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Liquid Biopsies for Early Cancer Detection: Beyond ctDNA

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ABSTRACT

Early cancer detection is key to better patient outcomes. Conventional diagnostic examinations are invasive and only give a snapshot of disease. Liquid biopsy, promising though it is, is hampered by problems of circulating tumor DNA (ctDNA) in the earliest stages of cancer by low sensitivity and abundance. This review covers new liquid biopsy biomarkers other than ctDNA, their biological rationale, sophisticated means of detection, and clinical utility in early cancer diagnosis on the basis of only original research and case reports. We discuss Circulating Tumor Cells (CTCs), Extracellular Vesicles (EVs) including exosomes and their varied cargo (proteins, microRNAs, long non-coding RNAs, exosomal DNA), cell-free RNA circulating in the blood (cfRNA), circulating proteins, and circulating metabolites. Each provides distinct tumor biology information, complementing ctDNA. Their potential is hampered by problems of low concentration of tumor-derived material, biological heterogeneity, and the strong need for standardized isolation and detection procedures. Future progress will rely on multi-omics integration and artificial intelligence to improve diagnostic accuracy, with the aim of translating these technologies into everyday clinical practice for population and personalized cancer screening.

Keywords: *Circulating metabolites, Biomarkers, ctDNA, Early cancer detection, Liquid biopsy.*

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INTRODUCTION

Global Cancer Prevalence and the Importance of Early Diagnosis: Cancer continues to be a leading cause of mortality throughout the world, demonstrating the imperative need for effective diagnostic methods. Despite ongoing advances in cancer chemotherapy, rates of survival too often remain low, primarily due to the fact that too often diagnoses are made in advanced stages of the disease. For instance, over 53% of all patients with lung cancer are diagnosed in a metastatic phase of the disease, in which the 5-year survival falls to a paltry 8.9%. This is emblematic of a fundamental issue in oncology: the asymptomatic presentation of nascent cancers. Silent growth is typical of many cancers, which are asymptomatic until advanced. When overt clinical symptoms do arise, the disease process will have reached stages at which therapeutic resections are less successful and the prognosis markedly worse. This inherent limitation of symptom-based diagnosis directly necessitates the development of highly sensitive, non-invasive methods for screening that identify cancer prior to symptom onset. Early detection is the key to maximizing survival rates for many cancers, as it offers the greatest survival advantage in oncology. The ability to identify malignant changes at their earliest stages may profoundly alter disease courses and drastically enhance patient outcomes.[1,2]

Limitations of Conventional Cancer Diagnostic Techniques: Traditional cancer diagnostic methods, such as "solid biopsies," are invasive and are associated with a range of inherent risks, such as infection, haemorrhage, and pain.

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Aside from being invasive, these biopsies are not typically amenable to repeated, continuous surveillance of cancer growth or recurrence. One of the major limitations of tissue biopsies is that they provide only a frozen-in-time view of the tumor at a point in time. The single view does not capture the dynamic and mutating genomic profile of the disease, which can fluctuate wildly, e.g., when the tumor develops resistance mutations to therapy, as cancer progresses. The absence of real-time capture of tumor dynamics means that a treatment plan based on a pilot biopsy can be made obsolete as the tumor changes, leading to suboptimal treatment choices.

Additionally, conventional clinical markers, including prostate-specific antigen (PSA) levels used in prostate cancer screening, prove to be insufficient for accurate risk stratification in the early stages. While PSA tests are operational, they prove to be of limited specificity, detecting only one-fifth of the affected population. This can lead to the overtreatment of indolent and, thus, unnecessary burden on the patient, and under treatment of aggressive disease, potentially leading to catastrophic outcomes. Even the latest imaging technologies, despite their sophisticated nature,

still under detect metastatic prostate cancer, hence, reflecting the diagnostic shortcomings in the domain of early and evolving disease. These inherent limitations of traditional methods underscore the essential imperative for diagnostic tools that can contribute to a more dynamic, comprehensive, and less invasive assessment of cancer.[3,4]

The Shift towards Liquid Biopsy Strategies: The limitations of conventional means of cancer diagnosis have brought significant progress toward liquid biopsy application. Liquid biopsy, in the form of analysis of circulating biomarkers in various bodily fluids like blood, urine, saliva, and cerebrospinal fluid, provides a seemingly appealing non-invasive option over the conventional tissue biopsies. The approach has various advantages, such as decreased invasiveness, decreased time demands, and overall patient safety improvement.

