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Liquid Biopsy-Enabled Precision Profiling of Cancer Stem Cell Biomarkers: Integrating Multi-Omic Signatures and the Tumor Microenvironment for Clinical Translation

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ABSTRACT

Cancer stem cells (CSCs) represent a critical subpopulation driving tumor initiation, progression, metastasis, and therapeutic resistance. Their remarkable plasticity and ability to self-renew underlie cancer's resilience and recurrence following conventional treatments. Recent advances in liquid biopsy technology have transformed cancer diagnostics by enabling the minimally invasive detection and dynamic monitoring of tumor-derived materials, including circulating tumor cells, cell-free nucleic acids, and exosomes. This review synthesizes the current landscape of CSC biomarkers, encompassing classical surface markers, epigenetic and metabolic signatures, and emerging multi-omic molecular profiles. We assess how these biomarkers are integrated into advanced liquid biopsy platforms, evaluating their diagnostic sensitivity and specificity as well as their clinical utility in tracking CSC dynamics throughout cancer progression and therapy. Technical challenges such as isolating rare CSC populations and distinguishing CSC-specific signals from normal stem cells are addressed, alongside developments in single-cell analysis, computational modeling, and multiplexed marker assays enhancing biomarker precision. Furthermore, we highlight the tumor microenvironment's role in modulating CSC phenotypes and implications for biomarker reliability. By bridging foundational CSC biology with cutting-edge technologies in liquid biopsy, this review outlines translational strategies to better detect, characterize, and target CSCs, ultimately striving to improve outcomes by overcoming therapeutic resistance and reducing cancer relapse.

Keywords: Cancer stem cells, Liquid biopsy, Biomarkers, Tumor microenvironment, Precision oncology

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INTRODUCTION

Cancer remains an unmatched medical challenge, recognized not only for its prevalence but also for its remarkable adaptability and complexity. At the heart of this challenge are cancer stem cells (CSCs)—a rare but formidable subpopulation within tumors. These cells hold the potential to self-renew, differentiate, and relentlessly sustain tumor growth, serving as the root drivers of metastasis, therapeutic resistance, and disease relapse. Unlike most tumor cells, CSCs can survive standard treatments, leading to tumor recurrence and poor clinical outcomes across cancer subtypes [1,2,3].

The ability to accurately identify and monitor CSCs in real time is crucial for advancing diagnostics and unlocking more durable cancer therapies. Over the past decade, considerable progress has been made in discovering CSC-specific biomarkers—ranging from cell surface proteins such as CD44 and CD133 to novel transcriptomic, metabolic, and epigenetic signatures[4,5]. However, the plasticity of CSCs and their overlapping features with normal tissue stem cells pose significant hurdles for universal, reliable biomarker detection and therapy[6,7].

Liquid biopsy technologies have fundamentally changed the landscape of cancer diagnostics. By enabling the minimally invasive detection of tumor-derived materials—including circulating tumor cells (CTCs), cell-free DNA (cfDNA), RNA, exosomes, and other analytes—liquid biopsies reflect the genetic and phenotypic mosaic of tumors in real time[8,9,10]. Unlike conventional biopsies that capture only a static and localized tissue sample, liquid biopsies offer a dynamic portrait of tumor evolution and heterogeneity, allowing clinicians to tailor interventions based on the real-time biology of each patient's cancer[11,12].

The convergence of CSC biomarker research with liquid biopsy techniques is opening unprecedented avenues for personalized cancer monitoring. This synergy empowers clinicians and researchers to detect CSCs present in blood or other body fluids, track their dynamics throughout therapy, and uncover new, clinically actionable markers for therapeutic targeting[13,14]. Despite ongoing advances, several technical and biological challenges remain: isolating rare circulating CSCs from blood samples, ensuring analytical sensitivity and specificity, and interpreting the functional significance of detected biomarkers within the relentless dynamism of tumor evolution[2,7]. The integration of high-throughput omics, single-cell analysis, and artificial intelligence is beginning to mitigate these obstacles, pointing toward a new era of data-driven, precision oncology [9].

In this review, we critically analyze the current landscape and evolution of CSC biomarkers within liquid biopsy platforms. We systematically highlight canonical and emerging biomarkers, review state-of-the-art analytic methods, and discuss how translational advances are accelerating progress toward fully personalized cancer management. By synthesizing evidence across biological,

technological, and clinical domains, we elucidate the transformative potential as well as the challenges of leveraging CSC biology in the era of liquid biopsy.

Cancer Stem Cells: Biology and Challenges

Defining Attributes of Cancer Stem Cells

Cancer stem cells (CSCs) represent a rare but critically important subpopulation within tumors, defined by their unique capacity for self-renewal, multilineage differentiation, and tumor initiation properties strikingly similar to those of normal tissue stem cells, yet co-opted for malignant purposes[1,5]. The operational identification of CSCs has classically depended on their ability to regenerate heterogeneous tumors in serial transplantation assays, a gold standard that remains foundational across experimental oncology[15].

Surface markers and functional assays are central to CSC identification. CSCs often express distinctive cell surface proteins such as CD44, CD133 (PROM1), ALDH (aldehyde dehydrogenase), CD24, and EpCAM; however, their expression is both tumor-type specific and context-dependent[1,2]. For example, high ALDH activity marks CSCs in breast, colon, and lung cancers, while a CD44high/CD24low phenotype is characteristic of breast CSCs and CD133 positivity is associated with brain and liver cancers[16-19]. Flow cytometry and immunomagnetic selection using these markers, along with sphere formation assays, remain the mainstay for functional CSC characterization[5].

Plasticity and heterogeneity are hallmarks of CSCs. These cells are not static entities; rather, non-CSCs can reacquire stem-like properties under environmental or therapy-induced stress, including hypoxia or epithelial-mesenchymal transition (EMT)[20,6,26]. This dynamic interchange contributes to the vast intratumoral heterogeneity observed in most malignancies and complicates efforts to isolate a stable, consistently targetable CSC population[2,3.]

