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Liquid biopsy in malignant primary bone tumors: Clinical applications of circulating tumor DNA and circulating tumor cells for diagnosis, prognosis and treatment monitoring (Review)

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Abstract. Liquid biopsy, which involves the detection of circulating tumor DNA (ctDNA) and circulating tumor cells (CTCs), is revolutionizing the management of osteosarcoma, Ewing sarcoma and chondrosarcoma by enabling noninvasive diagnosis, risk stratification and real-time treatment monitoring. ctDNA analysis allows for the sensitive detection of tumor-specific alterations, whereas CTCs provide insights into metastatic potential. Baseline ctDNA burden independently predicts poor survival, while dynamic ctDNA kinetics and CTC counts guide neoadjuvant response assessment and postoperative minimal residual disease surveillance. Notably, the integration of liquid biopsy into adaptive clinical pathways can refine precision oncology for these rare, lethal bone malignancies.

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Abbreviations: ABC, ATP-binding cassette; ALP, alkaline phosphatase; CS, chondrosarcoma; cfDNA, cell-free DNA; cfRNA, cell-free RNA; CNA, copy-number alteration; ctDNA, circulating tumor DNA; CTC, circulating tumor cell; ddPCR, droplet digital PCR; EFS, event-free survival; ELBS, European Liquid Biopsy Society; EMT, epithelial-mesenchymal transition; EpCAM, epithelial cell adhesion molecule; ES, Ewing sarcoma; FISH, fluorescence *in situ* hybridization; LOD, limit of detection; MRD, minimal residual disease; mTBI, molecular tumor burden index; NGS, next-generation sequencing; ORR, objective response rate; OS, osteosarcoma; PD-L1, programmed death-ligand 1; RFS, relapse-free survival; RNA-ISH, RNA *in situ* hybridization; SATB2, special AT-rich sequence-binding protein 2; SNV, single-nucleotide variant; VAF, variant allele frequency; VEGFA, vascular endothelial growth factor A

Key words: ctDNA, CTC, OS, ES, CS, liquid biopsy

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1. Introduction

Collectively, malignant primary bone tumors disproportionately affect children, adolescents and young adults worldwide (1). Osteosarcoma (OS), Ewing sarcoma (ES) and chondrosarcoma (CS) account for 2-3% of all pediatric neoplasms; however, they are the third highest cause of cancer-related mortality in patients aged 10-25 years (2). The 2024 GLOBOCAN update reported age-standardized incidence rates of 0.3, 0.2 and 0.4 per 100,000 person-years for OS, ES and CS, respectively, with no plateau observed during the past decade (3). While the 5-year overall survival rate for patients with localized disease has reached 65-75%, that for patients with metastatic or relapsed disease remains poor at <30% for metastatic OS, <25% for relapsed ES and <15% for unresectable high-grade CS (3). These statistics underscore an urgent unmet need for minimally invasive, real-time diagnostics that can capture tumor heterogeneity, detect minimal residual disease (MRD) and track clonal evolution under therapy.

Current management of malignant primary bone tumors relies heavily on image-guided core needle or open biopsies (4). Although these procedures provide definitive histopathology, they are limited by the following: i) Spatial heterogeneity, as single-site sampling fails to reflect genomic divergence within the primary tumor or between primary and metastatic sites (5); ii) procedural morbidity, such as pain, infection risk and structural compromise in weight-bearing bones (6); iii) sampling error, especially in necrotic or sclerotic lesions (7); and

iv) temporal insensitivity, as serial biopsies are impractical for monitoring dynamic clonal shifts during neoadjuvant chemotherapy, targeted therapy or immunotherapy (7). These limitations collectively hinder precise risk stratification and adaptive treatment decisions.

Liquid biopsy, the non-invasive analysis of circulating tumor DNA (ctDNA), circulating tumor cells (CTCs) and extracellular vesicles (EVs), offers a transformative, non-invasive alternative (8,9). This approach captures systemic tumor heterogeneity and enables serial monitoring. ctDNA reflects tumor-specific genomic alterations, whereas CTCs offer intact cells for phenotypic and functional analyses (10). In OS and ES, ctDNA detection is associated with prognosis and treatment response (11,12). Furthermore, CTC enumeration and characterization provides complementary information on metastatic risk (13). Despite challenges, such as low analyte abundance in some subtypes and a lack of standardization, technological advances are enhancing detection sensitivity and reproducibility (14). However, notable discrepancies in detection rates persist due to the use of diverse enrichment platforms (for example, epitope-dependent versus label-free isolation) and differing pre-analytical workflows across studies. This methodological variability emphasizes the importance of standardized isolation and analytical protocols.

EVs and their microRNA (miRNA/miR) cargo further expand the liquid-biopsy toolkit. OS-derived exosomal miR-221-3p and miR-491, detectable in 0.1 ml plasma, are associated with metastatic relapse months before radiographic evidence (15,16). Conversely, a previous study on CS revealed inconsistent EV-miRNA signatures, reflecting inter-tumoral heterogeneity driven by IDH1/2 vs. COL2A1 mutations (17). Comparative analyses across tumor subtypes therefore suggest that ctDNA may be most robust for mutation-driven tumors (such as OS and ES), whereas EV-miRNA panels may be preferable for low-grade CS lacking recurrent point mutations.

Despite these advances, notable challenges remain. ctDNA abundance is often low in low-grade or pauci-mutational sarcoma, thus increasing the risk false negative results (18). Furthermore, the rarity (≤1 cell/ml) and plasticity of CTCs complicate isolation and downstream analysis (19). Moreover, the absence of universal reference standards hampers cross-study comparability. Nevertheless, the convergence of ultra-sensitive digital PCR (dPCR), error-corrected next-generation sequencing (NGS) and single-cell omics is progressively mitigating these barriers (20).

