combining antigen-based capture with the advantages of microfluidics methods, such as CTC-iChip are being developed for increasing the isolation efficacy [45]; however, nowadays a robust and standardized platform to capture CTCs for clinical application remains a challenge.

Finally, it is important to remark that small volumes processed with the methods here described may be a serious limitation for the detection of these rare events, especially in cancer patients without metastases, in which the number of CTCs is expected to be very low. To solve this problem, some “in vivo” approaches such as GILUPI Nanodetector® [46] or Diagnostic leukapheresis (DLA) can be employed [47].

21.2.1.3 Single CTCs Isolation

After enrichment, the CTC fraction usually still contains a substantial number of leukocytes [29]. This background of leukocytes is seen in all CTC enrichment platforms being the posterior molecular analyses of CTCs a challenge. Therefore, after the detection of CTCs, there are some platforms that allow the isolation of pure CTCs at a single level by the use of micromanipulation or via dielectron force manipulation (such as the DEPArray system (Menarini, Silicon Biosystem, Bologna, Italy), among other strategies [48].

21.2.2 CTCs Characterization

After enrichment, a variety of approaches can be employed to distinguish and characterize the CTCs. Analysis of CTCs at the proteome, genome, and transcriptome level provides valuable information about the molecular heterogeneity of these cells and more precise characterization of the disease [49]. Furthermore, CTCs can also be used for functional studies "in vitro" and "in vivo" models, allowing to study the biological process and characteristics of CTCs as well as test the response to different therapies.

21.2.2.1 Protein Expression

After enrichment, immunohistochemical or immunofluorescent (IHC or IF) assays can be used to distinguish CTCs from nonspecifically captured cells. The most commonly used antibodies are cytokeratins combined with markers such as CD45 that identify the background blood cells [50]. In addition, other surface proteins can be analyzed by IF that could be key candidates for targeted therapies. Thus, certain protein expression in CTCs has been studied, such as PD-L1 in lung cancer patients [51] and ER and HER2 in breast cancer, among others [52]. In addition, a microfluidic western blot technology for proteomic phenotyping of CTCs has also been developed, however, the number of proteins included is scarce [53].

21.2.2.2 Genomic Analyses

Genomic analyses at the DNA level allow for the detection of driver mutations in enriched CTCs samples [54]. Real-time polymerase chain reaction (RT-PCR), digital droplet PCR (ddPCR), and next-generation sequencing (NGS) are the most employed methods; however, results obtained present a low sensitivity because of "masking" the tumor profile by wild-type DNA from leukocytes [55].

More comprehensive analyses can be carried out using CTCs isolated at a single level followed by amplification of the whole genome, providing a valuable tool in order to know more about the heterogeneity of the tumor [55], as well as to predict the response of therapy. For example, the genomic profile of single CTCs can be employed to generate a copy number abnormalities (CNA)-based classification that can differentiate chemosensitive from chemorefractory patients in small cell lung cancer [56]. In contrast, technical limitations of CTCs isolation efficiency and the difficulties of performing whole-genome analyses on rare cells have limited the number of CTCs genomic profile studies [50] in comparison with cfDNA studies.

In another hand, gene-expression analyses in CTCs could be useful to know the nature and extent of tumor heterogeneity, linking phenotypic differences with genetic and epigenetic aberrations [57]. However, RNA is less stable and more difficult to preserve in comparison to DNA. Hence, RNA degradation constitutes a major challenge for CTCs analyses in multicenter clinical studies. Until now few single-CTCs transcriptome studies have been performed.

21.2.2.3 Functional Analyses

CTCs can also be characterized in functional studies. Some strategies for CTCs isolation offer the possibility to isolate viable CTCs and apply innovative culturing technologies to study fundamental characteristics of CTCs such as invasiveness, kinetic activity, and responses to different therapies [28]. In vitro models have been successfully reported, however, to obtain a cell culture high number of CTCs are required, and few patients have the CTCs number required. So far, it has been possible to obtain a short-term and long-term expansion of CTCs from breast, colorectal, lung, and prostate cancer, among others [58]. These cell lines can be used for drug screening, but the process of establishing these cell lines is not yet rapid enough to enable studies to inform treatment decisions for the donor patient [59].

