Circulating biomarkers for diagnosis and response to therapies in cancer patients

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Abstract

Cancer presents a significant challenge to global health, driving worldwide concerted efforts to advance early detection, predict therapeutic response, and identify novel targeted therapies. Liquid biopsies emerge as promising avenues for discerning cancer biomarkers, offering less invasive approaches compared to conventional methods. Utilizing increasingly robust technologies, diverse bodily fluids can unveil genetic variants, epigenetic modifications, transcriptional alterations, and metabolomic signatures associated with cancer, thereby furnishing valuable insights for clinical management. This chapter intends to review the sources of cancer-related biomarkers found in circulation, prevalent techniques utilized for their identification, and the potential implications of different biomarker types on the management of cancer. Certain biomarkers currently used in clinical practice will be addressed, as well as potential biomarkers still in the study phase, and the inherent challenges in their practical implementation.

Introduction

Cancer stands as one of the primary contributors to morbidity and mortality on a global scale, posing a significant challenge to public health. It comprises a diverse array of illnesses distinguished by the unregulated growth of cells in constant transformation and evolution, with the potential to spread throughout the body, accumulating genetic and epigenetic changes that may lead to a lethal phenotype[1,2]. Therefore, early diagnosis and identification of pharmacological targets are crucial for improving survival rates and optimizing treatment options.

In this context, the investigation of cancer-related biomarkers has gained prominence in the field of modern oncology. Cancer biomarkers are any specific characteristics of cancer cells that can be identified in tissue biopsies, blood samples, or other patient fluids and can be employed to aid in tumor diagnosis, define disease prognosis, or predict patient therapeutic response to specific drugs. These can be molecular, cellular, physiological, or imaging-based characteristics[3,4].

Circulating cancer biomarkers are substances that can be detected in bodily fluids. These biomarkers circulate throughout the body and can either originate from cancerous cells or as a reaction of the body to the existence of cancer. The detection of circulating cancer biomarkers offers a unique opportunity to overcome limitations associated with more conventional methods, such as invasive biopsies and radiological imaging[5]. Furthermore, the analysis of circulating biomarkers can offer current insights into tumor behavior and how patients respond to treatment, allowing for a cancer patient care approach that is more tailored and accurate. This minimally invasive approach can also be repeated over time, enabling ongoing monitoring of disease advancement and treatment effectiveness[6]. Thus, the investigation of circulating biomarkers poses a crucial resource in the cancer diagnosis and treatment, with the potential to significantly impact clinical outcomes and consequently patient quality of life. Although some circulating protein biomarkers are frequently used as cancer indicators, circulating tumor cells (CTC), extracellular vesicles (EVs), circulating DNA, RNA-related molecules, and metabolites are emerging as types of biomarkers with great potential for clinical applicability[4, 6].

Therefore, this chapter aims to discuss the potential contribution of circulating biomarkers in the diagnosis and treatment of cancer, highlighting their possible clinical applicability and the

challenges associated with their implementation, as well as some detection technologies for cancer circulating biomarkers.

Liquid biopsy

Liquid biopsy is a method used to identify biomarkers present in bodily fluids. These samples can be collected from various sources, including blood, saliva, urine, sweat, breast milk, cerebrospinal fluid and others[3, 6] (Figure 1). Each of these biofluids harbors distinct biomarkers reflective of various physiological and pathological processes, presenting opportunities for early cancer detection, treatment response monitoring, and disease prognosis.

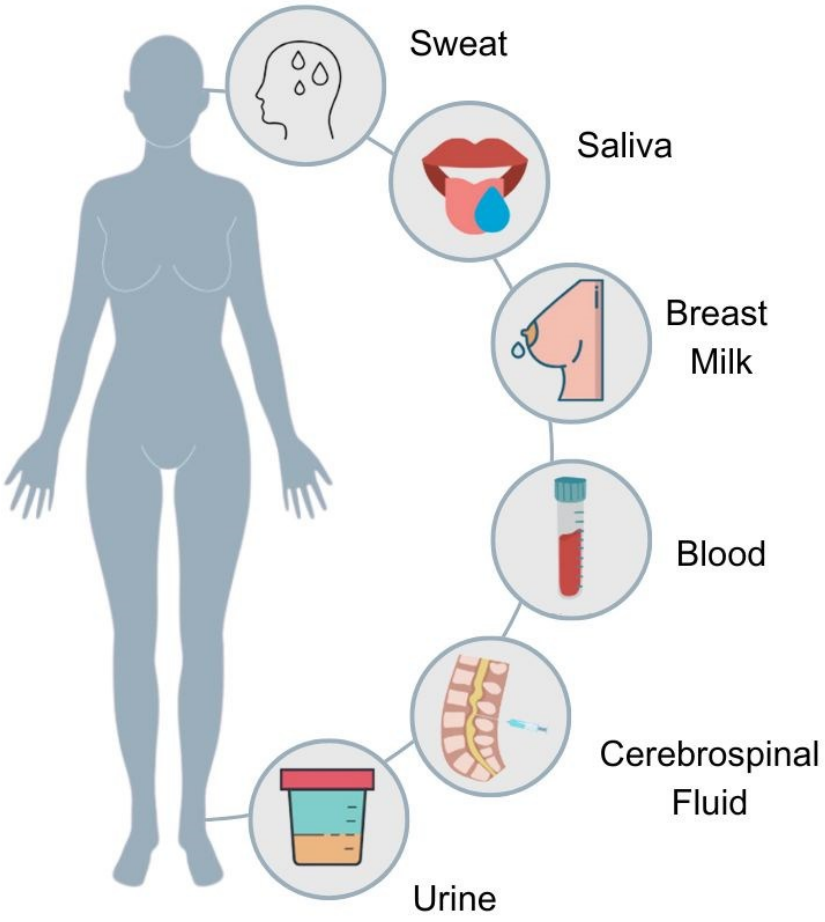

Figure 1. Liquid biopsy samples. Samples obtained through liquid biopsy comprise all elements present in bodily fluids, including blood components, urine, sweat, breast milk, cerebrospinal fluid, saliva, among others. These diverse biofluids typically offer an abundant reservoir of biomarkers for non-invasive diagnostic and management of cancer patients. Source: Created by the authors using BioRender.com.

Blood

In hematologic neoplasms, peripheral blood and bone marrow represent the primary specimen for identification, diagnosis, monitoring, and prognosis. The morphological analysis of blood cells constitutes the initial step in the identification and monitoring of hematologic neoplasms.

Moreover, white blood cells are employed to discern alterations in the karyotype and immunophenotype of cells, as well as to detect genetic mutations associated with each specific subtype of these malignant conditions[7, 8, 9].