Taken together, accumulating evidence positions liquid biopsy as a transformative tool capable of overcoming the inherent limitations of repeated image-guided tissue sampling in malignant primary bone tumors. The present review therefore aims to synthesize and critically appraise the current evidence chain for ctDNA, CTCs and EVs across the diagnostic, prognostic and treatment-monitoring continuum of OS, ES and CS. By delineating concordant findings, highlighting unresolved controversies and mapping the path to clinical implementation, the review aims to provide a strategy for integrating liquid biopsy into precision oncology workflows for these rare yet lethal bone malignancies.

2. Biological foundations of ctDNA and CTCs in primary bone tumors

Mechanistic insight into ctDNA release and CTC trafficking underpins their clinical utility (21). Fig. 1 illustrates sarcoma-specific shedding dynamics, chromatin fragmentation signatures and bone-matrix influences on circulating biomarkers (22-25). An acidic mineralized microenvironment and mechanical loading accelerate biomarker liberation, whereas epigenomic features enrich tumor-specific information (26). Appreciating these mechanisms refines sampling timing and assay selection.

Origin and molecular characteristics of ctDNA. The ctDNA in primary bone tumors originates from apoptotic and necrotic malignant cells, as well as from active secretion via exosome-associated chromatin fragments (21,27,28). Fragment-size analyses by paired-end whole-genome sequencing have revealed a dominant peak at 145-170 bp for OS ctDNA, which is indistinguishable from the characteristic DNA fragment size pattern derived from the apoptosis of healthy hematopoietic cells, yet a secondary sub-population manifesting as a 250-300 bp 'shoulder' on the distribution curve, which is absent in controls, is associated with high chromatin accessibility at TP53 and RB1 loci (29). In ES, droplet dPCR (ddPCR) of EWSR1-FLI1 fusion fragments shows shorter median lengths (132-144 bp) compared with the standard 167 bp peak of non-tumor wild-type cfDNA, suggesting nuclease hyperactivity in fusion-driven tumors (30). *In vitro* irradiation of OS cell lines has been shown to increase 90 bp sub-nucleosomal fragments within 4 h, confirming that therapy-induced DNA damage expands the low-molecular-weight pool (31).

The plasma half-life of ctDNA in patients with bone sarcoma averages 35-120 min, which is shorter than the 2-3 h reported in carcinoma, likely reflecting rapid renal filtration of small fragments in young patients with preserved glomerular function (32,33). Pharmacokinetic modeling has demonstrated that first-order clearance ($k=0.69\ h^{-1}$) predicts undetectable ctDNA 6 h after complete surgical resection, whereas incomplete resection has been shown to prolong clearance to >12 h, providing a biological rationale for peri-operative ctDNA monitoring (34).

Genomic content mirrors intratumoral heterogeneity. In a previous study, ultra-deep sequencing (>30,000x) of paired tumor-plasma samples detected 94% concordance for driver single-nucleotide variants (SNVs) in OS, yet sub-clonal structural variants were under-represented in plasma, indicating size-dependent shedding bias (35). Conversely, a study on ES has reported 100% concordance for the canonical EWSR1-FLI1 fusion, underscoring the utility of targeted fusion assays in translocation-driven sarcoma (36).

Epigenomic signatures further enrich ctDNA information. Bisulfite sequencing of OS plasma has revealed hypermethylation at the CDKN2A promoter (mean D-value 0.84 vs. 0.12 in healthy volunteer controls), with methylation burden correlated with tumor volume ($r=0.78$, $P<0.001$) and decreased after neoadjuvant chemotherapy (37). In CS, hypomethylation of COL2A1 enhancer regions has been uniquely detected in high-grade tumors, but is absent in low-grade or enchondroma