In another hand, CTC-derived explant (CDX) models have emerged recently. For their generation, CTCs are enriched from the blood of patients and injected into immunocompromised mice to generate tumors and expand the initial material. Thus, CDXs constitute a valuable tool for clinical drug development [60]. These CDXs have been successfully generated in small cell lung [61], colorectal [62], breast [63], and prostate cancer [64], among others. Their main limitation is the time required to develop the CDXs models, usually several months.

21.3 Strategies for cfDNA/ctDNA Characterization

Although the mechanisms by which this tumor DNA reaches the circulation are not fully described, there are currently two accepted processes to explain its release. The passive mechanism implies that cells release DNA into the circulation as a consequence of cell death phenomena (necrosis or apoptosis). In this sense, the usual size of ctDNA is 167 bp, in line with the size of nucleosomal DNA that normally appears in apoptotic phenomena, but fragments that represent nucleosomal dimers or trimers can also appear. The second mechanism that allows the appearance of DNA in circulation is associated with an active release by tumor cells and may constitute a communication mechanism, although this process is not known in detail [65, 66]. Once in circulation, cfDNA is eliminated in the liver, kidney, and spleen, with an approximate half-life in the circulation of 16 min [66]. In cancer patients, ctDNA is found in a variable but normally very low (1–0.01%) percentage in relation to all cfDNA, which is usually less than 1 ng/μL. As already mentioned, this fraction varies depending on the stage, location, or degree of vascularization of the tumor, but also other physiological conditions such as tissue damage or marked exercise. Thus, tumors with multiple metastatic locations and highly vascularized will have higher levels of ctDNA [67].

21.3.1 cfDNA Isolation and Quantification

Before describing detection techniques, it is important to focus on cfDNA isolation methods. For plasma isolation, the most recommended protocol includes double

centrifugation: the first centrifugation at 1200/1600×*g* for 10 min and then the second centrifugation at 5000/6000×*g* for another 10 min to ensure the elimination of any cellular debris. Once the plasma is isolated, it must be stored at −80 °C until its use and avoid several processes of freezing and thawing of the sample [51, 68]. For cfDNA isolation, we can use traditional extraction methods such as phenol-chloroform or alcoholic precipitation, which normally have very high yields. However, these approaches require more processing time than commercial extraction kits, which are mainly based on affinity columns, magnetic particle capture, capture by filtration, and methods based on the phenol-chloroform strategy. There are several studies that have compared the efficiency of different commercial isolation kits [68, 69]. The main differences observed between them are the recovery efficiency and the size of the isolated fragments. In some studies, the recovery results have been favorable to kits that use magnetic particles, such as MagNA Pure (Roche Diagnostics, Basel, Switzerland), compared to those that use affinity columns. One of the most widely used column-based isolation kits is the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), which has shown cfDNA recoveries of 80–90%. Another column kit that has shown good results is the NucleoSpin Plasma XS (Marcherey Nagel, Düren, Germany), which is capable of recovering DNA fragments >50 bp in very small volumes [70].

Four main strategies are commonly used to characterize the concentration and size of isolated cfDNA: spectrometry, fluorometry, electrophoresis, and PCR-based techniques. The most specific and sensitive of the four options is the assessment of cfDNA quantity by PCR-based strategies to detect conserved sequences in the genome [71].

21.3.2 ctDNA Characterization

CfDNA analyses allow to identify mutations of interest (including resistance mutations) to guide the therapeutic decisions in several cancer types [72], the detection of cancer at early stages and the presence of minimal residual disease [59, 73, 74] as well as the assessment of the tumor mutational burden [75].

Thus, after isolation, ctDNA can be assessed to investigate for molecular alterations by two different approaches: single-gene analysis (PCR-based methods) or genome-wide analysis (through NGS strategies) (Table 21.1). During the last years, the development and improvement of these technologies has allowed the implementation of ctDNA analyses into the clinical routine. Thus, four tests have been approved by the FDA for clinical use. Two PCR-based assays, the therascreen® *PIK3CA* PCR Kit (Qiagen, Hilden, Germany) for breast cancer patients [76] and the Cobas® *EGFR* Mutation Test v2 (Roche Molecular Systems, Inc., Basel, Switzerland) for NSCLC patients [77] and two NGS-based assays, the FoundationOne Liquid CDx test (Foundation Medicine, Inc., MA, EEUU) for patients with solid malignant neoplasm [78] and the Guardant360 CDx (Guardant Health, Inc., CA, EEUU) for NSCLC patients [79]. All of the kits allow identifying patients who may benefit from treatments based on specific targeted therapies.