Besides hematologic neoplasms, the DNA extracted from white blood cells is employed to identify hereditary genetic variations, contributing to the early detection and prognosis of cancer, including but not limited to BRCA1 and BRCA2 mutations, which are linked to an increased risk of developing breast and ovarian cancer[10]; genetic polymorphisms in the TP53 gene, which might elevate the risk of various cancer types, such as breast, colorectal, lung, among others [11]; and mutations in the MLH1, MSH2, MSH6, and PMS2 genes, which are linked with hereditary non-polyposis colorectal cancer predisposition syndromes[12, 13].

Not only white blood cells, but also plasma/serum can play an important role in identifying circulating biomarkers. From these blood components, proteins and other markers related to cancer can be identified, such as CEA in colorectal cancer, CA-125 in ovarian cancer, LDH in various types of cancer, among others[14].

Other potential cancer-related markers that can be found in blood and have gained prominence in the literature include microRNAs (miRNAs), vesicular biomarkers, CTC and circulating tumor DNA (ctDNA)[6, 14].

Saliva

Saliva also emerges as a valuable reservoir of cancer biomarkers. Collection of saliva samples, either directly or via buccal swabs, offers a convenient means of acquisition. Similar to plasma, saliva harbors EVs, DNA and RNA-related molecules, metabolites and proteins, which hold promise as biomarkers, particularly in cancers affecting the mouth, throat, and neck [15, 16]. For example, the identification of human papillomavirus (HPV) DNA may be utilized for monitoring treatment response and recurrence in cancers related to virus infection. Additionally, DNA mutations associated with oral and throat cancers may provide diagnostic insights[4, 17,18]. Furthermore, DNA extracted from saliva is mainly employed for genotyping hereditary genetic mutations, such as TPMT variant detection to assess risk prior to thiopurine treatment in some leukemia cases[3,19]. Oral samples also offer potential biomarkers for pancreatic cancer through the identification of salivary miRNA markers[20 - 22] as well as for lung cancer with identification of miRNA markers and DNA mutations[16, 23].

Urine

Urine has garnered significant attention within medical practice. This is an easy and valuable resource for biomarker analysis, particularly in urinary and male reproductive tract cancers diagnostics and treatment monitoring. For example, PCA3 messenger RNA (mRNA) stands out as a biomarker approved for clinical use for identification of early-stage prostate cancer from urine specimens. Furthermore, the detection of chromosomes 3, 7, and 17 anomalies, along with the absence of the 9p21 locus, is used for monitoring of tumor recurrence in bladder cancer[3,24].

Urine also acts as a reservoir for biomarkers related to other cancer types. Notable examples include the detection of B2M, utilized for prognostic determination and treatment response

monitoring in certain hematologic neoplasms such as multiple myeloma, chronic lymphocytic leukemia, and certain lymphomas. Additionally, the identification of urine catecholamines (such as VMA and HVA) assists in the diagnosis of neuroblastoma[24 - 26].

Expanding beyond established practices, numerous potential biomarkers have been detected in urine, including the oxidative DNA product 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), that shows promise in assessing therapy response in cancer patients[27 – 29], along with specific circulating extracellular vesicle-associated miRNAs that may prove useful in breast cancer diagnosis[30] as well as prostate cancer diagnosis and prognosis[31-33]. Moreover, urine appears to be an optimal medium for detecting ctDNA markers, with fewer interferents (such as proteins) compared to blood or serum[34].

Other samples

Other types of samples, though less frequent, hold significant potential in the identification of cancer-related biomarkers. These include, but are not limited to, sweat, breast milk, and cerebrospinal fluid.

Sweat, a less complex biological matrix compared to others, may contain substances present in blood and can be collected non-invasively. Recently, differences in the profile of volatile organic compounds were demonstrated between sweat specimens collected from healthy controls and patients with primary and metastatic cancer, thus highlighting sweat as a promising matrix for clinical studies aimed at cancer diagnosis[35].

Breast milk, although not a conventional sample, contains immune-responsive cells, soluble proteins, and exfoliated epithelial cells from mammary ducts. Studies have identified breast milk as a potential source for detecting EVs, ctDNA, and other types of biomarkers, thus highlighting its potential as a method for detecting breast cancer in its early stages [6, 36, 37].

Cerebrospinal fluid is a valuable sample obtained through invasive methods, serving as a source for pinpointing biomarkers, particularly concerning the central nervous system neoplasms and brain metastases from various primary tumor types, such as breast cancer, leukemia and others[38-41].

Detection of circulating biomarkers

Detection methods are continuously being developed and refined to improve sensitivity and specificity in identifying biological markers. Below, some methods/techniques currently employed for the identification of circulating biomarkers (Figure 2) will be briefly discussed:

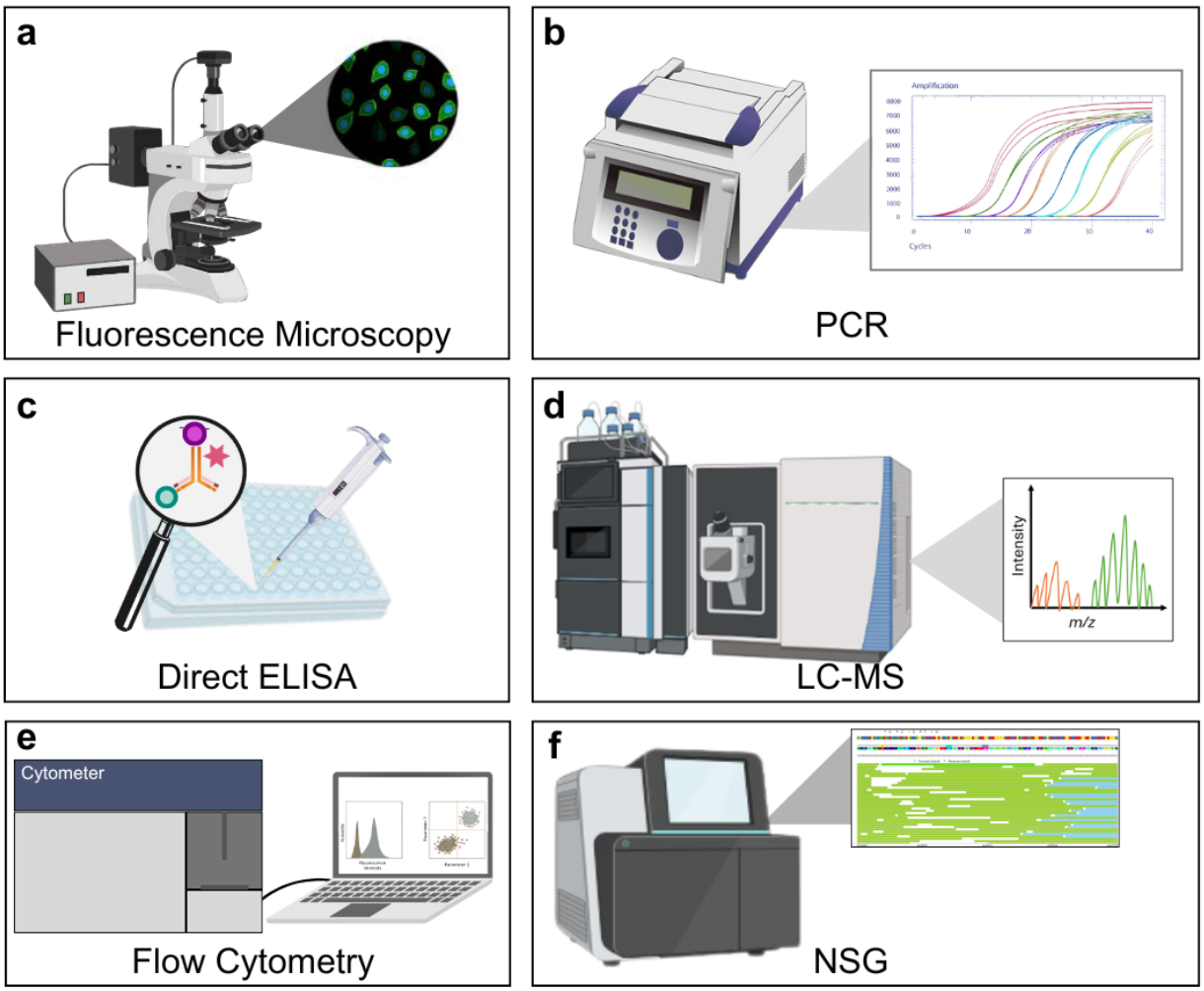

Figure 2. Techniques for Circulating Biomarker Identification. Examples of commonly used techniques in circulating biomarker identification, including fluorescence microscopy (**a**), polymerase chain reaction (PCR) (**b**), enzyme-linked immunosorbent assay (ELISA) (**c**), liquid chromatography coupled with mass spectrometry (LC-MS) (**d**), flow cytometry (**e**), and next-generation sequencing (NSG) (**f**). Source: Created by the authors using BioRender.com.

Optical imaging approaches

Optical imaging offers a high-resolution approach capable of capturing dynamic changes in biomarker expression. At the forefront of optical imaging techniques lies fluorescence-based imaging, leveraging the principles of immunofluorescence to target and visualize specific biomarkers associated with cancer cells or their microenvironment. Using fluorescently labeled antibodies or molecular probes, it is possible selectively bind to CTC, EVs, or other biomolecular targets, enabling their detection and quantification within complex biological samples[42-44].

Immunoassay

Immunoassays are widely utilized for the identification and quantification of proteins and biomolecules from blood or other bodily fluids. Notably, the enzyme-linked immunosorbent assay

(ELISA) is the predominant technique, which hinges on the binding between antibody and antigen molecules, both immobilized on a solid surface for efficient capture. During this process, an enzyme attached to the antibody-antigen complex triggers a colorimetric or fluorescent reaction upon substrate addition. Consequently, this reaction yields a measurable signal directly correlating with the concentration of the specific biomolecule in the specimen[45, 46]. Western blot is also a well-known immunoassay commonly utilized for the identification and quantification of specific proteins within a specimen, using antibodies. However, it can also be adapted to detect other molecules. In this process, proteins are separated by molecular weight on a gel, transferred to a membrane, and then detected using a primary antibody, followed by a secondary antibody for signal generation [47].

Flow Cytometry

In liquid biopsy applications, flow cytometry serves as a multiparametric technique widely employed for diagnosing and monitoring hematological malignancies, including the detection of Measurable residual disease (MRD; previously termed minimal residual disease), which refers to residual cancer cells post-treatment that often escape detection by conventional methods. By utilizing immunofluorescence principles and antibodies conjugated to fluorochromes targeting specific cell surface markers, flow cytometry enables the identification and quantification of distinct cell populations based on their immunophenotypic profiles. Abnormal expression of cellular markers aids in diagnosing various hematological neoplasms. Moreover, flow cytometry offers potential in detecting and characterizing CTC and demonstrates the capability to characterize individual EVs[47-50].

Polymerase Chain Reaction (PCR) techniques

PCR techniques are widely used in laboratories for amplifying specific regions of DNA. The PCR process initiates with denaturation, where the double-stranded DNA template undergoes heating to separate its two complementary strands. During annealing, primers designed to bind specifically to the target DNA sequence attach to their complementary sequences on the template DNA. Subsequently, DNA polymerase, a thermostable enzyme, extends the primers by synthesizing new DNA strands complementary to the template sequence in a process known as extension or elongation. This iterative cycle is replicated numerous times, typically around 20-40 cycles, resulting in the exponential amplification of the target DNA region. Consequently, even a minute amount of starting DNA can be amplified to detectable levels using various analytical techniques. The most common variations of PCR include real-time PCR (qPCR), which enables real-time quantification of DNA amplification; nested PCR, utilizing two pairs of primers to enhance sensitivity and specificity; reverse transcription PCR (RT-PCR), amplifying RNA rather than DNA; asymmetric PCR, producing more copies of one DNA strand; digital PCR (ddPCR), allowing precise quantification of DNA targets at low concentrations. This renders PCR a potent tool in identifying circulating biomarkers such as ctDNA and miRNAs[51, 52].

Mass Spectrometry (MS)

MS is a robust analytical technique that involves scrutinizing ions generated from molecules within a given sample. This is achieved through ionization, whereby sample constituents are converted into charged ions. These ions are subsequently sorted based on their mass-to-charge ratio (m/z)

and then detected. Regarding non-imaging techniques, liquid chromatography coupled with mass spectrometry (LC-MS) is prominent for its capability to segregate sample components before analysis, whereas gas chromatography coupled with mass spectrometry (GC-MS) excels, especially with volatile thermally stable compounds. Additionally, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) is invaluable for both solid and liquid samples, utilizing a laser to ionize molecules. These methods have found applications in pinpointing circulating cancer-related biomarkers, such as metabolites, proteins, lipids and modified nucleic acids, offering invaluable insights for diagnosis, prognosis, and treatment monitoring[47, 53].