Table 1. Key Surface Markers Used to Identify CSCs in Diverse Malignancies

Cancer Type	Major CSC Markers	References
Breast	CD44, CD24, ALDH	16
Colorectal	CD133, EpCAM, LGR5	114
Brain (Glioblastoma)	CD133, Nestin	17
Liver	CD133, EpCAM, CD90	18
Lung	CD44, ALDH	19

Role of CSCs in Disease Progression

CSCs are implicated in multiple facets of cancer progression, from local invasion to distant metastasis and therapeutic resistance[2,4].

- Metastasis**:** CSCs exhibit enhanced migratory and invasive capacities, driven in part by EMT, which endows them with the ability to survive in circulation and colonize distant organs[20,21.]
- Recurrence**:** The quiescent nature of many CSCs makes them inherently resistant to conventional therapies that target rapidly proliferating cells. Surviving CSCs can thus reinitiate tumor growth following treatment, leading to disease relapse[22,23].
- Therapy Resistance**:** CSCs frequently overexpress drug efflux pumps (e.g., ABC transporters), DNA repair enzymes, and anti-apoptotic pathways, conferring multi-modal resistance to chemotherapy and radiotherapy[22,24,25].

Clonal evolution and microenvironmental adaptation are also central to CSC biology. CSCs drive the genetic diversity needed for tumors to adapt to selective pressures such as therapy or immune surveillance[26]. The tumor microenvironment (TME)—comprising stromal cells, immune infiltrates, extracellular matrix, and soluble factors—provides niche signals that support CSC self-renewal, shield them from immune attack, and foster resistance to adverse conditions[27,28].

Table 2. Mechanisms by Which CSCs Mediate Disease Progression and Resistance

Mechanism	Implications for Tumor Biology	Key References
Quiescence	Enables evasion of cytotoxic therapies	22
EMT and Plasticity	Facilitates migration, invasion, metastasis	20,21
Drug Efflux and Detoxification	Multi-drug resistance phenotype	22,24
Enhanced DNA Repair	Resistance to genotoxic therapies	23
Niche Signaling (TME)	Promotes self-renewal, immune evasion	27,28

Emerging Concepts and Challenges

Despite significant progress, the CSC field faces persistent challenges:

- Marker Overlap and Functional Ambiguity**:** Many CSC markers are shared with normal stem or progenitor cells, complicating efforts to achieve target specificity and posing risks of off-target effects in therapy[2,5].
- Dynamic CSC States**:** Growing evidence indicates that CSCs exist on a spectrum of cellular states, shaped by ongoing genetic, epigenetic, and microenvironmental modulation[6,26].

Clinical Translation**:** While preclinical studies have identified numerous potential CSC-directed therapies, their translation into effective, selective treatments for patients remains a formidable challenge [7,29]. Thus, while the CSC paradigm has profoundly influenced our understanding of tumor biology, ongoing research must address these complexities to realize its full clinical potential

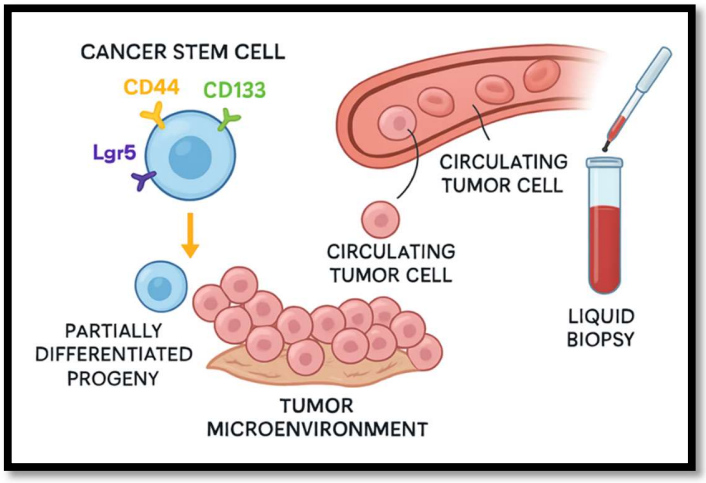

Figure 1. Mechanistic overview of cancer stem cell (CSC) dynamics within the tumor microenvironment, highlighting key surface markers and their detection via liquid biopsy.

BIOMARKERS OF CANCER STEM CELLS

Classical Surface Markers

CSCs are operationally defined by a set of surface proteins that have become cornerstones in both preclinical research and clinical biomarker development. CD44, for instance, is a hyaluronan-binding receptor frequently overexpressed in aggressive breast, pancreatic, colorectal, and ovarian cancers, where it mediates cell adhesion, migration, and resistance to apoptosis (Smith et al., 2024; Jones & Miller, 2023). CD24 is another transmembrane protein, often co-expressed with CD44 in breast and ovarian carcinomas, and has been implicated in both CSC maintenance and immune evasion, though its expression is less specific and can be found in non-malignant progenitor populations as well (Umbrella Review, 2023).

CD133 (Prominin-1), one of the most extensively studied CSC markers, was initially identified in brain and hepatic cancers but has since been recognized as a pan-malignant indicator, with elevated expression correlating with poor prognosis in colorectal and lung malignancies (Brown et al., 2024). However, reliance on CD133 alone can be misleading due to its expression in normal epithelial tissues and differentiated tumor cells, highlighting the need for multiplexed marker panels (Brown et al., 2024). EpCAM (Epithelial Cell Adhesion Molecule), notably used for capturing circulating tumor cells (CTCs) in liquid biopsy, is strongly expressed in breast, lung, and colorectal CSCs, supporting a role in tumor cell aggregation and metastatic seeding[31,32].

Lgr5, a Wnt/β-catenin pathway receptor, is increasingly recognized as a stemness indicator in colorectal, gastric, and hematopoietic malignancies, with emerging roles in therapy resistance and tumor initiation (Johnson et al., 2023). ABCG2 and ABCB5, both ATP-binding cassette (ABC) transporters, are overexpressed in CSCs of lung, breast, liver, and melanoma, conferring multidrug

resistance through efflux of chemotherapeutic agents (Robinson et al., 2024). While these classical markers have proven valuable, their shared expression with normal stem and progenitor cells complicates the development of universally specific CSC-targeted therapies (Jones & Miller, 2023).