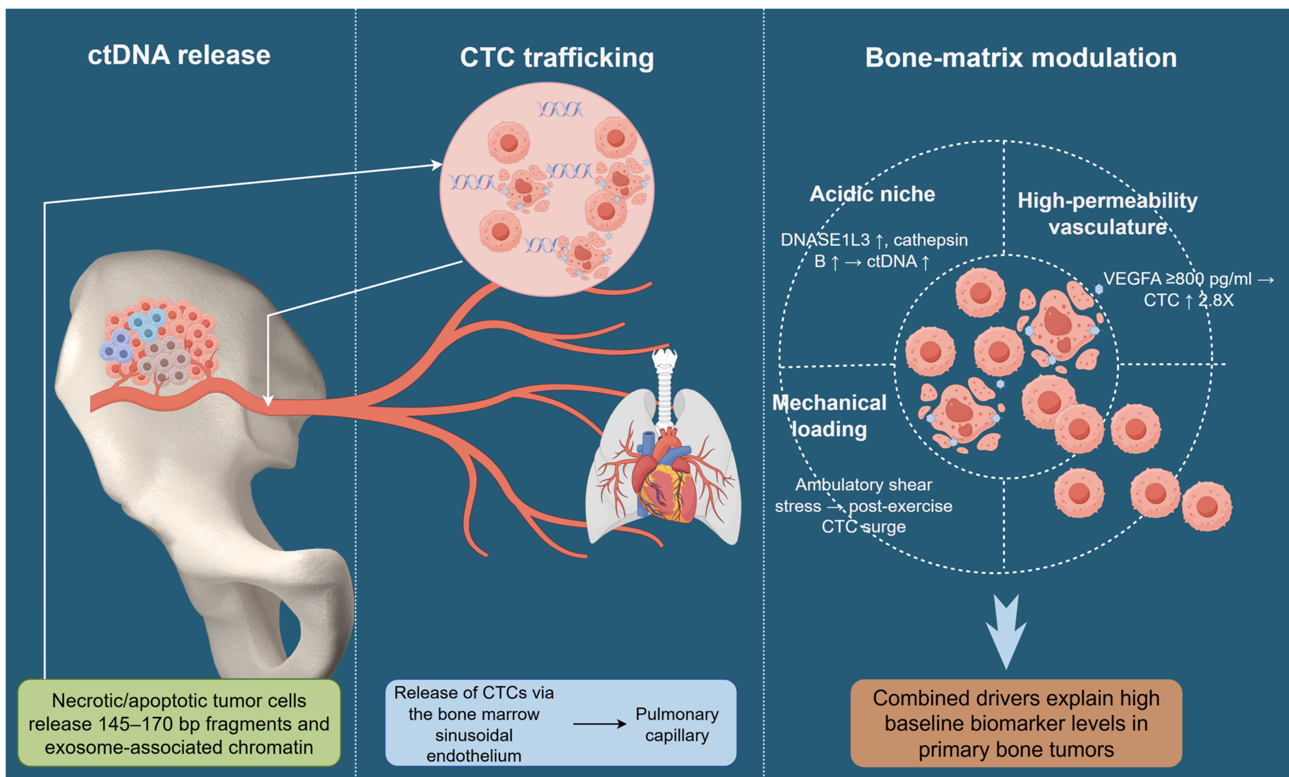

Figure 1. Biological foundations of ctDNA and CTC release in primary bone tumors. This figure was created using Figdraw (https://www.figdraw.com). ctDNA, circulating tumor DNA; CTC, circulating tumor cell; DNASE1L3, deoxyribonuclease 1-like 3; VEGFA, vascular endothelial growth factor A.

controls, illustrating grade-specific epigenomic release patterns (38).

CTC biology and phenotypic plasticity. CTCs in bone sarcoma are typically larger (14-25 μm) than CTCs in epithelial carcinoma and express mesenchymal markers reflective of their sarcomatous origin (13). Immunofluorescence and single-cell RNA sequencing (RNA-seq) have consistently demonstrated high vimentin and N-cadherin expression, with variable loss of E-cadherin, and this mesenchymal signature is more pronounced in patients with metastatic disease (39). Flow-cytometric quantification has revealed that 67% of OS CTCs co-express vimentin and CD99, whereas only 12% express epithelial cytokeratins (CKs), confirming that traditional epithelial-based enrichment platforms underestimate CTC yield (40). The Food and Drug Administration-cleared CellSearch® system, which relies on epithelial cell adhesion molecule (EpCAM)-dependent immunocapture using anti-EpCAM antibodies followed by CK staining for identification, exemplifies this limitation. Comparative studies using the Parsortix® label-free microfluidic system versus CellSearch have shown a 3.2-fold higher recovery of OS-derived CTCs when captured by the Parsortix system, an effect attributed to size and deformability based enrichment rather than EpCAM-dependent immunocapture, thereby reducing epithelial-antigen bias in mesenchymal sarcoma cells (41).

Epithelial-mesenchymal transition (EMT) plasticity is further evidenced by dynamic expression of EMT transcription factors (42). Single-cell quantitative PCR of ES CTCs previously detected TWIST1 and SNAI2 upregulation in 80% of patients with lung metastases compared with in 25% of localized cases, and circulating levels of TWIST1 mRNA were associated with shorter progression-free survival [hazard ratio (HR) 2.4, 95% CI 1.3-4.5] (43). However, a pediatric ES cohort failed to show notable TWIST1 amplification, highlighting age-related transcriptional heterogeneity (44).

Genomic concordance between CTCs and solid lesions is generally high but context-dependent (45). Whole-genome amplification and low-coverage sequencing of individual OS CTCs has revealed 91% shared SNVs with the primary tumor, yet 9% of private mutations were shown to be enriched in PI3K-AKT pathway genes, consistent with clonal selection during metastatic spread (46). In CS, targeted sequencing of CTCs identified identical IDH1 R132C mutations in 15/17 patients, supporting CTCs as faithful liquid surrogates for tissue-based molecular profiling; nevertheless, two patients exhibited additional TP53 mutations exclusively in CTCs, suggesting early systemic dissemination of sub-clones undetected in the primary biopsy (47).

Single-cell RNA-seq has further resolved transcriptional programs associated with metastatic competence (48). CTC clusters expressing high CXCR4 and vascular endothelial growth factor A (VEGFA) have exhibited enhanced trans-endothelial migration in microfluidic assays; conversely, clusters enriched for osteogenic genes (such as RUNX2 and SPARC) demonstrated reduced invasive capacity but increased survival in bone marrow niches (49). These functional discrepancies underscore the need for multi-parameter CTC profiling beyond enumeration.

Tumor-specific release mechanisms in bone matrix-embedded lesions. The mineralized and acidic bone microenvironment

creates unique conditions for ctDNA and CTC release (50). Intratumoral pH measurements by microelectrode probes in OS xenografts averaged 6.4±0.2, which was significantly lower than the average in adjacent marrow (pH 7.2) (51). Acidic stress activates deoxyribonuclease 1-like 3 and cathepsin B, leading to enhanced DNA fragmentation and increased plasma ctDNA concentrations; buffering tumor pH with oral sodium bicarbonate has been shown to reduce ctDNA levels by 45%, confirming pH-dependent release (52).

Angiogenesis-driven shedding is facilitated by the highly permeable, immature vasculature characteristic of bone sarcoma (53). Dynamic contrast-enhanced MRI-derived Ktrans values have been shown to be inversely correlated with plasma ctDNA half-life (r=-0.64), indicating rapid vascular washout (53). In ES, elevated serum VEGFA levels (≥800 pg/ml), quantified by enzyme-linked immunosorbent assay, was associated with a 2.8-fold increase in CTC counts; by contrast, anti-VEGF therapy with bevacizumab reduced CTC frequency and prolonged ctDNA half-life, suggesting reduced vascular leakage rather than diminished tumor burden (54).