Next-Generation Sequencing (NGS)

Next Generation Sequencing (NGS) stands as a potent sequencing method that enables the rapid analysis of large volumes of DNA or RNA sequences. The underlying principle of NGS entails fragmenting DNA or RNA molecules into smaller segments, affixing adapters, amplifying them, and subsequently sequencing millions of fragments simultaneously. Whole genome sequencing (WGS) and targeted sequencing are two approaches within the realm of NGS, each offering distinct advantages and applications. While WGS provides a comprehensive overview of the entire genome, targeted sequencing offers a more focused and cost-effective approach for studying specific genomic regions of interest. Techniques based on NGS can detect mutations, copy number alterations, and gene expression shifts, as well as identify single nucleotide variants, small insertions and deletions, gene fusions, and decipher intricate genomic rearrangements. In the realm of cancer research, NGS has proven important for the classification of certain hematologic neoplasms, as well as for the identification of MRD. Additionally, it has emerged as an invaluable tool for precisely pinpointing circulating tumor DNA (ctDNA), circulating tumor cells (CTCs), and extracellular vesicles (EVs)[54-56].

Types of circulating biomarkers

In general, circulating biomarkers may be associated with circulating cells or dissolved within the cytoplasm. Understanding the role and clinical relevance of these biomarkers is crucial for enhancing diagnostic and therapeutic strategies, as well as improving overall cancer management. Figure 3 shows the main types of circulating biomarkers.

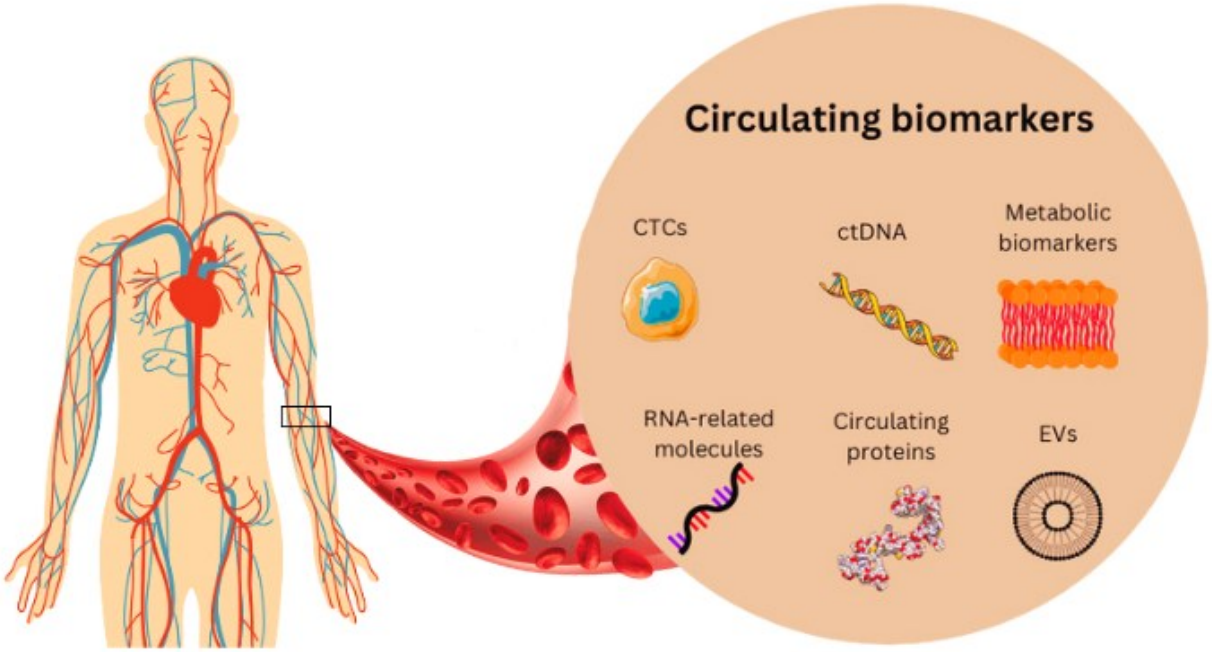

Figure 3. Main types of circulating biomarkers in cancer. Among the main types of circulating biomarkers are circulating tumor cells (CTC), circulating tumor DNA (ctDNA), metabolic biomarkers (such as enzymes, metabolites, lipids), RNA-related molecules, circulating proteins, and extracellular vesicles (EVs). These biomarkers can have vital functions in the detection, monitoring, and treatment of cancer, providing valuable insights for clinical practice in oncology. Source: Created by the authors using BioRender.com.

This topic provides information about the potential of these circulating biomarkers in oncological clinical practice.

CTC

CTC have emerged as a pivotal focus in cancer research, providing critical insights into disease progression and treatment efficacy. These cells, originating from primary tumors, disseminate through the bloodstream or lymphatic system, potentially initiating micro-metastases that may progress to macro-metastases, indicative of advanced cancer stages. The survival mechanisms inherent in CTC significantly contribute to their metastatic potential, particularly evident in CTC clusters, which demonstrate markedly higher metastatic capacity compared to individual CTC[57-59]. Consequently, CTC are actively under investigation as promising biomarkers across various cancer types, furnishing clinicians with non-invasive avenues for monitoring patients' disease trajectories and prognostication.

Since 2004, the Food and Drug Administration (FDA) has approved the detection, enumeration, and characterization of CTC in diverse clinical trials, predominantly encompassing breast, prostate, and colorectal cancer cohorts[60-62]. This milestone has catalyzed the advancement of circulating biomarkers in cancer research, where CTC analysis from peripheral blood has emerged as a pivotal tool for prognostic evaluation across a spectrum of malignancies. Notably,

in breast cancer, heightened CTC counts have been inversely correlated with overall survival rates[63]. Similarly, in colorectal cancer, elevated CTC levels correlate with diminished overall survival outcomes[64, 65]. Furthermore, the clinical significance of CTC as prognostic biomarkers extends to prostate cancer[66] and holds promise in predicting progression-free survival among patients with advanced gastric cancer[67].

In addition to their prognostic value, CTC have garnered attention for their potential to predict chemotherapy responses across various cancer types. Studies have revealed that the presence of CTC in breast cancer patients prior to chemotherapy and following treatment completion is associated with unfavorable prognostic outcomes and lower survival rates[68]. Similarly, in cases of prostate cancer, the detection of CTC was correlated to treatment resistance and and unfavorable prognostic outcomes [69,70]. CTC presence was also correlated with reduced treatment response and survival in colon cancer[71]. These findings extend to small-cell lung cancer, where studies have underscored the prognostic significance of CTC in predicting chemotherapy outcomes and patient survival[72,73]. Such observations underscore the multifaceted role of CTCs in cancer management, highlighting their potential also as predictive biomarkers to guide therapeutic interventions and improve patient outcomes.