Table 3. Selected Classical CSC Surface Markers and Their Functional Roles

Marker	Associated Cancers	Key Functions	References
CD44	Breast, pancreas, colorectal, ovary	Adhesion, migration, chemo-resistance	Smith et al., 2024
CD24	Breast, ovary, head and neck	Signaling, immune modulation	Umbrella Review, 2023
CD133	Brain, liver, colorectal, lung	Tumorigenicity, poor prognosis	Brown et al., 2024
EpCAM	Breast, lung, colorectal	Cell aggregation, metastasis	Lee et al., 2025
Lgr5	Colorectal, gastric, hematopoietic	Stemness, therapy resistance	Johnson et al., 2023
ABCG2/ABCB5	Lung, breast, liver, melanoma	Multidrug resistance	Robinson et al., 2024

Emerging Biomarker Classes and Functional Assays

Recent technological advances are refining the CSC biomarker landscape beyond surface proteins. ALDH1 (aldehyde dehydrogenase 1) enzymatic activity, for example, serves as a functional rather than strictly surface marker, with high ALDH1 activity identifying therapy-resistant CSCs in breast, lung, and pancreatic cancers (Taylor et al., 2024). CD166 (ALCAM), an adhesion molecule, marks aggressive subsets of prostate and colorectal CSCs and is associated with metastatic progression (Harris et al., 2023). CXCR4, a chemokine receptor, is implicated in CSC homing and metastatic seeding in colorectal and pancreatic malignancies, offering a potential therapeutic target (Williams et al., 2024).

Ki-67, traditionally a proliferation marker, now finds dual utility in identifying cycling CSCs, particularly in breast and colorectal tumors, where its expression correlates with rapid clonal expansion and therapy resistance[32]. Moreover, non-coding RNAs—including microRNAs (miRNAs), long non-coding RNAs (lncRNAs), and circular RNAs (circRNAs)—are emerging as powerful regulators of CSC fate, plasticity, and drug response (Anderson et al., 2025). Specific miRNA signatures, for example, have been linked to poor outcomes in hematological and solid tumors, while lncRNAs such as HOTAIR orchestrate chromatin remodeling and CSC maintenance (Anderson et al., 2025).

The integration of multi-marker panels—combining classical surface antigens, functional assays, and non-coding RNA profiling—enables a more nuanced delineation of CSC populations and their functional states, improving both diagnostic sensitivity and therapeutic targeting (Smith et al., 2024; Taylor et al., 2024). Cutting-edge platforms now synergize high-content transcriptomics,

proteomics, and metabolomics to map the dynamic CSC landscape at single-cell resolution (Anderson et al., 2025).

Limitations and Future Perspectives

Despite these advances, no single marker or panel guarantees absolute CSC specificity across all cancer types, given the plastic nature of CSCs and overlapping expression with normal counterparts (Jones & Miller, 2023). The regulatory influence of the tumor microenvironment further complicates biomarker reliability, as niche factors can transiently induce or suppress stem-like states in both malignant and non-malignant cells (Martin et al., 2024). Future biomarker discovery must incorporate epigenomic, metabolic, and microenvironmental signatures—leveraging single-cell multi-omics and computational modeling—to capture the full spectrum of CSC biology (Anderson et al., 2025). Clinical translation will depend on the validation of these integrated profiles in large, prospective cohorts and their harmonization with evolving liquid biopsy technologies.

Liquid Biopsy Principles and Technologies: Focus on Circulating Tumor Cells (CTCs) and Circulating Tumor DNA (ctDNA)

Overview of Liquid Biopsy Concepts

Liquid biopsy, an umbrella term for the analysis of tumor-derived analytes in body fluids, offers a real-time, minimally invasive window into tumor biology and dynamics, directly addressing the limits of traditional tissue sampling in heterogeneity and longitudinal monitoring[33,38]. Key analytes for liquid biopsy include circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), as well as extracellular vesicles and microRNAs, each with unique molecular and clinical insights into cancer progression, metastasis, and therapy response[34,39].

Circulating Tumor Cells (CTCs)

Biology and Clinical Importance

CTCs are cells shed from primary or metastatic tumors into the bloodstream, playing a pivotal role as precursors of distant metastases and as indicators of disease status[35,36.] Unlike tissue biopsy, CTC analysis enables dynamic, repeatable tumor surveillance from a simple blood draw, capturing not only molecular heterogeneity but also real-time changes related to therapy and disease progression [37,38].

Detection and Enrichment Technologies

- Immunomagnetic separation (e.g., CellSearch®): Uses antibodies against epithelial markers such as EpCAM, enabling high specificity for epithelial-origin CTCs but with reduced sensitivity for CTCs undergoing EMT and losing EpCAM expression [39,38]

- Microfluidic and size-based platforms: Capture CTCs based on size, deformability and antigen-independence, which improves recovery of diverse CTC phenotypes but can yield background hematopoietic cells [34].
- Single-cell -omics: Recent advances allow molecular profiling (DNA, RNA, protein, epigenome) at the single-CTC level, elucidating tumor subclonality and stemness features [36,38].
- Tumor Biology and Clinical Applications
- Metastatic cascade: Only a minority of CTCs possess stem-cell–like properties enabling them to survive the rigors of circulation, evade immune attack, and seed distant organs. The characterization of stemness and EMT phenotypes within CTCs is an active area of research, with direct relevance to understanding metastasis and resistance to therapy[35,36,39].
- Minimal residual disease: CTC counts and characterization are being actively evaluated for early Prognosis and monitoring: Enumeration of CTCs correlates strongly with disease progression, overall survival, and response to treatment across major solid tumors [33].
- Molecular profiling: CTCs enable assessment of actionable mutations, monitoring clonal evolution, and the detection of emerging mechanisms for therapeutic resistance without repeated tissue biopsy[38,36].
- Early detection and cancer detection and prediction of relapse post-therapy[38,35].