The technology of liquid biopsy, encompassing capture and molecular typing of disease-origin material, evolved rapidly from a conceptual level to translational application in the clinical environment in a period of less than a decade. The rapid advancement is enabled by its ability to provide a more fluid and holistic perspective on tumors. Liquid biopsy enables real-time monitoring of patients, immediate assessment of treatment response, and decision guidance on therapy. Such qualities enable clinicians to detect actionable mutations, track the efficacy of therapies, and detect mechanisms of resistance earlier than with traditional methods. By providing dynamic molecular information, liquid biopsy becomes the platform for developing and implementing highly individualized patient management strategies, thereby being a cornerstone of precision oncology. Such a transformation from empirical therapies to personalized therapies, enabled by real-time molecular surveillance, has the potential to revolutionize clinical outcomes.[5,6,7]

The History of ctDNA and the Call for Further Biomarker Research: Circulating tumor DNA (ctDNA) is an important biomarker in the field of liquid biopsies that has received significant attention due to recent advances in DNA technologies. These technology advances allow for the determination of tumor-specific characteristics, such as somatic mutations, viral DNA, or methylation patterns, found in the blood. Analysis of ctDNA has shown high utility in monitoring response to cancer therapy and in the assessment of minimal residual disease (MRD).

But while promising, ctDNA also suffers from several intrinsic limitations, especially when used for early cancer detection. One of the major limitations is the low tumor-specific DNA concentration in the blood, particularly in early-stage cancer or in tumors that shed little DNA. This lack of tumor-derived material directly impacts the analytical sensitivity and clinical specificity of ctDNA assays, and early detection is thus made unreliable. Furthermore, most of the currently available ctDNA tests in clinical practice are capable only of finding one or two characteristics of tumor DNA, making them less specific in general. While next-generation sequencing (NGS) platforms are raising sensitivity, low levels of ctDNA in early disease are proving to be a major hindrance to widespread early screening, as well as to continued calls for standardized protocols.

The ubiquitous problem of low prevalence at early stages of the disease is not specific to circulating tumor DNA (ctDNA); instead, it is a major stumbling block common to many liquid biopsy biomarkers, such as circulating tumor cells (CTCs). This intrinsic limitation demands a broader exploration of other constituents of liquid biopsies. Researchers now investigate other possible biomarkers of greater abundance at early stages of disease, such as exosomes, or those assayable via other molecular pathways, such as RNA modifications. The research includes circulating tumor cells (CTCs), extracellular vesicles (EVs), circulating free RNA (cfRNA), tumor-educated platelets (TEPs), and circulating proteins and metabolites. This multi-analyte approach, combined with the development of ultra-high-sensitivity assays and multi-omics approaches, aims to push the boundaries of low biomarker concentration at early stages of the disease and increase overall detection sensitivity. The current review provides a comprehensive overview of non-ctDNA liquid biopsy biomarkers, such as CTCs, EVs and their diverse cargo, cfRNA, circulating proteins, and circulating metabolites.[8-12]

METHODS

A systematic search of the literature was performed using databases such as PubMed, Web of Science, and Google Scholar, employing keywords such as "liquid biopsy," "early cancer detection," and individual non-ctDNA biomarkers (e.g., "circulating tumor cells," "extracellular vesicles," "cfRNA," "circulating proteins," "circulating metabolites").

Articles providing general overviews, those solely concerned with ctDNA, or studies predominantly addressing advanced cancer stages without explicit implications for early detection were excluded. Data related to biomarker categories, analytes, biofluid sources, isolation and detection strategies, and performance metrics were meticulously extracted and synthesized to determine their biological significance, technological requirements, and clinical effectivity in relation to early cancer detection. The ongoing focus on standardization in the literature is a reflection of the significance of rigorous and transparent methodologies for facilitating clinical application.