The study of CTC encounters substantial challenges, with the CellSearch System® currently standing as the sole FDA-approved method for CTC isolation. This system relies on specific markers, such as EpCAM-positive cells, prevalent in various cancer types, for CTC identification and isolation. Utilizing immunostaining and flow cytometry techniques post-blood sample collection, it facilitates CTC quantification. However, this system exhibits limitations including dependency on the expression of specific markers like EpCAM, potentially leading to underestimation of CTCs lacking these markers; limited sensitivity due to the rarity of these cells; and overlap with leukocytes in certain biophysical parameters, hindering precise identification. Some complementary approaches may surmount the limitations of the CellSearch System®, such as microfluidics-based isolation reliant on physical properties like size and deformability, development of single-cell sequencing techniques for molecular-level CTC characterization, and utilization of imaging technologies for CTC identification and characterization, offering comprehensive insights into CTC biology across different cancer stages[74-76].

ctDNA

Another type of cancer biomarker that can be found in liquid biopsies is ctDNA, which is discharged from tumor cells undergoing apoptosis or necrosis and can be detected in a variety of bodily fluids, including blood, urine, saliva, breast milk, and others. Unlike traditional tissue biopsies, liquid biopsies can capture this genetic information not only from the primary tumor but also from metastases. Moreover, ctDNA maintains crucial genetic and epigenetic characteristics of tumors, including mutations, DNA methylation patterns, rearrangements, deletions, insertions and others, providing a comprehensive overview of the genomic landscape during tumor progression[59, 77]. In this context, ctDNA has garnered considerable interest among researchers in the field of oncology as a potential prognostic biomarker and treatment response indicator. It has been demonstrated that ctDNA may serve as a significant tool in MRD in certain cancer types. Furthermore, various studies have demonstrated that ctDNA can be a valuable biomarker for

identifying individuals at high risk of recurrence and unfavorable outcomes among patients with non-small cell lung cancer (NSCLC)[78-84].

In breast cancer, the detection of ctDNA seems to be more prevalent in subtypes exhibiting negative hormonal receptor expression, a characteristic often indicative of a poorer prognosis. Furthermore, multiple studies have established a correlation between the presence of ctDNA in plasma and the onset of metastases in breast cancer. Analyses of ctDNA in breast cancer patients frequently reveal mutations in critical genes such as TP53, BRCA1, and BRCA2. Specifically, the presence of ctDNA mutations in TP53 or BRCA1 has been notably associated with an unfavorable prognosis in recurrence-free[85-89].

ctDNA can also serve as a crucial biomarker in other types of cancer. Various ctDNA biomarkers specific to gastric cancer have been identified[90]. For example, recently it was demonstrated that the combination of KRAS mutations in ctDNA and four protein biomarkers enhances the sensitivity and specificity of early detection of pancreatic cancer[91]. Additionally, in patients with renal cancer, the detection of ctDNA is linked to a reduction in survival rates. Elevated levels of ctDNA were also linked to the transition from non-muscle-invasive to muscle-invasive bladder cancer. [92].

As technology advances, liquid biopsy analysis of ctDNA holds promise for becoming a routine clinical practice, enabling non-invasive monitoring of tumor dynamics and treatment response across various bodily fluid sources. Currently, a few NGS panels and qPCR kits are FDA-approved for clinical use in detecting cancer-related genetic changes in plasma or serum [93, 94]. Some factors that may limit the applicability of ctDNA as a cancer biomarker include the broad low quantity of this genetic material secreted by tumor cells, as well as the heterogeneity in ctDNA fragments, and their relative instability with a half-life of less than two hours, necessitating rapid processing and rigorous pre-analytical procedures[90, 95].

RNA-related molecules

The human transcriptome encompasses a rich assortment of RNA species, including mRNA and non-coding RNA (ncRNA) variants such as antisense RNA, circular RNAs, miRNA, ribosomal RNA, and other RNA-related molecules[3, 94]. Studies in this field have unveiled the molecular underpinnings of diseases and key biological processes, particularly in cancer, elucidating aberrant transcriptional patterns and altered protein functions as direct causal factors[4, 95, 96]. In the cancer context, these entities display distinct expression patterns and wield substantial influence over carcinogenesis. Within this array, mRNA and miRNA have attracted considerable attention for potential as clinically relevant circulating biomarkers[3, 94]. miRNAs regulate gene expression by binding to target mRNA [97].

The mRNA involvement in cancer progression extends beyond its role as a template for protein synthesis, as its transcription, regulation, splicing, and translation can be affected. Insights into mRNA dynamics offer valuable avenues for investigating some cancers, shedding light on their underlying molecular mechanisms[3, 96, 98]. In this context, some mRNAs have shown diagnostic potential as circulating biomarkers on colorectal cancer, including Beta-catenin, PTGS2, JAG1, and GUCY2C[98,99]. Additionally, a recent study showed that mRNA levels of B2M, TIMP-1, and CLU were notably increased in the plasma of individuals diagnosed with metastatic colorectal cancer [100]. In thyroid cancer, it has been demonstrated that measuring mRNA levels of transcripts

specific to the thyroid could prove beneficial in the early identification of tumor recurrence [101]. Furthermore, levels of CCND1 mRNA expression appears to have prognostic value in breast cancer, identifying patients with poor overall survival. In lung cancer, elevated concentrations of EGFR mRNA have been demonstrated to correlate with advanced stages of cancer[102, 103].

Regarding miRNAs, these small ncRNAs are recognized regulators of gene expression post-transcriptionally and have emerged as central players in cancer initiation, progression, and metastasis. Dysregulation of miRNAs has been implicated in various facets of tumorigenesis, including cell proliferation, apoptosis, and angiogenesis. Their detectability and differential expression patterns in cancer patients render them promising candidates for non-invasive cancer-related biomarkers[104-106]. For example, studies have shown that miR-155 has significant potential as a prognostic biomarker in cases of lung cancer and certain types of leukemia, where it has been significantly associated with low overall survival and progression-free survival[107,108]. Furthermore, in B-cell malignancies, miR-155 showed potential not only as a prognostic biomarker but also as a diagnostic biomarker and therapeutic target[109]. In addition to this, other miRNAs have been described in the literature as potential biomarkers. miR-34a stands out as a promising indicator for diagnosing and/or predicting the prognosis of hepatocellular carcinoma, ovarian cancer, breast cancer, and various other types of cancer. [110-112]. In breast cancer, exosomal miR-3662, miR-146a, and miR-1290 hold predictive potential, suggested as diagnostic biomarkers and for preventive strategies[113]. Addionally, miR-10b appears to hold significant diagnostic value in breast cancer, prostate cancer, hepatocellular carcinoma, and glioblastoma[114-117].