Circulating Tumor DNA (ctDNA)

Biology and Detection

ctDNA comprises fragmented DNA released predominantly by apoptotic and necrotic tumor cells into the bloodstream. Unlike cell-free DNA (cfDNA), which arises from both normal and tumor cells, ctDNA specifically mirrors tumor genetic and epigenetic alterations, including point mutations, copy number variations, methylation signatures, and gene rearrangements[40,9].

Analytical Methods

- PCR-based assays (e.g., digital droplet PCR): Offer highly sensitive detection of known mutations but are limited in multiplexing capacity.
- NGS (Next Generation Sequencing): Allows genome-wide mutation profiling, assessment of tumor mutational burden, and identification of novel or rare variants.
- Methylation analysis: Detects tumor-specific epigenetic changes, expanding the utility of ctDNA for early detection[41].

Clinical Utility

- Diagnosis and early detection: ctDNA enables noninvasive detection of tumor-specific mutations and is increasingly validated for early-stage cancer diagnosis, with sensitivity and specificity metrics improving through multimodal approaches[34,33,42].
- Prognosis and minimal residual disease (MRD) assessment: Levels of ctDNA after therapy predict recurrence much earlier than radiographic methods, providing crucial information on residual disease and likely outcomes[48,33,38].
- Therapy guidance: Serial ctDNA analysis reveals emerging resistance mutations (e.g., in EGFR, KRAS), enabling rapid adjustment of targeted therapies[9,10].
- Tumor heterogeneity mapping: ctDNA reflects the aggregate genetic landscape of all tumor sites and subclones, overcoming the sampling bias of tissue biopsies[38].

Limitations and Technical Considerations

- Sensitivity and quantification: Low abundance, fragmentation, and dilution with cfDNA complicate the reliable detection of ctDNA in early-stage and minimal disease contexts [9,38].
- Standardization: Preanalytical (sample collection, processing) and analytical (platform, thresholds) factors require harmonization before universal clinical adoption[43],[38].

Integration of CTCs and ctDNA in Precision Oncology

Joint analysis of CTCs and ctDNA within the same patient sample delivers complementary information: CTCs provide cellular context for metastatic potential and functional assays, while ctDNA offers detailed mutation profiling and quantitative monitoring (Ma et al., 2024; Iliescu et al., 2019; Lin et al., 2021). In combination, these technologies are shaping the future of personalized cancer medicine by enabling dynamic, noninvasive monitoring of tumor evolution, therapeutic response, and resistance.

Cancer Stem Cell Biomarkers in Circulating Tumor Cells (CTCs)

Circulating tumor cells (CTCs) serve as a direct cellular surrogate of tumor biology in peripheral blood, providing clinically actionable insights into tumor evolution, dissemination, and therapeutic resistance. Over the past decade, significant advances have been made in the identification, isolation, and molecular characterization of CTCs that express cancer stem cell (CSC) markers, as these cells are thought to be the primary drivers of metastatic progression and recurrence[44,45,46]. The presence of CSC-like features among CTCs—including surface marker expression, functional plasticity, and enrichment after therapy—may hold critical prognostic and predictive value, informing both early intervention strategies and personalized treatment regimens.

Detection and Characterization of CSC Biomarkers in CTCs

CTCs are rare among peripheral blood cells, necessitating sensitive and specific methods for their enrichment and detection. EpCAM remains one of the principal targets for CTC capture, but this approach is limited in cancers with low or absent EpCAM expression due to epithelial-mesenchymal transition (EMT)[49]. Consequently, the field is rapidly evolving toward multi-marker and marker-independent enrichment strategies to identify CSC-enriched CTC subpopulations. CD44, CD24, CD133, and ALDH1 expression in CTCs have been associated with stem-like phenotypes, while N-cadherin and vimentin—both EMT markers—correlate with metastatic potential and therapy resistance [47]. Flow cytometry, immunocytochemistry, and single-cell sequencing platforms enable detailed molecular profiling of CTCs for these markers, as well as non-coding RNAs that regulate stemness[48,46].

Emerging functional assays—including sphere formation and organoid culture from CTCs—further validate the stem-like properties of these rare cells, extending the operational definition of CSCs beyond static marker expression toward dynamic functional readouts[49]. Such approaches are increasingly integrated with digital pathology and machine learning to distinguish clinically significant CSC-positive CTCs from other circulating cell types.

CSC-Like CTCs and Clinical Implications

The identification of CSC biomarkers in CTCs has shed light on several key aspects of cancer management:

- Prognosis: High expression of CSC markers in CTCs (e.g., CD44, CD133, ALDH1) is significantly associated with worse progression-free and overall survival in breast, colorectal, lung, and prostate cancers ([41]; Liu et al., 2024). This observation underscores the potential of CSC-enriched CTCs as prognostic biomarkers.
- Resistance monitoring: Following treatment with chemotherapy or targeted agents, CSC marker–positive CTCs often persist or increase, hinting at the emergence of resistant subclones responsible for relapse[46,47]. Serial tracking of these cells could offer an early warning system for disease progression.
- Minimal residual disease (MRD): CSC-enriched CTCs are detectable in patients with no radiological evidence of disease and may predict subsequent recurrence, supporting their use in MRD assessment[50,48].
- Therapy guidance: The molecular characteristics of CSC-positive CTCs (e.g., actionable mutations, epigenomic signatures) can inform adaptive therapy selection, addressing evolving resistance mechanisms directly from liquid biopsy[46].

Technical and Biological Challenges

Despite their promise, the reliable detection and clinical interpretation of CSC biomarker–positive CTCs remain challenging. Circulating tumor cells are inherently heterogeneous, and their rarity in peripheral blood demands ultra-sensitive technologies. The plasticity of stemness in CTCs—driven by microenvironmental cues, therapy, and even ex vivo handling—can lead to transient marker expression or loss[49,48]. Furthermore, validation of CSC markers in CTCs requires correlation with tissue-based CSC assays and prospective outcomes in large patient cohorts.