Circulating Tumor Cells (CTCs) as Early Detection Biomarkers

Biological Foundations and Clinical Implications: Circulating Tumor Cells (CTCs) are tumor cells that break off from primary tumors or metastases and become incorporated into the circulatory system, and are a key component of tumor metastasis. CTCs were first described in 1869. CTCs may have more aggressive genetic alterations than primary cells.

CTCs are very rare (about one cell per 105 to 106 peripheral blood mononuclear cells), particularly in non-metastatic tumors (usually fewer than one CTC per milliliter), that are a major technical barrier to detection in early disease. Their heterogeneity, e.g., EpCAM-negative mesenchymal CTCs, renders them even more difficult to detect, requiring numerous and very sensitive means of isolation and characterization. 3.2 Early-Stage Disease Isolation and Detection Technologies CTC detection involves enrichment to separate them from normal blood cells, measurement, and quantitation, using either biological properties (surface markers) or physical properties (size, density).[13, 14]

Immunoaffinity-based methods: These agents act against epithelial cell surface antigens, such as EpCAM. The CellSearch, System, the 'Gold Standard' and sole FDA-approved CTC enumeration technology for metastatic breast, colorectal, and prostate cancer (mainly prognostic), employs immunomagnetic enrichment with anti-EpCAM antibodies. Manufacturers quote high recovery (≥85%), but independent reports vary (42-90%). Its inability to enumerate EpCAM-negative or non-cytokeratin-expressing CTCs, which are more aggressive, is a serious drawback. Manual validation also adds variability. Adnatest®, MagSweeper, and others microfluidic chips are some other systems.[13, 14]

Label-Free (Physical Property-Based) Techniques

Label-free technologies take advantage of CTCs' biophysical characteristics. Microfluidic platforms, or "lab-on-a-chip" devices, provide benefits such as decreased sample volume and increased sensitivity.

Filtra Cell (FMSA) is micropillar size-based enrichment, and it is capable of processing 8 mL of blood in 20 minutes and was employed to recover CTCs from patients with breast, colorectal, and NSCLC. A clinical trial (NCT01722903) indicated that perioperative sampling can improve the recovery of CTCs in colorectal cancer.

Screen Cell employs filters (7.5 or 6.5 µm pore size) for size separation and obtains near 100% recovery rates and fast processing (50 seconds/mL). ScreenCell has been applied in clinical trials (EXPEVIVO-CTC, NCT03797053; ESO-CTC, NCT02610764), but as yet, no results are published.

Clinical Utility and Case Reports for Early Detection of Diabetes

Although FDA-approved for prognostic use in metastatic disease, CTC detection in early cancer is an area under investigation. In early breast cancer, CTCs are not frequent (~20% detectable), and 10-year follow-up showed they are not an effective long-term prognostic marker owing to methodological constraints and cost. In lung cancer, CTC count was higher in patients with cancer but was not discriminatory enough for early detection (AUC 0.598). However, detection of CTCs associated with greater recurrence (HR 2.97) and mortality (HR 3.53) risk in resectable NSCLC, suggesting prognostic and micro metastasis indication. In conclusion, detection of CTCs has significant limitations for early cancer detection in routine use owing to low concentration and heterogeneity and requires further methodological advancements and standardization.[13, 14]

Extracellular Vesicles (EVs) as Early Detection Biomarkers

Biological Foundations and Clinical Significance: Extracellular Vesicles (EVs) are lipid bilayer-defined, nanoscale structures secreted from nearly all cell types, including cancer cells, and present in all body fluids in high numbers. Exosomes (40-160 nm) are a common subtype, produced by endosomal budding. Tumor-derived EVs are emerging as a "treasure chest" for cancer biomarkers because they deliver a rich cargo (DNA, RNA, proteins, lipids, and metabolites) that mirrors parental cell characteristics. A liberating benefit is that they are present in high numbers (up to 1011/mL, many orders of magnitude more than CTCs), and thus, potentially, more readily accessible to detect, particularly in early stages of tumors when other biomarkers are in short supply. The lipid bilayer shields their enclosed content from degradation, allowing the recovery of complete oncogenic information. Tumor-derived exosomes play central roles in cancer initiation, progression, and metastasis, and thus a colossal potential as new cancer biomarkers in liquid biopsy for early detection.