The literature provides various other examples of circulating mRNA and miRNA as potential cancer biomarkers; however, their detection remains challenging. Precise analysis demands quantitative approaches with high levels of sensitivity, specificity, and robustness. Methods utilized for such analyses encompass NGS, PCR techniques, Northern blotting, and microarray assays[97, 118].

EVs

EVs represent membranous lipid bilayer particles released from cells, lacking autonomous replication ability. Their classification can be based on physical traits such as size and density, biochemical composition, or cellular source. Recently, the International Society for Extracellular Vesicles (ISEV) updated the "Minimum Information for Studies of Extracellular Vesicles (MISEV)" guideline, advocating for the terminology "small EVs" (<200 nm) and "large EVs" (>200 nm) for size characterization purposes. Specific caution is advised against employing terms like "exosomes," "microvesicles," or "microparticles" due to the semantic implications associated with these terms. "Exosomes" typically denote derivation from the endosomal system, while "microparticle" or "microvesicle" imply a plasma membrane origin[119].

Recent advancements in research has uncovered the crucial involvement of EVs in initiating, advancing, and spreading cancer, exhibiting biological functions ranging from intercellular communication to tumor microenvironment modulation. These vesicles facilitate the transfer of aberrant genetic material, such as mutated DNA or oncogenes, between cells during cancer

initiation, potentially fostering malignant transformation. Moreover, EVs provide pro-inflammatory and pro-angiogenic signals favoring the formation of a tumor-permissive microenvironment.

Regarding cancer progression, EVs have a pivotal function in enhancing migration, invasion, and resistance to anti-tumor therapies by transporting bioactive molecules, including growth factors, cytokines, and miRNAs. This cargo enhances cell proliferation, angiogenesis, and the activation of pro-tumoral signaling pathways, thereby fueling tumor progression[44, 120].

Given their multifaceted roles and the diversity of molecules they carry, EVs have emerged as promising circulating cancer biomarkers, holding significant potential for diagnostic and prognostic applications in cancer management [44, 119].

In prostate cancer, for instance, the ExoDx Prostate® has gained commercial availability and FDA approval as a urinary test for predicting the probability of high-grade prostate cancer. This assay RT-qPCR assesses three genes (PCA3, ERG, and SPDEF) found on EVs[121, 122]. Additionally, protein combinations within urinary EVs have shown potential for distinguishing between benign tumor and prostate cancer patients, with specific combinations demonstrating utility in discerning high- and low-grade prostate cancer cases[123]. The upregulation of oncogenic miRNAs in EVs from prostate cancer patients, including miR-21, miR-141, and miR-375, also highlights their significance as diagnostic markers and their roles in tumor growth, metastasis, and immune activation[124].

Moreover, plasma EVs from breast cancer patients with lymph node metastasis exhibited decreased expression levels of miR-363-5p compared to those without metastasis, suggesting this miRNA as a potential biomarker to identify lymph node metastasis[125]. Additionally, other vesicular miRNAs have been reported in the literature as potential biomarkers for breast cancer metastasis detection. For example, miR-21 and miR-218-5p are associated with bone metastasis; miR-573-3p, miR130a, and miR-181c are associated with brain metastasis; and miR-105, miR-200c, miR-141, and miR-7641 are associated with metastasis without organ specificity[126].

In addition to prostate and breast cancer, EVs have been reported in the literature as potential biomarkers related to other types of cancer. Different EGFR mutations have been identified in plasma EVs from a large cohort of NSCLC patients, which could be useful as diagnostic markers for this type of cancer[127]. In the case of colorectal cancer, vesicular expression of CPNE3 in plasma samples from patients correlated positively with overall survival[128]. Additionally, regarding treatment response, elevated levels of PD-L1 mRNA detected in circulating EVs from melanoma and NSCLC patients prior to undergoing anti-PD-1 treatment showed a correlation with partial or complete remission post-therapy. [129]. Numerous other examples, not cited here, can be found in the literature.

Various techniques are employed for the isolation, identification, and characterization of EVs in the research field. Isolation methods include differential ultracentrifugation, size exclusion chromatography, polymer-based precipitation, and immunocapture. Following isolation, EVs can be identified using transmission electron microscopy, fluorescence microscopy, or flow cytometry. Characterization techniques such as infrared spectroscopy, MS, and proteomic analysis are utilized to determine the biochemical and molecular composition of EVs. The integration of these

methods enables a comprehensive approach to studying the physical properties, composition, and function of EVs across a range of biological and clinical contexts[130-133].

Nevertheless, it is imperative to acknowledge that the practicality of utilizing EVs as cancer biomarkers is still encumbered by some limitations, predominantly associated with the procedures of isolation, due to risk of introducing contaminants or inducing alterations in EV composition; purification, because of the diversity in purification methodologies adopted across studies, dovetailing the reproducibility and comparability of outcomes; and quality assurance. Additionally, the absence of standardized protocols in this domain contributes to the variability in EV-centric biomarker investigations[48, 134].

Circulating proteins

Proteinaceous alterations in cancer encompass a plethora of changes occurring at the proteomic level, including but not limited to aberrant protein expression, post-translational modifications (PTMs), and isoform variations. These alterations can stem from genetic mutations, epigenetic dysregulation, or signaling pathway aberrations intrinsic to cancer development and progression.

One prominent example is the dysregulation of oncogenes or tumor suppressor genes, leading to the overexpression or under expression of specific proteins critical for cellular processes like proliferation, apoptosis, and DNA repair. Additionally, PTMs such as phosphorylation, glycosylation, and ubiquitination can modulate protein function, stability, and subcellular localization, thereby influencing cancer phenotypes. Furthermore, alternative splicing events can generate protein isoforms with distinct functions or activities, contributing to the complexity of the cancer proteome.

In this context, circulating proteins have been investigated as biomarkers for a variety of clinical purposes, and some of them have already been approved by the FDA. This type of biomarker can assist in early diagnosis, differentiate between tumor subtypes, predict patient prognosis, and monitor treatment efficacy over time [135-137].

For instance, the serum quantification of PSA as well as HK2 stands as a cornerstone in the diagnostic approach to prostate cancer, alongside clinical data, such as age, digital rectal examination, and multicore prostate biopsy. Additionally, serum PSA analysis is routinely performed in these patients for the purpose of monitoring cancer progression, assessing treatment response. However, it is important to highlight that these are not cancer-specific biomarkers. It is an organ-specific proteins that may be overexpressed in other non-malignant conditions as well[138-140].