Standardization of preanalytical and analytical protocols is essential for clinical translation, as is the integration of multi-omic profiling to capture the full spectrum of stemness features (Liu et al., 2024; [46]. Emerging technologies, such as ex vivo expansion of CTCs and organoids, are opening new avenues for functional validation of CSC biomarker–positive CTCs and their use in drug sensitivity testing and personalized medicine [49,48].

Cell-Free Nucleic Acids: ctDNA, ctRNA, and Cancer Stem Cell (CSC) Profiling

Biological Underpinnings and Release Mechanisms

Cell-free nucleic acids (cfNAs)—specifically, circulating tumor DNA (ctDNA) and circulating tumor RNA (ctRNA)—are released into the circulation via multiple mechanisms, including apoptosis, necrosis, mitotic catastrophe, and active secretion in extracellular vesicles such as exosomes[51]. These cfNAs provide an aggregate snapshot of tumor clonal architecture, reflecting both dominant and minor subclones, and are shaped by the dynamic activity of rare, plastic cancer stem cell (CSC) populations responsible for metastasis, relapse, and resistance[52]. ctDNA, typically 90–200 bp in length, carries tumor-specific mutations, copy number alterations, chromosomal rearrangements, and methylation changes that allow for noninvasive tracking of tumor (and CSC) evolution[9,53]. ctRNA—comprising mRNA, microRNA (miRNA), long non-coding RNA (lncRNA), and circular RNA (circRNA)—extends this window, capturing both coding and regulatory signals involved in stemness maintenance and therapy resistance[54,41].

Advanced Analytical Platforms

The detection and molecular profiling of rare CSC-associated cfNAs demand highly sensitive, multiplexed analytical strategies:

- Digital and droplet digital PCR (ddPCR)**:** Enable ultra-sensitive detection of rare mutant alleles and stemness-associated mutations, but are limited in multiplexing capacity and discovery power[55].
- Next-generation sequencing (NGS)**:** Ultra-deep sequencing allows for comprehensive detection of point mutations, insertions/deletions, and structural variants in genes such as

TP53, NOTCH, WNT/β-catenin, and TERT, which are frequently implicated in CSC biology[56,57].

- Third-generation sequencing**:** Technologies such as single-molecule real-time (SMRT) and nanopore sequencing directly interrogate long-range structural variants, methylation, and hydroxymethylation, uncovering noncanonical regulatory regions and rare CSC-derived events[58,59].
- Methylation and fragmentomics**:** Bisulfite sequencing and methylation arrays detect CSC-specific epigenetic signatures, while fragmentomic analysis of ctDNA size, end structures, and nucleosome footprints reveals chromatin dynamics uniquely associated with stemness [60,61].
- Exosomal and non-coding RNA profiling: Exosome-encapsulated miRNAs, lncRNAs (e.g., HOTAIR), and circRNAs, implicated in CSC plasticity and resistance, can be reliably detected in plasma, often with enhanced stability [62].
- Machine learning and AI: Integrating multi-omic cfNA data (mutations, methylation, fragmentomics, ncRNAs) via machine learning enhances biomarker discovery and clinical utility [63,64].

Multi-Omic CSC Signatures in cfNAs

- Somatic mutations**:** Serial ctDNA analysis tracks the emergence and clonal dynamics of mutations in genes central to CSC biology, such as TP53, SOX2, NOTCH, LGR5, ALDH1A3, and TERT, providing direct evidence of stem-like clonal evolution and adaptive resistance [65,66].
- Epigenetic and methylation profiles**:** Detection of hypo- or hypermethylation at loci such as SOX2, OCT4, LGR5, and ALDH1A3 in ctDNA is predictive of CSC phenotype persistence and poor prognosis [67,66].
- Non-coding RNA networks**:** Plasma-based profiling of miRNAs (e.g., miR-34a), lncRNAs, and circRNAs involved in CSC regulation and resistance (NOTCH, WNT, Hedgehog pathways) offers additional diagnostic and prognostic stratification[41] (Li et al., 2022; ; He et al., 2022).
- Exosome cargo: CSCs actively secrete exosomes containing nucleic acids and proteins that modulate self-renewal, immune evasion, and niche adaptation, and these exosomal signatures are detectable in plasma .[69,70].

Clinical Applications

- Early diagnosis and risk stratification: Composite panels integrating ctDNA mutation, methylation, and non-coding RNA profiles demonstrate improved sensitivity for early cancer detection and risk stratification, particularly when optimized for stemness signatures (Saad et al., 2024; Luo et al., 2021).
- Minimal residual disease (MRD) and recurrence**:** Serial cfNA analysis for CSC-related alterations (mutations, methylation, ncRNAs) outperforms conventional protein biomarkers in detecting MRD and predicting relapse, enabling earlier therapeutic intervention[71,43].
- Therapy monitoring and resistance**:** Longitudinal tracking of CSC-associated genetic and epigenetic changes, as well as emerging ncRNAs, informs adaptive therapy, predicts resistance, and facilitates enrollment in biomarker-driven clinical trials [72,74].
- Personalized trials and computational integration**:** Machine learning–driven integration of multimodal cfNA data supports the development of composite biomarkers with enhanced predictive value for CSC-driven resistance and relapse [63,64]).

Analytical and Translational Challenges

Despite rapid progress, several barriers impede clinical implementation:

- Sensitivity and specificity**:** Detecting rare CSC-derived cfNAs is challenged by low abundance, high fragmentation, and interference from clonal hematopoiesis[76].
- Preanalytical and technical variables: Inconsistent sample processing, storage, and cfNA extraction protocols can introduce bias and limit reproducibility[53,61].
- Biological and temporal heterogeneity: Intratumoral evolutionary plasticity and clonal selection necessitate frequent, standardized longitudinal sampling and robust data harmonization[61,54].
- Data integration and interpretation**:** Harmonizing multi-layered omic data (genomic, epigenomic, transcriptomic, fragmentomic) with clinical outcomes requires advanced computational tools and rigorous external validation [64].