Methods for Isolation and Characterization

Safe EV isolation without changing their properties is paramount.

The gold standard is ultracentrifugation (UC), though this is time-consuming and can lead to protein contamination.

Density gradient (DG) centrifugation is used to separate EVs according to density, yielding more concentrated and uniform EVs, but is constrained by technical skill and expense.

Immunoaffinity capture methods selectively bind EVs through specific surface markers, increasing EV markers and related proteins.

Size exclusion chromatography (SEC) separates EVs according to size and has proven to be able to purify EVs from most body fluids to screen renal and prostate cancer.

Exosomal Cargo as Biomarkers: Heterogeneous contents of exosomes are a treasure trove for the discovery of candidate biomarkers.

Exosomal Proteins: Exosomal proteins are great diagnostic biomarkers and are commonly utilized as immunoaffinity binding sites for the isolation of exosomes. Tumor-derived exosomes have an elevated expression of tetraspanins (CD9, CD63, CD81, CD82), which are engaged in cargo selection and exosome biogenesis. Quantitative proteomics characterizes differentially expressed proteins that participate in cancer. For instance, the ExoScreen assay identifies CD63 and CD9 surface expressions in patients with colorectal cancer.

Exosomal Nucleic Acids (evDNA, miRNAs, lncRNAs): Exosomes contain different nucleic acids such as RNA and DNA.

Exosomal DNA (evDNA) can be a perfect non-invasive cancer tissue biomarker for human cancer: MicroRNAs (miRNAs) are small non-coding RNAs (19-22 nucleotides) of major regulatory functions in genes, often deregulated in cancer. Exosomal miRNAs are highly stable in bodily fluids and hence excellent liquid biopsy candidates. Data indicate miRNAs, either singularly or in panels, improve diagnostic and predictive ability. In lung cancer, a six-miRNA panel had good AUCs (0.78-0.86), rising to 0.96-0.99 with nodule diameter. Exosomal miRNAs in prostate cancer had better diagnostic ability than PSA. Disadvantages are a lack of standardization and requirement for higher miRNA specificity, often necessitating panels.

Long non-coding RNAs (lncRNAs) are also being identified as potential candidates for early, non-invasive cancer diagnosis in exosomal cargo with aberrant expression associated with oncogenesis. Serum exosomal lncRNA FOXD2-AS1 was diagnostic in colorectal cancer (AUC 0.736, 0.758 for early-stage), and exosomal lncRNA-GC1 separated gastric cancer patients (AUC > 0.86), better than traditional markers.

Case Reports/Clinical Utility for Early Detection: Exosomes are of great potential for cancer early detection, monitoring, and treatment decision-making. Scientists intend to take advantage of EVs for the early detection of cancers, depending on their molecular cargo for information on the presence of cancer prior to symptoms or large tumors. A systematic review of prostate cancer indicated disease-specific alteration in exosomal miRNA, protein, and lipids in urine and blood, where exosomal miRNAs were better in diagnostic utility compared to PSA. Promising as it is, there are still issues to be addressed, such as standardizing exosome isolation, establishing quantitative cut-offs, and building probabilistic multi-omic models of tissue origin. However, exosome diagnostics yield real-time, dynamic information, improving personalized medicine and potentially revolutionizing cancer diagnostics.[15-18]

Cell-free RNA (cfRNA) circulating as early biomarkers for detection (along with exosomal RNA)

Clinical Significance and Biological Basis: Cell-free RNA (cfRNA) is also of enormous potential in disease diagnosis and personalized medicine, yielding complementary information on gene expression or epigenetic modifications. Purified in 1999 (mRNA) and 2008 (miRNA), cfRNAs are better biomarkers in the sense that they are abundant, have unique tumor signatures, and can modulate genes. Release into body fluids is expected to be through apoptosis and/or necrosis, as for cfDNA.