Other proteins are also well-established in clinical practice as cancer-related biomarkers. For example, CA-15-3 and CA125 exhibit elevated levels in the serum of breast cancer and ovarian cancer patients, respectively, thereby underscoring their significance as biomarkers for monitoring disease recurrence and evaluating therapy response[141]. Similarly, CA199 is elevated in pancreatic cancer, providing diagnostic and prognostic utility in this population[142].

In urine samples, both BTA and fibrin/fibrinogen are utilized in clinical practice for monitoring bladder cancer, and Fibrin/fibrinogen are also used to assess treatment response. Additionally,

immunoglobulins can be identified in both urine and blood, assisting in the diagnosis, evaluation of treatment response, and identification of recurrence in patients with multiple myeloma [24].

While some circulating protein markers are currently utilized in clinical practice, the investigation of potential circulating proteins as cancer-related biomarkers continues to be a prominent area of research. It's plausible to expect that numerous other circulating cancer biomarkers exist at comparable levels, awaiting discovery through systematic exploration[137].

For instance, serum IL6 and YKL-40 have been recognized as potential novel prognostic biomarkers in biliary tract cancer. Elevated levels of these proteins prior to treatment, as well as escalating levels during treatment, have been correlated with reduced overall survival[143]. Additionally, it was demonstrated that elevated serum levels of BAG6 in NSCLC were correlated with unfavorable overall survival outcomes in treatment-naive patients[144].

Furthermore, while PD-L1 is typically assessed in tissue samples, it was demonstrated that high levels of PD-L1 in serum may serve as a prognostic indicator for poor outcomes in hepatocellular carcinoma patients[145]. This protein has also been proposed as a biomarker for predicting the survival benefits of adjuvant cytokine-induced killer cell immunotherapy in this patient population[146].

Similarly, it was demonstrated in the literature that PD-L1 expression can be accurately and quantitatively evaluated in CTC and platelets utilizing the FDA-approved CellSearch® assay in patients with metastatic breast cancer, suggesting that the expression of CTC and/or platelet PD-L1 could predict the benefit of PD-1/PD-L1 pathway-directed immunotherapy[147, 148].

Researchers have also identified the precise HER-2 status in CTC from metastatic breast cancer and proposed the utilization of this protein for monitoring treatment response in patients undergoing anti-HER2 therapy[149].

The examples mentioned above are just a few samplings of what is being investigated, with numerous studies in literature exploring proteomic signatures related to different types of cancer.

In biomarker research, various techniques are utilized for the identification and quantification of circulating proteins. Absorption spectrophotometry methods, including the Bradford assay and Bicinchoninic Acid (BCA) assay, enable indirect protein quantification. ELISA is commonly employed to detect specific proteins in complex samples, while Western blotting facilitates protein expression analysis. Additionally, MS and chromatography techniques serve as powerful tools for protein identification and separation[47, 137, 150].

Some limitations encountered in the research of circulating protein biomarkers include, but are not limited to, sample purification; low specificity of some biomarkers; low sensitivity and specificity of certain protein identification methods; the influence of biological factors on protein expression; and sample volume, which can impact result robustness. Overcoming these limitations will require further research, utilization of more sensitive and specific detection techniques, as well as validation in large patient cohorts[47].

Metabolic biomarkers

Cancer cells display distinct metabolic patterns closely associated to dysregulated oncogenes and tumor suppressor genes. In this regard, researchers have identified certain "oncometabolites," that are endogenous metabolites aberrantly accumulated, which are implicated in initiating and/or sustaining cancer development[151].

Different metabolic pathways can be targets of oncometabolite research, such as glycolysis, Tricarboxylic Acid (TCA) cycle, amino acid metabolism, nucleotide metabolism, lipid metabolism and others[4]. For instance, the accumulation of 2-HG, associated IDH1 and IDH2 mutations, has been observed in the blood of acute myeloid leukemia patients. This accumulation is associated with the TCA cycle, but it may interfere with various metabolic and epigenetic pathways, thereby promoting tumorigenesis[152, 153].

The identification of circulating oncometabolites can also prove useful in cases of solid tumors. For example, succinyl-adenosine and succinic-cysteine, two metabolites also associated with the TCA cycle, have been described as excellent plasma biomarkers for the early diagnosis of fumarate hydratase-deficient renal cell carcinoma. They accurately indicated the status of fumarate hydratase mutation and tumor mass [154]. Additionally, the high enzymatic activity of the IDO in serum, which is related to the tryptophan pathway, has been linked in the literature to advanced stages of lung cancer[155] and as an immune escape mechanism in cases of ovarian cancer[156].

These kinds of biomarkers can also be identified in other types of samples. It has already been demonstrated that increased levels of polyamine, which are organic compounds derived from amino acids, can be detected both in serum and urine, serving as valuable indicators for cancer diagnosis and tracking tumor progression in lung and liver cancers. [157]. Overall, these metabolites are recognized for their role in fostering tumor growth and aggressiveness[158]. Furthermore, a recently published systematic review highlighted that blood and urine-based metabolites that may become useful for prostate cancer diagnosis include lipid classes, fatty acids, amino acids, and volatile organic compounds[159].

Thus, “oncometabolic” profiling provides valuable insights into the current biological state of cells in the context of cancer. The approaches currently employed to investigate metabolic alterations include LC-MS, GC-MS, and nuclear magnetic resonance spectroscopy[160]. However, the clinical application of oncometabolites as cancer biomarkers presents certain limitations since metabolic pathways are intricately influenced by environmental factors and the metabolites have a short half-life. Furthermore, due to the complex network of metabolites, panel analysis is considered more representative than isolated metabolites. Then, more extensive studies are required to broadly enable the use of oncometabolites in clinical practice[160-162].

Discussion

The investigation of circulating biomarkers represents an expanding area in modern oncology. For decades, researchers have sought to deepen their understanding of the biological, biochemical, and molecular foundations underlying tumor development, aiming to identify increasingly specific markers to aid in the diagnosis, treatment, and monitoring of various types of cancer[163].

The use of circulating biomarkers in clinical practice offers several significant advantages. Liquid biopsies are generally less invasive than tissue biopsies, which reduces risks and discomfort for patients[5]. Additionally, it enables the early cancer detection, continuous monitoring of treatment response, and disease progression, potentially leading to quicker and more effective therapeutic interventions[6].

Although a few circulating cancer biomarkers are well established in oncological practice today, many potential new biomarkers are in the early or advanced stages of research, showing promising results[163-165].