Table 21.1 Summary of the most common strategies for the ctDNA analysis

Method	Platform	Sensitivity	Specificity	Limitations
PCR-based	RT-PCR	1–0.1%	99%	Detects only known mutations; medium sensitivity
	ddPCR	0.01–0.1%	100%	Detects only known mutations; limited in multiplexing
	BEAMing	0.01%	100%	Detects only known mutations
Genome wide analyses	NGS panels	> 0.4	> 99%	High ctDNA input; bioinformatic interpretation
	WGS/ WES	0.02%	80–90%	High ctDNA input; bioinformatic interpretation; higher risk of false positives

However, due to the low concentration of ctDNA in total cfDNA, ctDNA analyses involve a challenge for detecting genetic alterations (point mutations, CNAs or small indels) at the early stages of tumor development [80]. Epigenetic analyses on cfDNA have increased relevance to improving ctDNA detection in the early phases of the disease.

21.3.2.1 PCR-Based Techniques

PCR-based techniques were the first assays that allow to detect single or a low number of point mutations using highly sensitive and specific techniques with a rather fast and cost-effective rate. Real-time PCR (RT-PCR) was the first assay employed, reporting specific known mutations, but with a limited sensitivity (0.1–1%) [81]. In the last years, new technologies such as digital PCR (dPCR) methods, which include droplet digital PCR (ddPCR) and BEAMing (beads, emulsions, amplification and magnetics), showed high concordance with results obtained in tumor tissue [8, 82], and improved the sensitivity (0.01–0.1%) and specificity (100%).

Nevertheless, the main limitation of ctDNA analyses using PCR-based techniques is the requirement of previous information about the tumor type and the mutations characterizing this tumor. Therefore, PCR-based techniques are commonly employed to select targeted therapies, monitor the patients' evolution or detect resistant mutations during the treatment.

21.3.2.2 NGS

The second approach is focused on a genome-wide analysis of CNAs or point mutations through next-generation sequencing (NGS) strategies. Based on the assay panel size, there are single-locus/multiplexed assays, targeted sequencing, and genome-wide sequencing [83]. Genome-wide characterization allows a more complete and patient-specific genotyping to assess tumor heterogeneity and to follow the clonal evolution across the treatment [83]. The principal limitations of NGS-based strategies are the high cfDNA input requirement and general present lower specificity (80–99%) [67, 81].

Among these approaches, ctDNA can be analyzed by specific panels covering a high number of targeted genes (by NGS panels) or analyzing the total genome by whole-genome sequencing (WGS) or whole-exome sequencing (WES). WES and WGS based methods allow the detection of all possible aberrations in DNA, although it has limited analytical sensitivity in cfDNA applications. This phenomenon could be due to the efficiency by which the genetic regions of interest can be captured/enriched from cfDNA and the higher error rate of sequencing reactions [84].

21.3.2.3 Epigenetic Alterations

In addition to genetic alterations, different types of epigenetic marks have been explored in cancer as specific to the malignant process. These marks have been mainly explored in tissue samples but their interest in cancer diagnosis and monitoring using liquid biopsy has increased exponentially during the last 5 years [85].

DNA methylation is the most studied epigenetic modification. This covalent modification consists of the incorporation of a methyl group to the 5′ carbon of cytosines in cytosine-phosphate-guanine (CpG) dinucleotides to generate 5-methylcytosine (5mC) [86]. The detection strategies of DNA methylation patterns can be divided into sodium bisulfite conversion dependent or independent [87]. The most used are the bisulfite conversion dependent and are based on the fact that after sodium bisulfite treatment, 5mC cannot be converted into uracils [88].

For interrogating DNA of both CTCs or cfDNA different techniques have been successfully applied such as methylation-specific PCR (MSP), methylation-sensitive high-resolution melting (MS-HRM), quantitative methylation-specific PCR (qMSP) and digital PCR (dPCR) such as methyl-BEAMing and droplet digital PCR (ddPCR) [89–92]. These PCR based approaches are directed to analyze a low number of CpG, while other strategies like methylation microarrays [93, 94] or genome-wide bisulfite-based approaches based on NGS provide a more comprehensive view of the methylome using both cfDNA from cancer patients or DNA isolated from CTCs, even at the single-cell level [95, 96].