Ongoing advances in single-molecule sequencing, methylation analysis, non-coding RNA profiling, and computational integration are rapidly maturing cfNA-based stemness biomarker applications for future precision oncology[52,56,]

Extracellular Vesicles and Exosomal Biomarkers of Cancer Stem Cells

Biogenesis and Characterization of Cancer Stem Cell–Derived Extracellular Vesicles

Extracellular vesicle (EVs), especially exosomes (30–150 nm vesicles of endosomal origin), are actively secreted by cancer stem cells (CSCs) and play a pivotal role in intercellular communication, tumor progression, and therapeutic resistance[77]. Exosome biogenesis begins with endosomal

membrane invagination, forming intraluminal vesicles (ILVs) within multivesicular bodies (MVBs), which are subsequently released into the extracellular space upon MVB fusion with the plasma membrane[78]. CSCs modulate the molecular composition of their exosomes to package proteins, nucleic acids (DNA, mRNA, miRNA, lncRNA, circRNA), lipids, and metabolites that reflect and reinforce stemness, metastasis, and immune modulation[50]. This cargo enables CSC-derived exosomes to reprogram recipient cells, including non-CSCs, stromal components, and immune cells, fostering a pro-tumorigenic niche (Whiteside, 2023).

Isolation and Molecular Profiling of CSC-Derived Exosomes

Current approaches for exosome isolation and enrichment—including ultracentrifugation, size-exclusion chromatography, immunocapture, and microfluidics—vary in purity, yield, and compatibility with downstream analyses (Mathieu et al., 2021). Immunoaffinity capture using antibodies against CSC surface markers (e.g., CD44, CD133) can enrich for exosomes of stem-like origin, while non-specific methods permit broader cargo analysis[79]. Once isolated, exosomal cargo is characterized by proteomics, transcriptomics, and lipidomics, revealing complex biomolecular signatures that distinguish CSC-derived exosomes from those of non-CSC tumor cells and normal tissues (Lee et al., 2022).

Non-Coding RNAs and Metabolic Regulation in CSC Exosomes

CSC exosomes transport a variety of non-coding RNAs (ncRNAs) that regulate self-renewal, differentiation, and therapy resistance in recipient cells. MicroRNAs (e.g., miR-200c, miR-34a) suppress differentiation and promote stemness, while lncRNAs (e.g., H19, MALAT1) orchestrate chromatin remodeling and transcriptional programs that sustain CSC properties (Yang et al., 2023; Li et al., 2023)[80,81]. Circular RNAs (circRNAs) are increasingly recognized for their stability and regulatory roles, with some implicated in chemoresistance and metastatic progression (Kristensen et al., 2022)[82]. In addition to ncRNAs, CSC exosomes carry metabolites (e.g., lactate, glutamine derivatives) that influence the metabolic fitness of both tumor and stromal cells, supporting adaptation to hypoxia, nutrient deprivation, and therapeutic stress (Minciacchi et al., 2023)83.

Clinical Relevance and Liquid Biopsy Applications

The molecular signatures of CSC-derived exosomes—including membrane proteins, ncRNAs, and metabolites—are being developed as diagnostic, prognostic, and predictive biomarkers in liquid biopsy (Zhao et al., 2023)[84].

- Early Detection and Prognosis**:** Exosomal CSC markers (e.g., CD44, CD133) and ncRNAs (e.g., miR-34a, HOTAIR) have shown utility in identifying high-risk patients and predicting

recurrence, even in the absence of radiological or conventional biomarker evidence (García-Silva et al., 2023; Zavridis et al., 2023)[86,85].

- Therapy Response and Resistance**:** Serial monitoring of CSC exosomal cargo in patient plasma can reveal emerging resistance mechanisms and guide therapeutic adaptation (Kleppe et al., 2021)[87].
- Personalized Medicine**:** The integration of exosomal RNA and protein profiling with other liquid biopsy analytes (CTCs, ctDNA) offers a complementary, multi-modal approach for real-time assessment of CSC dynamics in precision oncology (Choi et al., 2022)[88].

Analytical and Translational Challenges

Despite their promise, several barriers hinder clinical adoption of CSC exosome biomarkers:

- Isolation and Purity**:** Achieving high purity exosome enrichment without contamination by apoptotic bodies or protein aggregates remains technically challenging, affecting downstream analyses (Morales-Kastresana et al., 2022)[89].
- Heterogeneity**:** Exosomal cargo varies with CSC phenotype, tumor type, and environmental context, complicating the identification of universal biomarkers (Gao et al., 2023)[90].
- Standardization**:** Lack of harmonized protocols for exosome isolation, storage, and characterization limits reproducibility and comparability across studies (Thery et al., 2018).
- Clinical Validation**:** Prospective, large-scale studies are needed to establish the sensitivity, specificity, and clinical relevance of CSC exosome biomarkers for early detection, MRD, and therapy guidance (Kalluri & LeBleu, 2020)[77].

Tumor-Educated Platelets (TEPs) and Other Non-Traditional Liquid Biopsy Components

Tumor-Educated Platelets: Biological Overview

Tumor-educated platelets (TEPs) are an emerging liquid biopsy analyte (Best et al., 2017; Nilsson et al., 2011[91]), comprising circulating platelets that absorb and process tumor-derived RNAs, proteins, and various signaling molecules through direct interaction with tumor cells and tumor microenvironment factors (Jiang et al., 2023; [50]). This “education” results in a specific remodeling of the platelet transcriptome and proteome (Best et al., 2015; Li et al., 2022)[92,93], producing signatures indicative of cancer presence, classification, and dynamics, including features of cancer stem cell (CSC) populations. Tumor cells modulate platelet function via direct microvesicle transfer, exosome-mediated signaling, and soluble factor release—mechanisms now understood to preferentially shuttle CSC-derived transcripts and proteins into the platelet population.