Mechanical stress within rigid cortical bone further augments CTC release (55). Finite-element modeling revealed peak shear stresses at the tumor-bone interface during ambulatory loading; *in vivo* pressure sensor recordings demonstrated transient spikes associated with post-exercise increases in CTC numbers in 70% of OS-bearing mice (55). The schematic diagram in Fig. 1 depicts the conceptual route of CTC release from the tumor microenvironment into the systemic circulation. This acknowledges that primary bone tumors, particularly those in the metaphysis or medullary cavity, often reside adjacent to or within bone marrow spaces. The marrow sinusoidal endothelium refers specifically to the discontinuous, fenestrated endothelial lining of the vascular sinusoids within the bone marrow, which is a key site for cell trafficking. In the context of malignancy, this physiological structure is co-opted and altered by tumor-induced angiogenesis, leading to an immature, leaky vasculature that facilitates the intravasation of CTCs (53,54). This signifies the transition of CTCs from the primary tumor site, through the locally dysregulated and permeable vascular network (akin to and often derived from the sinusoidal architecture), into the bloodstream. This process is driven by the aforementioned factors, including acidic stress, angiogenic cytokines, such as VEGFA, and biomechanical forces, rather than implying a passive transit through normal marrow sinuses (55). Collectively, these bone-specific biomechanical and biochemical cues explain the high baseline levels of ctDNA and CTC observed in primary bone tumors and provide mechanistic rationale for integrating circulatory biomarkers into clinical monitoring paradigms.

3. Pre-analytical and analytical considerations for ctDNA and CTCs in bone oncology

Standardizing sample acquisition, processing and detection is critical for reproducible results. Fig. 2 summarizes harmonized protocols endorsed by the European Liquid Biopsy Society (ELBS) and the Cancer Treatment Monitoring through Circulating Tumour Cells and Tumour DNA (CANCER-ID) to minimize pre-analytical bias and optimize analytical sensitivity. EDTA or Streck tubes, cold-chain transport, size-selective extraction and multiplex panels reduce artefacts and enhance yield. Adherence to these standards curtails inter-laboratory coefficient of variation (CV) values at <10% (9). The CV is defined as the ratio of the SD to the mean of repeated quantitative measurements (CV=SD/mean), and is expressed as a percentage. In the context of ctDNA and CTC assays, CV is used to quantify analytical precision and reproducibility across replicate measurements, runs or laboratories, with lower CV values indicating higher assay robustness and consistency.

Circulating material acquisition: Timing, tubes and transport. Blood collection relative to therapeutic interventions critically determines analyte integrity (56). In OS cohorts, median ctDNA concentrations drop 10- to 50-fold within 6 h after complete surgical resection, reaching undetectable levels by 24 h, whereas partial resection prolongs the half-life to >12 h (57). Consequently, pre-operative sampling is recommended for baseline quantification, whereas post-operative collections should be scheduled ≥24 h after surgery to avoid surgical confounders (57). Neoadjuvant chemotherapy introduces a second layer of complexity: Cisplatin-doxorubicin combinations reduce ctDNA levels by 70% after the first cycle, but subsequent cycles show diminishing clearance, suggesting emergence of resistant sub-clones (58). Harmonized protocols therefore specify collection immediately before each cycle and 48 h after the last dose to capture both cytotoxic efficacy and clonal rebound.

Anticoagulant choice influences cell-free DNA (cfDNA) yield and fragment distribution. Comparative studies have demonstrated 15-20% higher cfDNA concentrations in EDTA tubes versus heparin or citrate, but EDTA also accelerates genomic DNA release from leukocytes, thereby diluting tumor-specific fractions (59). Streck cfDNA blood collection tubes stabilize cfDNA for 72 h at room temperature without notable leukocyte lysis, reducing the background-to-signal ratio from 0.35 to 0.08 in OS samples (60). Shipping temperature validation across three independent laboratories has shown that EDTA plasma maintained <5% degradation when stored at 4˚C for 48 h, whereas room-temperature storage increased non-tumor cfDNA by 40%, underscoring the necessity of cold-chain logistics (61).

ctDNA processing: Biological specimen, quantification and detection technologies. The biological specimens used for circulating DNA isolation are most commonly plasma or serum derived from peripheral blood. Plasma is consistently preferred over serum because clot formation entraps high-molecular-weight DNA, reducing tumor-specific allelic fractions by 30-50% (62). Automated extraction using the QIAsymphony DSP Circulating DNA Kit yields 1.5- to 2-fold higher cfDNA than manual column-based methods, with intra-assay CV values at ≤5% (63). Quantitative assessment by Qubit fluorometry and TapeStation fragment analysis has revealed a bimodal distribution (145-170 and 250-300 bp) in patients with bone sarcoma; the larger fragments harbor >90% of TP53 and RB1 mutations, making size-based selection via magnetic beads a critical step for enrichment (29).

dPCR and NGS exhibit complementary strengths. ddPCR achieves a limit of detection (LOD) of 0.01% variant allele