21.4 Advances in Circulating Extracellular Vesicles Analyses

The extracellular environment contains a large number of mobile membrane-limited vesicles secreted from different cells called “extracellular vesicles” (EVs) [97–99]. Although current research focuses primarily on two major types of EVs (exosomes and microvesicles (MVs)), EVs also include other vesicular structures such as large apoptotic bodies (Abs) as well as retrovirus-like particles (RLPs), exosome-like vesicles and membrane particles [97, 99, 100].

EVs represent a tool for intercellular communication in the body [97, 101–104] being present in a variety of body fluids including blood, urine, saliva, cerebrospinal fluid, lymphatics, tears, saliva and nasal secretions, ascites, and semen [101, 105, 106], which make EVs an interesting cancer biomarker. They carry different types of cellular content such as lipids, proteins, metabolites, receptors, effector molecules,

and nucleic acids like DNA and RNA (mRNA and microRNA) [107, 108]. This content can be translated to another cell [107–110] promoting different mechanisms including tumor progression by favoring angiogenesis and tumor cell migration in metastases [111–113]. Actually, EVs have shown to be valuable tools as biomarkers for longitudinal monitoring, defining tumor type, stage, progression, and treatment response [114, 115].

Exosomes are small EVs that generally possess a diameter of ~40–100 nm and a buoyant density of 1.13–1.19 g/mL [101, 116–118]. They are generated through a double invagination of the plasma membrane and the following formation of intracellular multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) [119]. These vesicular bodies are sorted by the endosomal network to their appropriate destinations, including lysosomal degradation, recycling, or exocytosis, releasing his ILVs content as exosomes [98, 106, 120, 121]. On the other hand, microvesicles arise through direct outward budding and fission of the plasma membrane, in a process called ectocytosis which produces microvesicles, microparticles, and large vesicles in the size range of ~50 nm to 1 μm in diameter [106, 113].

21.4.1 Isolation Methods

Currently, available purification methods are not capable of fully discriminating between exosomes and MVs [97, 122]. This lack of sufficient specificity and sensitivity makes more challenging their implementation in routine clinical practice [123, 124]. In fact, nowadays, there is no consensus on a "gold standard" method for EV isolation and purification [125]. Therefore, it is of utmost importance to improve and establish guidelines for EV isolation and analysis [125, 126], since depending on the method employed the amount, type and purity of the EVs recovered is different. Here we summarize some of the options for their isolation and characterization (Fig. 21.2).

21.4.1.1 Ultracentrifugation Techniques

Differential Ultracentrifugation

This is the most commonly employed EVs isolation method [127–129]. It is based on a succession of differential centrifugal forces to separate the particles: firstly, a low centrifugal force (300–400$\times g$) to sediment a main portion of the cells, a 2000$\times g$ to remove cell debris, a 10,000$\times g$ to remove the aggregates with a buoyant density higher than the EVs and a final high force (100,000$\times g$) that concentrate EVs in the resulting supernatant [126, 130]. However, this protocol is not unified and can vary depending on the volume and viscosity of the sample, which can affect the speed of centrifugation and the time needed for the obtention of the EVs [130, 131]. This strategy needs costly instrumentation, is time-consuming, requires a large amount of sample, and the recovery is normally low and contaminated with non-vesicular