Detection methods and issues: Asessment of cfRNA quantity and viability is difficult because of its inherent instability, the presence of RNase enzymes in the blood, and poor control of RNA stability when handling a sample. In spite of these drawbacks, sequencing of circulating RNA makes it possible to acquire target gene expression data non-invasively, possibly eliminating invasive biopsies.

New cfRNA Variants for Early Detection

Circulating mRNA (cf-mRNA): First isolated in 1999 from plasma of nasopharyngeal carcinoma patients, cf-mRNAs are typically short and the most scarce cfRNA species and hence hard to detect. However, the presence of high levels of them in oncological patients hints at a possible role in cancer diagnosis and monitoring.

Cell-free microRNAs (cf-miRNAs): The best-characterized family of cfRNA, the cf-miRNAs, were first found in the blood of prostate cancer patients. They are unexpectedly stable in most body fluids, making them viable targets for the majority of cancers. Studies show that they can distinguish between healthy patients and cancer patients for the majority of cancers, including breast, colorectal, gastric, and endometrial cancers.

Circulating Small Nuclear RNA Molecules (cf-snRNAs): Some cf-snRNAs have been investigated as cancer biomarkers. The "U" family is well characterized, with U2 snRNA having changed levels in pancreatic and colorectal adenocarcinomas, and U6 snRNA being overexpressed in breast cancer serum.

Long Non-coding RNAs (lncRNAs): LncRNAs are new novel biomarkers for early cancer detection. Original research (2025) showed long-read nanopore sequencing of full-length plasma cell-free RNA (cfRNA) uncovers a heterogeneous cfRNA transcriptome, allowing precise machine learning-based diagnosis of precancerous states (e.g., high-grade dysplasia Barrett's esophagus) and adenocarcinoma of the esophagus. This proves RNA liquid biopsy's potential for early disease discovery and targeting. In addition, a University of Chicago study showcased a new RNA modification-based assay utilizing RNA as opposed to DNA, with nearly 95% accuracy in the detection of earliest stages of colorectal cancer. The test utilizes RNA modification levels, including that of gut microbes whose activity varies with tumors, indicating earlier detection compared to DNA-based tests due to greater abundance of microbial RNA in early disease. This represents the first application for RNA modifications as an emerging cancer biomarker, proving more reliability and sensitivity for early-stage detection.[19-23]

Circulating Proteins as Novel Early Detection Biomarkers

Biological Foundations and Clinical Implications: Proteomics, or high-throughput protein analysis, is an important area for early cancer detection, as DNA changes result in altered protein expression. Though challenging because of proteome heterogeneity and the inability to assess low-abundance proteins, scientists are discovering pan-cancer protein signatures that can be employed to supplement nucleic-acid strategies. The universality of proteins and direct relationship to cellular function make them suitable targets for dynamic, real-time disease monitoring.

6.2 Detection Technologies Enzyme-linked immunosorbent assays (ELISA) are strong for the detection of single, individual protein biomarkers. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) with mass spectrometry (MS) has emerged as a mainstay method of discovery but is demanding of protein and is confronted with analyzing low-abundance proteins. More sophisticated methodologies are Multidimensional Protein Identification Technology (MudPIT) is employed to enhance sensitivity, while protein microarrays enable multiparametric analysis. Aptamer-based arrays (e.g., SomaScan) and sensitive proximity assays are employed to discover pan-cancer protein signatures and very low-abundant proteins as robust early-stage biomarkers.

New Proteomics-Directed Strategies for Early Detection: New multi-cancer early detection screening tests based on proteomes were established using plasma proteins in recent original research. Sex-specific 10-protein panels were very accurate (AUC 0.98 in men, 0.983 in women), detecting 93% of cancers in men and 84% in women at stage I with 99% specificity. Tissue of origin was detected in more than 80% of cases using 150-protein panels. The most striking finding was that the most informative biomarkers for early-stage detection were plasma proteins of low abundance, highlighting the necessity for highly sensitive assays. The study also demonstrated strong evidence of cancer protein signatures being sex-specific, with poor correlation across protein profiles in men and women.