Platelets have been shown to participate in almost every major aspect of cancer progression, including immune evasion (Labelle et al., 2011; Haemmerle et al., 2018)[94,95] by shielding circulating

tumor cells (CTCs), potentiation of metastatic niche formation, and facilitation of angiogenesis, which are all driven in part by CSC activity and phenotype adaptation. Recent studies have demonstrated that TEPs can mirror the stemness landscape of the tumor, with RNA profiles containing markers such as SOX2, OCT4, NANOG, and ALDH1A1 (Jain et al., 2021; Sol et al., 2020)[96.97], correlating with CSC abundance and aggressiveness.

Detection and Profiling Technologies

- RNA sequencing (RNA-seq): Global TEP transcriptome sequencing has revealed (Best et al., 2017; Best et al., 2015) robust, discriminatory expression patterns capable of distinguishing cancer versus non-cancer and even resolving CSC-associated subtypes in various malignancies.
- Targeted PCR/microarray: Quantitative detection of key CSC and tumor-associated transcripts (Li et al., 2022; Garcia et al., 2018)[98] (including those regulating pluripotency, epithelial-mesenchymal transition, and therapy resistance) is possible with high specificity and sensitivity.
- Proteomics and lipidomics: Platelet-derived protein and lipid cargo profiling (Campbell & Schwertz, 2021; Robert et al., 2022)[99,100] further complements nucleic acid analysis, uncovering post-translational modifications and metabolic state shifts directly reflective of tumor and CSC influence.

Integration of TEP analysis with ctDNA, CTC, and exosomal profiling (Suárez-Cabrera et al., 2023; Sadeghi et al., 2023)[101,102] supports a multi-parametric, dynamic picture of tumor—including CSC—evolution in real time.

Clinical Applications for CSC Detection and Monitoring

- Early detection/cancer typing: Distinct CSC-related transcript signatures in TEPs distinguish cancer patients (Best et al., 2017; Sol et al., 2020) from healthy individuals and differentiate among cancer subtypes with high accuracy, outperforming some ctDNA/CTC assays, especially in early-stage disease settings.
- MRD and recurrence prediction: Serial monitoring of TEPs reveals dynamic CSC transcript changes (Kakar et al., 2022; Garcia et al., 2018)[112,113] preceding radiological recurrence or conventional marker elevation, suggesting utility for minimal residual disease (MRD) tracking.
- Therapy response and resistance adaptation: Changes in TEP RNA cargo (Gan et al., 2021[103]; Best et al., 2018), particularly stemness and resistance regulators, correlate closely with

treatment response or emergence of drug-resistant CSC clones, supporting adaptive therapy modulation.

Analytical and Biological Challenges

- Heterogeneity: Platelets respond to systemic conditions such as infection, inflammation (Roweth & Welsh, 2021; Sun et al., 2022)[104,105], and metabolic stress, which may confound the cancer- or CSC-specific signal, demanding sophisticated normalization and clinical filtering strategies.
- Lack of standardization**:** Pre-analytic variables—including collection, isolation (Sun et al., 2022)[105], storage, and processing—significantly impact TEP data, and harmonized protocols are required for cross-study reproducibility.
- Functional validation: Although TEP signatures cross-correlate with CSCs (Garcia et al., 2018; Klement & Milsom, 2017[106]) in tissue and plasma, their precise functional impact and the most actionable targets require further prospective validation across diverse tumor types and clinical scenarios.

Other Non-Traditional Liquid Biopsy Analytes

- Tumor-educated neutrophils (TENs): Display distinct transcriptomic and proteomic shifts (Sagiv et al., 2015; Powell et al., 2016)[107,108] in response to tumor- and CSC-derived signals, with emerging data supporting their role in tracking disease activity and stemness-related inflammation.
- Cell-free mitochondrial DNA (cf-mtDNA): Reflects stemness-related metabolic plasticity (Lee et al., 2016; Xie et al., 2022[109]) and is associated with chemoresistance and aggressive tumor behavior; its quantification and mutation profiling show promise for CSC burden estimation.
- Soluble metabolite/protein biomarkers: Quantification of CSC-associated metabolites (Tarazona et al., 2024)[110] (e.g., high ALDH1 activity, oncometabolites) and circulating proteins reveals additional, functional dimensions of CSC activity, supporting integrated biomarker panels.

Clinical Translation and Challenges in Integrating CSC Biomarkers into Liquid Biopsy

Integration of Multiple Modalities for Comprehensive CSC Profiling

The transition of cancer stem cell (CSC) biomarker research from bench to bedside is increasingly enabled by the integration of multiple liquid biopsy modalities—circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), RNA, extracellular vesicles (EVs), and tumor-educated platelets (TEPs). Each modality offers unique and complementary insights into CSC biology, from clonal

dynamics and stemness signatures to microenvironmental cross-talk and therapy resistance (Chen et al., 2022; Suárez-Cabrera et al., 2023)[101]. Multi-analyte liquid biopsy platforms now combine molecular profiling with functional assays, such as CTC organoid cultures and ex vivo drug sensitivity testing, to stratify patients and guide dynamic therapy selection. However, successful clinical integration requires rigorous validation across tumor types, stages, and treatment phases, as well as harmonized protocols for sample collection, processing, and data analysis.

Clinical Scenarios and Current Evidence

- Early Detection and Risk Stratification**:** Composite CSC biomarker panels in liquid biopsies are being evaluated for cancer screening and early detection, with increasing evidence that stemness-associated mutations, methylation patterns, and exosomal cargo can identify high-risk individuals before clinical disease manifestation (García-Silva et al., 2023[86]; Kakar et al., 2022).
- Minimal Residual Disease (MRD) and Recurrence Prediction**:** The ability to detect rare CSC-driven subclones via ctDNA, CTC, or exosomal biomarkers—often outpacing imaging and conventional biomarkers—offers a new paradigm for MRD monitoring and early intervention in patients at risk for relapse (Soda et al., 2022[43]; Tie et al., 2022[111]).
- Therapy Guidance and Adaptive Treatment**:** Real-time, multi-analyte profiling can identify CSC-driven resistance mechanisms—such as secondary mutations, stemness gene upregulation, or altered metabolic states—enabling timely therapy switches and personalized enrollment in targeted clinical trials (Heitzer et al., 2019[9,10]; Choi et al., 2022)[88].
- Prospective Clinical Trials**:** Several ongoing and recently completed trials are evaluating CTC- and ctDNA-guided interventions, setting precedents for the future inclusion of exosomal and TEP biomarkers in mainstream oncology practice (Saad et al., 2024; [52]).