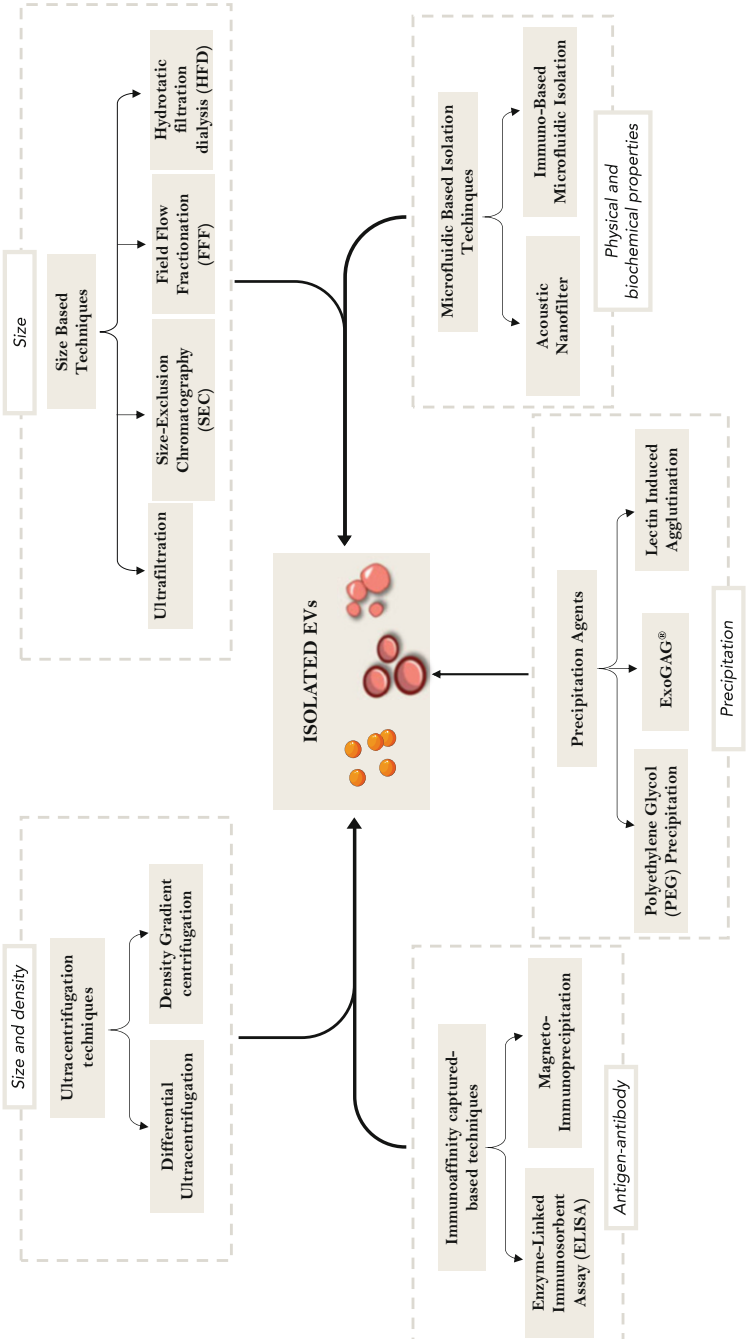

Fig. 21.2 Different strategies for EVs isolation and characterization

materials [132], which affect the purity of the samples in terms of the omics, RNA, and functional EVs analysis [133].

Density Gradient Centrifugation

This is a stricter strategy based on size and density [126] where the separation occurs in the presence of a preconstructed density gradient, typically made of sucrose or iodoxinol [134], resulting in differences in the osmotic pressure which can potentially affect the EVs [134, 135]. Density Gradient centrifugation is very effective in separating EVs from protein aggregates and non-membranous particles. Although it reports higher purity, it counts with limitations associated with ultracentrifugation [136] such as low recovery [137].

21.4.1.2 Size-Based Techniques

Ultrafiltration

This is the most commonly used size-based technique and consists of the separation of particles using semipermeable membranes with defined pore size or molecular weight cut [134]. While the larger particles are retained, the smaller ones passed through the filter into the filtrate [138]. Ultrafiltration is less time-consuming than ultracentrifugation and does not require special equipment [139]. However, the use of shear force may result in the deformation, clogging, or trapping in the unit or breaking up of large vesicles which may potentially skew the results of downstream analysis [139–141].

Size-Exclusion Chromatography (SEC)

This technique lies in sorting vesicles and other molecules based on their size by filtration through a gel. The gel is composed of spherical beads which contain pores of a specific size distribution through which small particles can penetrate. When the sample enters the gel, small molecules slow down the movement into the pores, causing them to elute later, while large molecules are excluded from entering the pores [142, 143]. Despite SEC methods enabling more accurate EVs purification [144] and preserving vesicle integrity and biological activity, they require run times of several hours, are not easily scalable, and cannot be used for high throughput applications [145].