Clinical Implications for Early Detection: This proteome-based screening test demonstrated strong performance compared to other technologies, including ctDNA tests. It greatly surpassed current multi-cancer screening technologies (e.g., Galleri, CancerSEEK) in the detection of cancer at every stage (I, II, III), demonstrating enhanced sensitivity at stage I alone while maintaining a specificity rate of 99%. It also demonstrated significantly higher accuracy in identifying the tissue of origin. In comparison to the US Preventive Services Task Force-recommended four screening tests (colonoscopy, Pap test, mammography, low-dose CT), this test demonstrated greater sensitivity for cancer detection at early stages. These results have significant implications for cancer screening policy, indicating a potentially more effective methodology that would change guidelines and be added to routine health checks because of its high sensitivity, specificity, and projected low cost. Further validation in larger population groups remains necessary.[24,25]

Metabolites as Circulating Biomarkers of Early Detection

Biological Rationale and Clinical Relevance: Metabolomics dysregulation is also centrally involved in cancer genesis, with dysregulated metabolic pathways in cancer cells. As a result, circulating metabolites are now recognized as potent potential biomarkers for cancer diagnosis and dissecting disease biology, with implications for more effective screening strategies and identification of lethal cases.

Detection Methods: Investigation is primarily composed of untargeted pre-diagnostic circulating metabolomics analyses, measuring a broad range of metabolites in biofluids like plasma in order to assess associations with cancer risk. Heterogeneity of methods between studies (sampling, designs, platforms, data handling) has been a classic reproducibility challenge.

Clinical Implications for Early Detection: A systematic review and meta-analysis of 12 pre-diagnostic untargeted circulating metabolomics studies examined up to 408 metabolites' correlation with four prostate cancer (PCa) outcomes. Significant correlations were found in the analysis: three metabolites for total PCa, eleven for high/very high-risk PCa, and nineteen for fatal PCa. Implicated metabolites in high/very high-risk PCa were predominantly lipid-enriched. These results signify the high potential of metabolites to individualize the risk of fatal PCa, which could be pivotal in the development of risk-stratified screening policies and drug/dietary modifiable targets for PCa prevention.[26,27]

DISCUSSION

The field of early cancer detection by liquid biopsy is quickly broadening beyond ctDNA, spurred by its own limitations in early disease under conditions of low abundance and sensitivity issues. This review identified several promising non-ctDNA biomarkers, each with their distinct biological insights and with different strengths and challenges of translating to the clinic.

Circulating Tumor Cells (CTCs) provide us with real-time information on metastatic potential, but their virtual invisibility in early cancer (e.g., ~20% are detectable in early breast cancer) is a monumental barrier to early detection in the population at large. While extremely promising as prognostic markers, they are not yet suitable for early diagnosis in routine practice because of technical limitations, cost, and the requirement for standardization.

Extracellular Vesicles (EVs), specifically exosomes, are promising with their very high density in body fluids (various orders of magnitude over CTCs) and their lipid bilayer protective coat, that preserves heterogenous content (proteins, evDNA, miRNAs, lncRNAs). They are thus of interest for early detection when other biomarkers are limited. Diagnostic utility with exosomal miRNAs in lung and prostate cancer, and lncRNAs in colorectal and gastric cancer, has been demonstrated. Standardization of EV isolation and characterization, and determination of quantitative cut-offs, are however required for clinical utility.

Circulating Cell-Free RNA (cfRNA) yields dynamic information concerning gene expression and function. Although less frequent, cf-miRNAs are also stable and cancer/healthy patient discriminatory. A novel RNA modification-based microbial RNA assay was very sensitive (~95%) for the identification of early colorectal cancer and is proposed to be more sensitive and accurate than DNA-based assays since

microbial RNA is more present in early disease. Instability of cfRNA and availability of RNase remain challenges.

Circulating proteins give a direct link to cellular function. Novel proteomics-informed multi-cancer screening tests have shown very high accuracy (AUC 0.98) in the detection of early-stage cancers with high specificity (99%), outperforming the performance of some existing multi-cancer screening tests and traditional methods at stage I. The key results are the use of low-concentration proteins and sex-specific biomarkers, which necessitate the employment of highly sensitive assays.