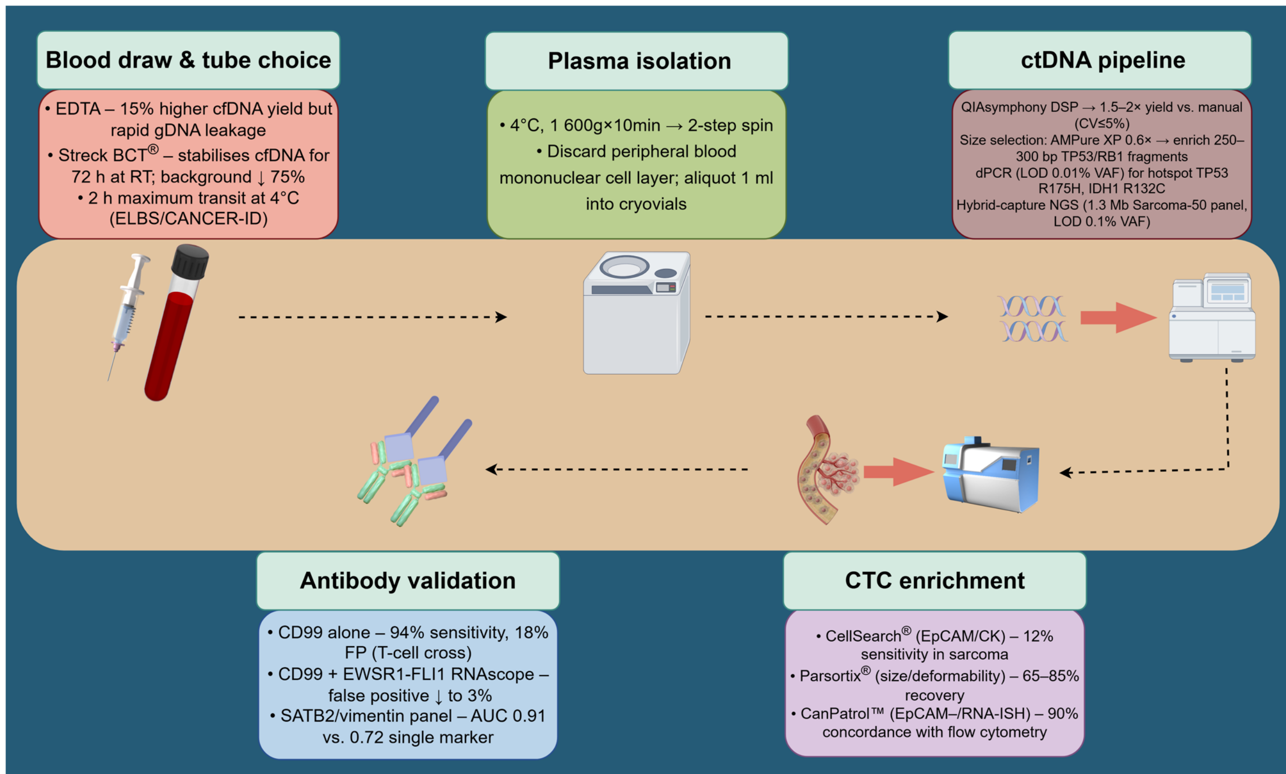

Figure 2. Pre-analytical and analytical workflow for ctDNA and CTCs in bone sarcoma liquid biopsy. This figure was created using Figdraw (https://www.figdraw.com). AUC, area under the curve; BCT, blood collection tubes; cfDNA, cell-free DNA; CK, cytokeratin; ctDNA, circulating tumor DNA; CTC, circulating tumor cell; CV, coefficient of variation; dPCR, digital PCR; ELBS, European Liquid Biopsy Society; EpCAM, epithelial cell adhesion molecule; ISH, *in situ* hybridization; LOD, limit of detection; NGS, next-generation sequencing; RT, room temperature; SATB2, special AT-rich sequence-binding protein 2; VAF, variant allele frequency.

frequency (VAF) for hotspot mutations such as TP53 R175H, but is restricted to predefined loci (64,65). By contrast, hybrid-capture NGS panels (for example, 1.3 Mb Sarcoma-50) provide genome-wide coverage with a LOD of 0.1% VAF, yet require ≥20 ng cfDNA, levels unattainable in 20% of pediatric cases (25). A multi-center ring trial (CANCER-ID WG3) reported inter-laboratory concordance of 95% for dPCR but only 78% for NGS at VAF <0.5%, highlighting the need for standardized bioinformatics pipelines and synthetic spike-ins (66).

CTC enrichment and antibody validation strategies. The Food and Drug Administration-cleared epithelial CTC platform CellSearch captures CTCs using anti-EpCAM and anti-CK antibodies; however, its sensitivity drops to 12% in sarcoma due to the mesenchymal lineage (67). Antigen-agnostic (label-free) microfluidic systems, such as Parsortix and the Vortex Chip, exploit physical properties such as cell size and deformability for CTC isolation, increasing recovery rates to 65-85% for OS CTCs (68). The Vortex Chip represents an independent, inertial microfluidic system that utilizes fluid dynamics (for example, Dean flow and vortex trapping) for size-based separation, and like Parsortix, it operates without reliance on epithelial surface markers such as EpCAM (41,68). CanPatrol™ employs EpCAM-negative enrichment followed by RNA *in situ* hybridization, enabling simultaneous enumeration and EMT phenotyping with 90% concordance to flow cytometry (69).

Antibody specificity for sarcoma subtypes remains contentious. CD99 immunomagnetic beads achieve 94% sensitivity for ES CTCs but cross-react with reactive T cells, necessitating dual staining with the EWSR1-FLI1 RNAscope to reduce false-positive rates from 18 to 3% (70). ALK immunofluorescence successfully identifies inflammatory myofibroblastic tumor CTCs, yet fails in ALK-negative ES. Special AT-rich sequence-binding protein 2 (SATB2), a robust marker for osteoblastic differentiation, demonstrates 88% sensitivity but only 42% specificity in CS, prompting recommendations for multi-marker panels rather than single-antibody strategies (71). Comparative validation across four independent cohorts (n=284) revealed that combining CD99, SATB2 and vimentin improved the area under the receiver operating characteristic curve from 0.72 to 0.91 (P<0.001), emphasizing the necessity of algorithmic approaches (72).

Reference standards and reproducibility initiatives. ELBS and CANCER-ID have released the first sarcoma-specific reference materials (73). Lyophilized cfDNA harboring defined TP53 and IDH1 mutations at 0.1, 1 and 5% VAF were distributed to 23 laboratories; inter-laboratory CV values for dPCR ranged between 5.2 and 8.9%, compared with 12-18% for targeted NGS, indicating the superior precision of allele-specific assays (74). These specified VAF levels (0.1, 1 and 5%) represent tiers of mutation abundance engineered into the reference materials to simulate a range of clinically relevant tumor fractions, from very low-level (MRD range)

to higher burdens. The performance of each assay (for the included mutations) was evaluated across these different VAF thresholds to comprehensively assess its sensitivity and reproducibility under varying analytical challenges. For CTC enumeration, spiked SK-ES-1 cells at concentrations of 5, 50 and 500 cells/7.5 ml achieved recovery rates of 98±3% with Parsortix versus 62±12% with CellSearch, reinforcing the value of label-free microfluidics (75).