Field-Flow Fractionation (FFF)

In this separation technique, a force field is applied perpendicular to a sample flow, to enable separation based on different sizes and molecular weights. When the perpendicular force field is applied, analytes in the sample are driven toward the boundary. Brownian motion creates a counteracting motion such that smaller particles tend to reach an equilibrium position further away from the boundary. This type of separation spans a broad size range and could be applied to a wide variety of eluents [136].

Hydrostatic Filtration Dialysis (HFD)

In HFD, based on the traditional dialysis separation method, the sample is forced throw a dialysis tube with a mesh of membrane with a molecular weight cutoff of 1000 kDa by hydrostatic pressure. As a result, larger particles like exosomes and other EVs remain in the tube where they can be collected. Apart from showing an efficient enrichment of the vesicles in comparison with the differential centrifugation protocol, it counts with a superior cost-efficiency with a faster workflow too [146].

21.4.1.3 Precipitation Agents

Polyethylene Glycol (PEG) Precipitation

By introducing a water-excluding polymer, such as polyethylene glycol (PEG) into the sample, exosomes can be settled out of biological fluids [139]. The water molecules "tides-up" causing exosomes, and the rest of the less soluble molecules, to precipitate out the solution [139]. This isolation method is quick, easy to use, requiring little technical expertise or any specialized equipment [134, 141]. Furthermore, it is compatible with a large number of samples. However, although it could be an easy option to integrate into clinical usage, its lack of selectivity, causes PEG polymers to be not exclusive to EVs and have other contamination substances [128, 133, 141].

Lectin Induced Agglutination

As an alternative to PEG, lectins are a family of proteins that bind carbohydrate moieties of other particles at a very high specificity [122]. Like PEG precipitation methods, the lectin precipitation methods are not time-consuming and do not need much expertise but have the problem of other soluble components. Hence, Lectin-induced exosome agglutination was explored for urinary exosome isolation [147].

It is also important to remark that several commercially available kits based on precipitation agents have been produced like ExoQuick (System Biosciences, CA, EEUU) [148] and ExoSpin (Cell Guidance System, Cambridge, UK) [149], which are based on PEG precipitation or ExoGAG (NasasBiotech, A Coruña, Spain) [123] that is a reactive that bonds with the glycosaminoglycans (GAGs) presented in the surface of EVs.

21.4.1.4 Immunoaffinity Captured-Based Techniques

They rely on the use of antibodies to capture the EVs based on the presence of lipids, proteins, and polysaccharides exposed on their surface [138, 150]. The fact that these techniques are primarily marker-dependent could be a constraint because the specificity of the assay relies on the specificity of the antibody used and thus tend to underestimate counts [134]. On the other hand, it presents a higher EVs purity than other methods based on other techniques [141]. Some examples of immunoaffinity capture-based techniques not exclusive to EVs are the Enzyme-Linked Immunosorbent Assay (ELISA) used to isolate exosomes from urine, plasma, and serum and Magneto-Immunoprecipitation that in comparison with ELISA has a higher isolation efficiency [122].

21.4.1.5 Microfluidic Based Isolation Techniques

Microfluidic-based isolation techniques are presented as a way to establish the use of EVs in clinical practice; however, its implementation is obstructed by issues such as scalability, validation, and standardization. They consist of the isolation of EVs based on their physical and biochemical properties simultaneously [134]. With their use, significant reductions in sample volume, reagent consumption, and isolation time are obtained because they can reproduce numerous laboratory processes on a microscale with high accuracy and specificity [149].

21.4.2 cEVs Cargo Profiling

EVs have a tremendous potential to be used in the field of liquid biopsy due to the molecules enclosed in them, which turn them into a useful circulating biomarker [117]. These molecules are basically DNAs, RNAs, multiple proteins, and metabolites [151] (Fig. 21.3). The identification of EVs-RNAs has been improved in the last years. The RNA cargo includes protein-coding transcripts (mRNAs) and many types of non-coding RNAs, including miRNA, long non-coding RNAs (LncRNAs), circular RNAs (circRNAs), small nucleolar RNA (snoRNAs), small nuclear RNAs (snRNAs), transfer RNA (tRNAs), ribosomal RNAs (rRNAs), and piwi-interacting RNAs (piRNAs) [152, 153]. Besides, EVs harbor different types of DNA, including single-stranded (ssDNA), double-stranded (dsDNA), mitochondrial DNA (mtDNA), and even viral DNA [154]. Importantly, the analysis of dsDNA in exosomes reflects the mutational status of parental tumor cells, thus is potentially