Circulating Metabolites reflect metabolic dysregulation in cancer cells. Research found specific metabolites associated with prostate cancer risk, including lethal PCa, and suggested their potential utility in risk-stratified screening and prevention targets. Methodological heterogeneity remains a challenge.[13-15, 17-22, 24-27]

In brief, the non-ctDNA biomarkers are all complements to ctDNA, each with its own advantage: CTCs for cellular data, EVs for dense and protected cargo, cfRNA for gene expression kinetics, proteins for functional changes and multi-cancer detection, and metabolites for metabolic changes. A multi-analyte approach, with all these heterogeneous biomarkers, holds the highest promise for enhancing early cancer detection. However, the common problem with all these new biomarkers is the urgent need for standardization of sample collection, processing, and analysis protocols, and rigorous validation by large-scale clinical trials, to ensure their reliable and reproducible entry into routine clinical practice.

Future Considerations

Multi-Omics Integration: The future of liquid biopsy for cancer early detection is in the integration of multiple "omics" data, from fragmented DNA to multi-omics, ultra-high-sensitivity testing. The integration of various types of biomarkers—CTCs, EVs, cfRNA, proteins, and metabolites—yields a more complete picture of tumor biology. The integrated method raises sensitivity and specificity, overcoming early disease low abundance, and uncovers tumor heterogeneity and resistance earlier.

Artificial Intelligence and Machine Learning: Machine learning (ML) and artificial intelligence (AI) will revolutionize analysis and interpretation of large multi-omics liquid biopsy samples. AI-powered biomarker detection improves early diagnosis and personalized medicine. ML algorithms learning from multi-omics data forecast tumor behavior, discover new biomarkers, and design optimized treatment plans. AI improves CTC detection specificity/accuracy, enables proteomics, and cfRNA analysis. AI-powered precision medicine integrating with liquid biopsy is revolutionizing cancer therapy in precision and personalization.[28,29]

Standardization and Regulatory Frameworks: There remain significant hurdles in standardizing approaches and traversing regulatory environments. Lack of standard operating procedures for sample acquisition, manipulation, and analysis across differing biomarker types undermines reliability and reproducibility and, as a result, clinical translation. While ctDNA testing is Medicare-covered and part of the NCCN guidelines, other emerging biomarkers must undergo rigorous multi-center validation to gain the regulatory approval. It is necessary to meet these criteria for new biomarkers to gain regulatory clearance and become the bedrock of clinical practices.

Clinical Translation and Population-Wide Screening: Liquid biopsy is evolving from a diagnostic tool mostly applied in advanced-stage cancer to a potential universal diagnostic tool. The development of highly sensitive assays, like the proteomics-based plasma test, heralds a future where liquid biopsy can revolutionize cancer screening guidelines and become a standard part of routine health assessments. Its high sensitivity for the detection of early tumors in asymptomatic patients, coupled with its high specificity, makes it a well-suited candidate for mass population screening, thus improving patient outcomes through earlier and more precise therapeutic interventions. In addition, the estimated cost-effectiveness of these next-generation assays is expected to sustain their integration into healthcare infrastructures.[30,31]

CONCLUSION

This review highlights the exceptional progress and immense promise of liquid biopsy biomarkers beyond circulating tumor DNA (ctDNA) for early cancer diagnosis. While ctDNA is critical for monitoring, early-stage disease detection is constrained by its capabilities, necessitating alternative and complementary biomarkers. The collective evidence from original studies and case reports suggests that these biomarkers outside of ctDNA are indispensable complements to ctDNA. Their integration into multi-omics platforms, and with artificial intelligence and machine learning, is estimated to overcome analytical obstacles, enhance diagnostic accuracy, and provide complete, real-time molecular profiles for early-stage diseases. Their complete clinical realization is dependent upon continued rigorous research, establishing standardized protocols, and implementing regulatory pathways. Finally, the development of liquid biopsy techniques outside of ctDNA is predicted to transform cancer screening, allowing for earlier, less invasive, and more tailored treatment, thereby significantly improving patient outcomes globally.

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