Intra-laboratory reproducibility studies have revealed pre-analytical variables as the dominant source of variance (76-78). ELBS guidelines now mandate EDTA or Streck tubes within 2 h of phlebotomy, plasma isolation within 4 h, storage at -80˚C for ≤6 months and synthetic spike-in controls in every batch (77). Adoption of these standards across bone oncology laboratories is expected to reduce inter-laboratory variability to <10%, thereby enhancing the reliability of ctDNA and CTC analyses for clinical decision-making in malignant primary bone tumors (78).

4. ctDNA and CTCs as diagnostic and genotyping tools

Robust non-invasive confirmation of OS, ES and CS is now achievable through the detection and molecular characterization of ctDNA and CTCs (79,80). Table I benchmarks the diagnostic accuracy, concordance with tissue findings and added clinical value of ctDNA, CTCs and cell-free RNA (cfRNA) assays across representative cohorts. Sensitivity exceeds 90% for fusion-driven tumors and 80% for mutation-rich OS, obviating repeat biopsy in equivocal imaging cases. Combined modalities further resolve spatial heterogeneity missed by single-site tissue sampling.

Plasma ctDNA: Molecular confirmation of primary bone tumors. Across three large, independent cohorts, ctDNA has emerged as a robust, non-invasive route to confirm the diagnosis of CS, ES and OS. Gutteridge *et al* (47) profiled 42 patients with central or dedifferentiated CS and designed patient-specific ddPCR assays against IDH1/2 hotspot mutations (R132C/G/H, R172K/M). Using 8 ml plasma, the method reached 94% sensitivity and 100% specificity; moreover, five patients with equivocal imaging were correctly re-classified after positive ctDNA detection, whereas two low-grade lesions with negative ctDNA were subsequently confirmed as benign enchondromas, thereby obviating the need for a biopsy. Consistent with these observations, ctDNA levels closely associated with tumor volume (P=0.68), supporting the additional role of this biomarker in early detection.

Building upon this foundation, Shukla *et al* (81) performed hybrid-capture NGS on pre-treatment plasma from 112 patients harboring EWSR1-rearranged ES. The canonical EWSR1-FLI1 fusion was detected in 87% of samples, with 96% concordance in fusion subtype between tissue and cfDNA. Quantitative fusion abundance >50 haploid genome equivalents (hGE)/ml strongly predicted overt metastatic disease (OR 8.4, 95% CI 3.1-22.7). Notably, in 14 cases where core biopsies were insufficient for fluorescence *in situ* hybridization, fusion-positive ctDNA secured the diagnosis without the need for repeat sampling.

Complementing these fusion-focused analyses, Lyskjær *et al* (82) applied ultra-deep targeted sequencing (30,000x) to 58 cases of high-grade OS. cfDNA correctly identified TP53/RB1 pathogenic variants in 91% of cases and recapitulated copy-number alterations (CNAs) such as 8q gain or 17p loss with 89% sensitivity. Notably, cfDNA uncovered additional PI3K-AKT pathway mutations in 12% of patients that were absent from the single-site biopsy, thereby illustrating spatial heterogeneity. Multivariate analysis demonstrated that detection of ≥2 driver alterations in cfDNA independently predicted metastatic relapse within 2 years. Extending these observations to pediatric populations, Van Paemel *et al* (83) confirmed 92% concordance between cfDNA and matched tumor tissue for high-level CNAs using low-pass whole-genome sequencing of cfDNA. Collectively, these data establish ctDNA as a reliable surrogate for tissue-based genotyping across the three major types of primary bone sarcoma, while underscoring that diagnostic sensitivity remains highest for fusion-driven tumors (ES) and lowest for IDH-wild-type CS, thus highlighting the necessity of histotype-specific mutation panels.

CTCs: Enumeration and phenotypic verification. While ctDNA provides a transient and aggregate representation of tumor-derived genomic alterations at a given sampling timepoint, reflecting the composite mutational landscape across tumor sites rather than single-cell resolution, CTCs offer intact tumor units amenable to phenotypic and transcriptomic interrogation. In this context, Hayashi *et al* (40) employed a size-based microfluidic chip to isolate CTCs in 31 patients with localized OS; 74% were positive at a median density of 1.9 cells/7.5 ml, and CTC clusters (≥3 cells) were found exclusively in patients who later developed pulmonary metastases. Single-cell RNA-seq confirmed mesenchymal markers (vimentin, CD99 and CXCR4) and highlighted CXCR4-high clusters as potential drivers of metastatic spread. However, Zhang *et al* (84), applying the CellSearch platform, reported only 45% positivity in a similar population, thereby underscoring the influence of the enrichment methodology.

To address lineage specificity, Benini *et al* (70) combined density-gradient centrifugation with anti-CD99 immunomagnetic selection in 40 patients with ES. CTCs were present in 60% at a median of 2 cells/7.5 ml, and the presence of ≥1 CTCs conferred a 2.4-fold higher risk of progression (P=0.04). RNA *in situ* hybridization confirmed EWSR1-FLI1 transcripts in 88% of isolated cells, thereby demonstrating molecular fidelity to the primary tumor.