It is noteworthy that significant limitations still hinder the full implementation of these biomarkers in the diagnosis and monitoring of oncology patients. Biological variability among patients, influenced by factors such as age, sex, comorbidities, and concurrent treatments, complicates the standardization of tests. Additionally, the low sensitivity and specificity in detecting certain biomarkers pose significant challenges, as many biomarkers are common to multiple cancer types and not all methods can detect biomarkers at very low concentrations, which can lead to false results. Furthermore, even methods with high sensitivity are not free from biases related to sample collection and storage, as well as the analytical and post-analytical processes [5, 163–167]. Other important limiting factors include high costs and the need for sophisticated equipment, especially in resource-limited settings, as well as the technical complexity of some methods for identifying circulating biomarkers, which require specialized expertise for execution and interpretation of results[47, 55, 168].

However, the future outlook for circulating biomarkers is promising. Technological advancements, such as the development of new multiplexed biosensors and point-of-care devices, have the potential to enhance sensitivity and specificity, streamline methodologies, and potentially reduce costs[169, 170, 171]. Additionally, the application of artificial intelligence and machine learning algorithms can aid in analyzing large volumes of biomarker data, identifying patterns, and improving diagnostic accuracy[172, 173, 174].

Conclusion

In this chapter, it was discussed that a variety of liquid biopsy samples provide valuable insights into tumor dynamics, facilitating the acquisition of crucial information for early diagnosis, patient stratification, disease progression monitoring, and assessment of patient response to treatments. Although several methods are commonly used to identify different types of circulating biomarkers, technologies are increasingly being refined to enhance detection sensitivity and specificity. Despite ongoing technical and clinical challenges, the identification of novel biomarkers from liquid biopsies has the potential to revolutionize clinical practice, enabling a more personalized and precise approach to cancer patient care.

Summary

Table 1 summarizes the circulating biomarkers discussed throughout this chapter and highlights some of their current or potential applicability in oncology.

Table 1. Summary of circulating biomarkers and potential oncological applicability.

Circulating biomarker	Type	Application
BRCA1 and BRCA2	ctDNa	Breast and ovarian cancer[10]
TP53	ctDNA	Breast, colorectal and lung cancer[11]
MLH1, MSH2, MSH6 and PMS2	ctDNA	Colorectal cancer[12,13]
HPV DNA	ctDNA	Cancers related to virus infection[17,18]
HER2-status	CTC	Breast cancer[149]
PCA3	mRNA	Prostate cancer[24]
B2M	mRNA	Hematologic neoplasms and metastatic colorectal cancer[24, 26, 100]
TIMP-1	mRNA	Metastatic colorectal cancer[100]
CLU	mRNA	Metastatic colorectal cancer[100]
VMA and HVA	mRNA	Neuroblastoma[24,25]
CCND1	mRNA	Breast cancer[103]
EGFR	mRNA	Lung cancer[102]
Beta-catenin	mRNA	Colorectal cancer[99]
miR-155	miRNA	Lung cancer and leukemia[107,108]
miR-34a	miRNA	Hepatocellular carcinoma, ovarian and breast cancer[110-112]
miR-3662	miRNA	Breast cancer[113]
miR-146a	miRNA	Breast cancer[113]
miR-1290	miRNA	Breast cancer[113]
miR-10b	miRNA	Breast, prostate, and hepatocelular cancer, and glioblastoma[114-117]
ExoDx Prostate (PCA3, ERG, SPDEF)	EVs	Prostate cancer[121,122]
miR-21	miRNAs in EVs	Prostate cancer and metastatic breast cancer[124,126]
miR-141	miRNAs in EVs	Prostate cancer and metastatic breast cancer[124,126]
miR-375	miRNAs in EVs	Prostate cancer[124]
miR-363-5p	miRNAs in EVs	Breast cancer[125]
miR-218-5p	miRNAs in EVs	Breast cancer associated with bone metastasis[126]
miR-573-3p	miRNAs in EVs	Breast cancer associated with brain metastasis[126]
miR130a	miRNAs in EVs	Breast cancer associated with brain metastasis[126]
miR-181c	miRNAs in EVs	Breast cancer associated with brain metastasis[126]
miR-105	miRNAs in EVs	Metastatic breast cancer[126]
miR-200c	miRNAs in EVs	Metastatic breast cancer[126]
miR-7641	miRNAs in EVs	Metastatic breast cancer[126]
CPNE3	EVS	Colorectal cancer[128]
PD-L1	miRNAs in EVs and Circulating proteins	Melanoma, hepatocellular carcinoma, lung and breast cancer[129, 145, 1477, 148]
PSA	Circulating proteins	Prostate cancer[138-140]
HK2	Circulating proteins	Prostate cancer[138-140]
CA-15-3	Circulating proteins	Breast cancer[141]

CA-125	Circulating proteins	Ovarian cancer[141]
CA-199	Circulating proteins	Pancreatic cancer[142]
IL-6	Circulating proteins	Biliary tract cancer[143]
YKL-40	Circulating proteins	Biliary tract cancer[143]
2-HG	Circulating proteins	Acute myeloid leukemia[152,153]
IDH1 and IDH2	Circulating proteins	Acute myeloid leukemia[152,153]
Succinyl-adenosine and Succinic-cysteine	Circulating proteins	Renal cell carcinoma[154]
IDO	Circulating proteins	Lung and ovarian cancer[155,156]
CEA	Circulating proteins	Colorectal cancer[14]

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Statements and Declarations

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Abbreviations

8-oxo-dG 8-oxo-7,8-dihydro-2'-deoxyguanosine

BCA Bicinchoninic Acid

CTC Circulating tumor cells

ctDNA Circulating tumor DNA

ddPCR Digital PCR

ELISA Enzyme-linked immunosorbent assay

Evs Extracellular vesicles

FDA Food and Drug Administration

GC-MS Gas chromatography coupled with mass spectrometry

HPV Human papillomavirus

ISEV International Society for Extracellular Vesicles

LC-MS Liquid chromatography coupled with mass spectrometry

MALDI-MS Matrix-assisted laser desorption/ionization mass spectrometry

miRNAs MicroRNAs

MISEV Minimum Information for Studies of Extracellular Vesicles

MRD Measurable residual disease

mRNA Messenger RNA

MS *Mass Spectrometry*

ncRNA Non-coding RNA

NGS *Next-Generation Sequencing*

NSCLC Non-small cell lung cancer

PCR *Polymerase Chain Reaction*

PTMs Post-translational modifications

qPCR Real-time PCR

RT-PCR Reverse transcription PCR

TCA Tricarboxylic Acid

WGS Whole genome sequencing

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