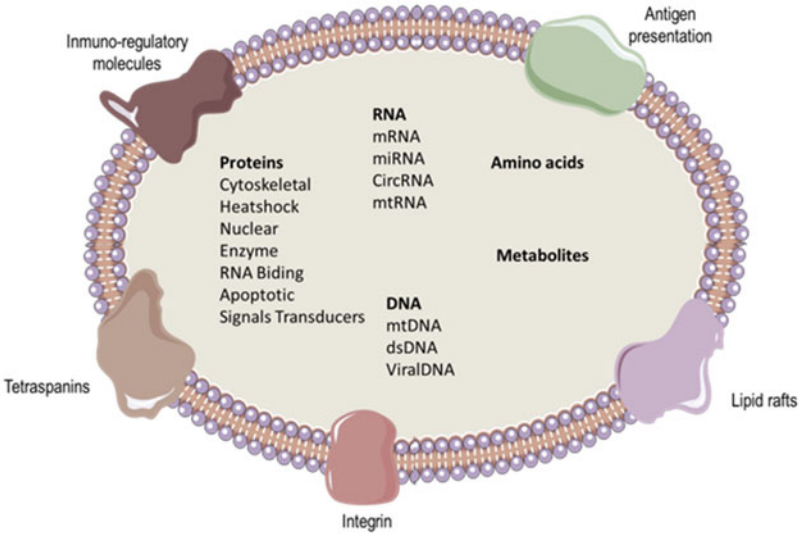

Fig. 21.3 Representation of EVs structure and molecular content

useful for early detection of cancer and metastasis and also for tumor phenotyping [155, 156].

The protein content of cEVs has been also explored to find diagnostic and prognostic biomarkers. Current tools used to study EV-proteins include Western blot, enzyme-linked immunosorbent assays (ELISA), flow cytometry, and mass spectrometry, among others. Thus, for example, higher levels of ANXA2 were described in cEVs isolated from plasma samples of patients with EC than healthy controls. The presence of therapeutic targets such as PD-L1 is also feasible in the fraction of cEVs although its clinical meaning is not totally understood [157, 158]. Also, Melo et al. demonstrated the interest of Glypican-1 (GPC1) positive exosomes for identifying early and late-stage pancreatic cancer from healthy individuals or patients with benign disease [23].

21.5 Alternative Circulating Biomarkers

In recent years the potential of tumor-educated blood platelets as a non-invasive tumor biomarker has been demonstrated [159, 160]. Platelets are involved in the progression and spread of various solid cancers, and their RNA molecular signatures can provide specific information about the presence, location, and molecular characteristics of the tumors [161]. Preliminary studies indicate that platelet RNA may complement the information obtained with other non-invasive biomarkers for cancer diagnosis, potentially improving early-tumors detection and facilitating dynamic monitoring of the disease [161]. In fact, recent advances in the characterization of platelet-mRNA using high-throughput techniques revealed that, in the presence of malignant disease, there was an increase from 10 to more than 1000 altered mRNAs in platelets. In fact, clinically relevant fusions such as *EML4-ALK* rearrangements have been described in platelets from patients with non–small cell lung carcinoma (NSCLC) [162]. Besides platelets can intake plasma proteins that promote tumor growth and vascularization, such as basic fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF) [163, 164].

For platelets isolation, there are some important points that should be taken into account. Many drugs can interfere with platelet studies (for example, antihistamines, aspirin, non-steroidal anti-inflammatory drugs). Furthermore, systemic factors such as chronic or transient inflammatory diseases, or cardiovascular events and other noncancerous diseases, can also influence the platelet mRNA profile. Therefore, for blocking platelet activation during the isolation procedure, strong mechanical forces should be avoided and platelet inhibitors such as Citrate or HEPES can be also used. The recommended isolation method is double centrifugation. The first centrifugation at 150–300$\times g$ to obtain platelet-rich plasma and the second to collect the platelet fraction is generally performed at 300–800$\times g$.