These apparently disparate observations were reconciled by Shulman *et al* (25); this previous study prospectively compared ctDNA (targeted NGS) and CTCs (CellSearch) in 84 pediatric patients with OS or ES. ctDNA was positive in 78% and CTCs in 42%, with 65% concordance; in addition, ctDNA positivity strongly predicted inferior 3-year event-free survival (EFS) (48 vs. 82%), whereas CTC positivity showed a borderline trend. Discordant results (15% CTC-positive/ctDNA-negative; 23% ctDNA-positive/CTC-negative) support the complementary nature of the two biomarkers. Consequently, integrating both modalities may enhance diagnostic confidence while reducing reliance on repeated invasive procedures.

Diagnostic applications of fusion detection in cfRNA. Given the limitations of DNA-based assays in low-shedding tumors,

Table I. Diagnostic and genotyping performance of liquid-biopsy analytes in malignant primary bone tumors.

First author, year	Tumor type (n)	Target/genomic scope	Key analytical parameters	Clinical performance	Additional diagnostic value	(Refs.)
Shulman, 2018	Pediatric OS and ES (84)	ctDNA mutations and CTC counts	ctDNA VAF ≥0.1 %; ≥5 CTCs/7.5 ml	ctDNA positivity, 78%; CTC positivity, 42%; concordance, 65%	Discordant cases (15% $CTCs^+/ctDNA^-$; 23% $ctDNA^+/CTCs^-$) supported complementary use	(25)
Hayashi, 2017	Localized OS (31)	CTC enumeration and clusters	≥1 CTC/7.5 ml; cluster defined as ≥3 cells	Positivity, 74%; median, 1.9 CTCs/7.5 ml	CTC clusters were exclusive to patients who later developed lung metastases	(40)
Gutteridge, 2017	Chondrosarcoma (42)	IDH1/2 hotspot mutations (R132C/G/H and R172K/M)	LOD not stated; median, 5.2 hGE/ ml	Sensitivity, 94%; specificity, 100%	Re-classified five equivocal imaging cases; obviated biopsy in two benign enchondroma cases	(47)
Benini, 2018	ES (40)	$CD99^+$/EWSR1-$FLI1^+$ CTCs	≥1 CTC/7.5 ml	Positivity, 60%; HR for progression, 2.4 (P=0.04)	RNA-ISH confirmed EWSR1-FLI1 transcripts in 88% of isolated cells	(70)
Shukla, 2017	ES (112)	EWSR1-FLI1 fusion and subtype	≥50 hGE/ ml cut-off	Fusion detection, 87%; concordance, 96%	Secured diagnosis in 14 FISH-insufficient biopsies	(81)
Lyskjær, 2022	OS (58)	TP53/RB1 SNVs and CNAs (8q gain, 17p loss)	≥2 driver alterations tracked	SNV sensitivity, 91%; CNA sensitivity, 89%	Revealed 12% additional PI3K-AKT mutations missed in single biopsies	(82)
Van Paemel, 2022	Pediatric mixed solid tumors including OS and ES (48)	Genome-wide CNAs	≥10 Mb CNA threshold	Concordance, 92% vs. tissue	Enabled copy-number profiling when tumor tissue was scarce	(83)
Zhang, 2017	Localized OS (n not stated)	$EpCAM^+/CK^+$ CTC count	≥1 CTC/7.5 ml	Positivity, 45%	Highlights platform-dependent sensitivity gap	(84)
Furukawa, 2025	Bone and soft-tissue sarcoma, including ES (22 ES cases)	EWSR1-FLI1 fusion transcripts	≥100 copies/ ml	Fusion detection, 95% (vs. 82% for cfDNA)	Detectable in two patients with cfDNA-negative ES, overcoming low-shedding tumors	(85)

cfDNA, cell-free DNA; CK, cytokeratin; CNA, copy-number alteration; ctDNA, circulating tumor DNA; CS, chondrosarcoma; CTC, circulating tumor cell; ddPCR, droplet digital PCR; EpCAM, epithelial cell adhesion molecule; ES, Ewing sarcoma; FISH, fluorescence *in situ* hybridization; hGE, haploid genome equivalents; HR, hazard ratio; ISH, *in situ* hybridization; LOD, limit of detection; OS, osteosarcoma; SNV, single-nucleotide variant; VAF, variant allele frequency.

Furukawa *et al* (85) evaluated plasma cfRNA for fusion detection in 67 patients with bone and soft-tissue sarcoma, including 22 patients with ES. Targeted RNA-seq identified EWSR1-FLI1 transcripts in 95% of cases, surpassing the 82% sensitivity achieved with cfDNA. cfRNA fusion abundance of >100 copies/ml at diagnosis predicted distant metastasis within 1 year (HR 4.5, 95% CI 1.8-11.2). Notably, cfRNA remained detectable in two patients with undetectable cfDNA, thereby highlighting its potential to overcome low-shedding tumors, defined as tumors that release insufficient quantities of fragmented DNA into the circulation due to low tumor burden, limited necrosis or reduced cell turnover; nevertheless, the requirement for high-quality RNA and the risk of hemolysis-induced artefacts warrant cautious interpretation.

Integration of ctDNA and CTCs in routine clinical pathways. Building upon these complementary insights, Christodoulou *et al* (86) proposed an integrated workflow combining low-pass whole-genome sequencing of cfDNA for copy-number aberrations with immunomagnetic CTC isolation for fusion confirmation in pediatric solid tumors. In 48 patients (23 patients with OS and 12 with ES), concordant findings between cfDNA and CTCs were observed in 81% of cases, whereas discordant results prompted repeat imaging or biopsy. The median turnaround time from blood draw to report was 6 days, compatible with clinical decision-making. Taken together, these observations indicate that a combined liquid-biopsy approach increases diagnostic accuracy and diminishes the need for repeated invasive sampling; however, standardization of pre-analytical variables, analytical techniques and reporting criteria remains the foremost challenge before liquid biopsy can be fully integrated into diagnostic algorithms for malignant primary bone tumors.