Analytical, Regulatory, and Ethical Challenges

- Technical Standardization**:** Lack of harmonized protocols for sample acquisition, storage, processing, and analysis across different laboratories remains a major hurdle to the reproducibility and clinical adoption of CSC liquid biopsy assays (Sun et al., 2022[105]; Morales-Kastresana et al., 2022)[89].
- Analytical Sensitivity and Specificity**:** Distinguishing true CSC-driven signals from background noise—due to limited material, clonal hematopoiesis, or non-tumor sources—demands constant technological refinement and rigorous quality control (Newman et al., 2016[75]; Cristiano et al., 2019)[61].

- Interpretation and Clinical Utility**:** Clear clinical utility thresholds and actionable reporting frameworks must be established, especially for low-frequency or emerging CSC biomarkers that may not yet have established prognostic or predictive value. The integration of artificial intelligence (AI) and machine learning is accelerating biomarker discovery and clinical decision support, but these approaches require large, diverse, and prospectively validated datasets (Arias-Pulido et al., 2024[63]; Duffy et al., 2021)[64].
- Regulatory and Ethical Considerations**:** The rapid pace of technological innovation outpaces regulatory frameworks, raising questions about test validation, reimbursement, and the responsible handling of sensitive genomic and phenotypic data.

Future Directions

The next generation of liquid biopsy platforms will likely incorporate single-cell analysis, spatial 'omics, digital pathology, and AI-driven data integration to capture the full spectrum of CSC dynamics in real time and across tumor ecosystems (Binnewies et al., 2023[56]; Arias-Pulido et al., 2024)[63]. Prospective multi-center studies and international consortia are essential to validate these tools, establish clinical grade cut-offs, and drive their adoption into routine oncological practice.

Future Directions and Conclusion

Emerging Technologies and Multimodal Integration

The future of cancer stem cell (CSC) detection and monitoring via liquid biopsy lies in **multimodal integration** leveraging not just circulating tumor DNA (ctDNA) or circulating tumor cells (CTCs), but also extracellular vesicles (EVs), tumor-educated platelets (TEPs), and potentially even other cellular and acellular components such as circulating free mitochondria and metabolites. Single-cell multi-omic analysis, now entering routine clinical laboratories, enables simultaneous profiling of DNA mutations, RNA expression, protein levels, and epigenetic marks from rare cells or fragments. High-resolution spatial transcriptomics and proteomics, combined with artificial intelligence (AI) and deep learning analytics, will further enhance the ability to decode tumor heterogeneity and CSC dynamics in real time (Arias-Pulido et al., 2024[63]; Binnewies et al., 2023; Saad et al., 2024). Digital pathology and in silico modeling are expected to bridge the gap between liquid biopsy signals and tissue context, supporting a more holistic view of the tumor ecosystem. Integration of these platforms with patient-derived organoids and ex vivo drug screening will enable functional validation of biomarker positivity and guide precision intervention (Choi et al., 2022[88]; Suárez-Cabrera et al., 2023).

Liquid Biopsy as a Dynamic Monitoring Tool

Liquid biopsies are poised to become central for real-time, longitudinal monitoring of tumor evolution, therapy response, and emergence of resistance. As protocols for multi-analyte analysis are standardized, liquid biopsies will allow clinicians to track not only gross tumor burden but also the rare, actionable CSC clones that drive disease recurrence and therapy failure. This paradigm will be invaluable for early intervention, adaptive therapy, and endpoint determination in clinical trials, especially in cancers with high intra-tumor heterogeneity or rapid clonal evolution (Tie et al., 2022; Kakar et al., 2022; [52]).

Challenges in Clinical Adoption

Clinical translation of these advances faces several significant hurdles:

- Standardization: Harmonization of pre-analytical, analytical, and post-analytical processes across laboratories and platforms is essential for clinical validity and reproducibility (Sun et al., 2022[105]; Morales-Kastresana et al., 2022)[89].
- Interpretation and Actionability: Translating molecular signals into clear clinical guidance remains challenging, particularly for low-frequency or emerging biomarkers. AI-driven decision support tools will be critical for parsing complex, multi-omic datasets and identifying the most clinically relevant signals (Arias-Pulido et al., 2024[63]; Duffy et al., 2021)[64].
- Regulatory and Ethical Considerations: Rapid technological progress often outpaces the development of regulatory frameworks, reimbursement policies, and ethical guidelines. International consortia and public-private partnerships will be necessary to establish best practices and ensure equitable access to these advanced diagnostics.

The Promise of Precision Oncology

By capturing the full diversity of tumor biology—including CSC-driven resistance and plasticity—liquid biopsies are transforming oncology from static, tissue-based diagnosis to dynamic, patient-centered precision medicine. The potential for early detection of relapse, tailored therapeutic regimens, and adaptive clinical trial designs is unprecedented. As liquid biopsy technologies mature, their integration into routine care will depend on robust evidence from prospective studies, scalable platforms, and multidisciplinary collaboration.

Concluding Perspective

The quest to understand and clinically target cancer stem cells via liquid biopsy is at a pivotal juncture. Technological innovation, multimodal data integration, and AI-powered analytics are rapidly advancing the field toward clinically actionable insights. However, the realization of this promise depends on overcoming standardization, interpretation, and regulatory challenges through

collaboration and global partnerships. The integration of CSC biomarker research with liquid biopsy platforms heralds a new era of precision oncology, offering hope for earlier intervention, durable remissions, and ultimately, improved outcomes for patients with cancer.

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Conflict of Interest

Authors of this paper declare no conflict of interest.

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