On the other hand, in the era of immunotherapy, several works have described the interest of analyzing the immune cells present in the bloodstream. The isolation and characterization of these cells are preferentially performed by flow cytometry and the selected cell fraction can be analyzed by different strategies to characterize the

proteins and DNA/RNA content. Of note, a correlation between the neutrophil to lymphocyte ratio has been described as a mark of the immunotherapy activity in terms of survival rates [165, 166]. Besides, the T-cell receptor (TCR) repertoire, which consists of the number of T cells with specific TCRs, has also been described as a predictor biomarker in patients under immunotherapy treatment) [167]. The analysis of PD-1 expression on circulating lymphocytes has been linked to better immune responses in melanoma and renal cell carcinoma [168]. Among the different subpopulations of immune cells CD8+/CD73+ subset of lymphocytes has been associated with worse survival and poor clinical benefits in patients with melanoma under immunotherapy [169]. Also, in melanoma low levels of myeloid-derived suppressor cells were associated with better response to immunotherapy [170].

21.6 Challenges for the Clinical Application

The possibility of finding non-invasive circulating biomarkers that provide comprehensive information about the molecular characteristics of each tumor is of incredible interest for oncologists [171]. However, 20 years after the field of liquid biopsy started to grow only ctDNA analyses are being used in a clinical context. Numerous studies have shown the potential of new technologies for detecting genetic alterations associated with ctDNA, with promising preliminary clinical results. However, the implementation of liquid biopsy analyses is being slow due to the need for very high-sensitive technologies and more economic sources to cover the PCR or NGS-based studies. Besides, liquid biopsy tests lack standardized workflows, and this impacts reproducibility and, therefore, on the robustness of the tests [171]. Preanalytical steps, including sample collection, processing, and storage, are important factors conditioning this reproducibility [172, 173]. The specificity is also a critical point, since, for example, the detection of mutations in cancer-associated genes is not a guarantee of their tumoral origin. Thus, the existence of clonal hematopoiesis should be taken into consideration when interpreting NGS results on cfDNA analysis in order to avoid false positives [174]. In addition to genetic alterations, epigenetic marks will play an important role to translate the cfDNA analyses to diagnosis or screening scenarios [85]. Besides, fragmentomics also appears as a promising strategy to identify tumors specific patterns in cfDNA from cancer patients [175].

Although ctDNA has emerged as the leading circulating biomarker, the analysis of other circulating biomarkers such as CTCs and cEVs can provide more biological information about tumor dissemination and the development of resistance mechanisms. In addition, the field of CTCs should go behind the enumeration and validate the CTC phenotyping as a surrogate of the solid tumor. For that, techniques should improve their versatility and sensitivity to be able to have more CTCs numbers for molecular characterization [18]. In this context, single-CTCs characterization is opening new perspectives for the definition and interpretation of tumor heterogeneity and its biological impact on tumor aggressiveness. For advancing in cEVs validation as a clinical tool the implementation of easy and reproducible

techniques is a clear challenge in the close future [176]. Besides, the development of novel strategies for cEVs isolation which cover EV subgroups in a pure fraction will be also a key point for the field development [21, 176].

Overall, the incorporation of liquid biopsy analyses into the clinical context requires the generation of guidelines and harmonized procedures. This will allow the development of interventional clinical trials to demonstrate the clinical benefit of including liquid biopsy for the management of cancer patients.

21.7 Conclusions

The application of liquid biopsy-based biomarkers is being broadly explored in many clinical contexts to manage cancer patients due to its minimal invasiveness and its value to obtain comprehensive and dynamic information about tumors. Several technologies have been developed during the last 20 years to address the study of different circulating elements, mainly CTCs, cfDNA, and cEVs. Sensitivity and reproducibility are two of the most valuable characteristics which are mandatory to characterize the tumoral material present in body fluids. The analysis of CTCs needs still improvement in these two aspects, and for this reason, CTCs studies are mainly focused on translational research to understand the dissemination process although different clinically relevant markers can be characterized in this tumor circulation population. Fortunately, cfDNA analyses, through PCR or NGS-based approaches, are nowadays being incorporated into the clinical practice to select targeted therapies in advanced tumors opening new avenues for personalized treatments. Other circulating elements such as cEVs or educated platelets represent promising biomarkers to complement the current alternatives to address the study of liquid biopsies in oncology.

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