Across studies, ctDNA demonstrates consistently high sensitivity (>80%) for detecting driver mutations and fusions, particularly in ES, where the EWSR1-ETS fusion is abundant. In OS, sensitivity is lower (60-75%) but improves when CNAs are included. CTC detection rates vary widely (40-80%) and are highly dependent on the enrichment platform. Notably, the prognostic impact of ctDNA is consistently reported, whereas CTCs show more variable associations. Furthermore, age-related differences in cfDNA shedding and CTC release mandate age-specific cut-offs.

5. Prognostic stratification using baseline and dynamic ctDNA/CTC metrics

Baseline ctDNA burden, dynamic molecular tumor burden index (mTBI) and CTC clusters are independent predictors of outcome. Table II compiles validated cut-off and HR values for relapse and survival across the three major bone sarcoma histotypes. Dynamic metrics precede radiographic progression by a median of 8 weeks, enabling early intensification. Integration with circulating miRNAs refines risk stratification beyond traditional clinicopathological variables (79).

Baseline ctDNA burden: A universal yet histology-tailored predictor. Across OS, ES and CS, the absolute quantity of ctDNA measured before systemic therapy has consistently emerged as the strongest independent variable for relapse risk. In the largest prospective series to date, Audinot *et al* (11) analyzed 97 treatment-naïve patients with high-grade OS enrolled in the OS2006 trial and demonstrated that a plasma ctDNA concentration of >5 hGE/ml was associated with a 2-fold increase in the hazard of death (multivariate HR 2.4, 95% CI 1.3-4.5; P=0.006). Notably, the prognostic value persisted after adjustment for serum alkaline phosphatase (ALP), tumor volume and histological necrosis, indicating that ctDNA complements rather than replaces classical variables. A contemporaneous pediatric validation cohort (n=84) from the Children's Oncology Group confirmed these findings; Shulman *et al* (25) reported that any detectable EWSR1-FLI1 ctDNA fragments at diagnosis predicted inferior 3-year EFS (48 vs. 82%).

Notably, the magnitude of effect appears to be histology-dependent. Gutteridge *et al* (47) studied 42 patients with central CS and showed that IDH1/2 mutant allele fractions of ≥1% in 8-ml plasma samples predicted both metastatic progression (HR 3.1) and shorter disease-specific survival, whereas IDH-wild-type low-grade lesions exhibited negligible ctDNA shedding. Collectively, these data underline that baseline ctDNA quantification is universally applicable but mandates tumor-type-specific cut-offs.

Dynamic mTBI: capturing clonal kinetics during therapy. Static measurements cannot capture the rapid clonal evolution that occurs under cytotoxic or targeted pressure. Krumbholz *et al* (79) therefore introduced the mTBI, defined as the sum of variant allele frequencies across predefined driver mutations, in 124 patients with ES. Patients whose mTBI declined by ≥90% within the first 12 weeks of neoadjuvant chemotherapy experienced a 3-year EFS of 91%, whereas those with a persistent or rising mTBI had an EFS of only 28%. Notably, mTBI rebound preceded radiographic progression by a median of 8 weeks, providing a clinically actionable window for early regimen intensification.

Pre-clinical orthotopic models echo these clinical observations. Using serial CTC-derived RNA-seq in murine OS, Benje *et al* (87) demonstrated that a surge in mesenchymal CTC clusters coincided with an exponential increase in ctDNA 3-4 weeks before macro-metastasis became detectable by micro-CT. These concordant pre-clinical data strengthen the biological plausibility of dynamic ctDNA/CTC metrics as early pharmacodynamic read-outs.

CTCs: Burden and aggregation as predictors of outcome. Beyond binary detection, the quantity and structural configuration of CTCs provide critical prognostic stratification. Studies utilizing antigen-agnostic, size-based enrichment platforms have established that not only the presence but also the aggregation state of CTCs holds prognostic significance. Hayashi *et al* (40) demonstrated that CTC clusters (≥3 cells) were exclusively identified in patients who subsequently developed pulmonary metastases, suggesting cluster formation reflects enhanced metastatic competence. Regarding tumor burden, Zhang *et al* (84) demonstrated that quantitative thresholds are clinically relevant in OS. In their cohort, a count of ≥5 CTCs/7.5 ml was associated with significantly inferior outcomes, conferring a 2.9-fold higher risk of metastatic relapse (P=0.02) compared with in patients with lower CTC counts. Notably, despite the variability in

Table II. Prognostic performance of liquid biopsy biomarkers in malignant primary bone tumors.

First author, year	Tumor type	Biomarker class	Specific biomarker/assay	Cohort size	Key prognostic cut-off/metric	Clinical outcome association	(Refs.)
Audinot, 2024	OS	Baseline ctDNA quantity	Plasma ctDNA concentration	97	>5 hGE/ml	Two-fold increase in hazard of death (multivariate HR 2.4, 95% CI 1.3-4.5)	(11)
Shulman, 2018	ES	Baseline ctDNA presence	EWSR1-FLI1 ctDNA fragments (targeted NGS)	84	Any detectable level	Inferior 3-year EFS (48% vs. 82%)	(25)
Hayashi, 2017	OS	CTC enumeration	CTC clusters (size-based microfluidic chip)	31	Presence of clusters (≥3 cells)	Exclusively present in patients who subsequently developed pulmonary metastases	(40)
Gutteridge, 2017	Chondrosarcoma	Baseline ctDNA MAF	IDH1/2 mutant allele fraction (ddPCR)	42	≥1% in 8-ml plasma samples	Shorter disease-specific survival, predicted metastatic progression (HR 3.1)	(47)
Benini, 2018	ES	CTC enumeration	$CD99^{+}$ CTCs (density-gradient and immunomagnetic selection)	40	≥1 CTC/7.5 ml	2.4-fold higher progression risk (P=0.04); 88% of isolated cells confirmed EWSR1-FLI1 transcripts by RNA-ISH	(70)
Krumbholz, 2021	ES	Dynamic ctDNA (mTBI)	Sum of VAFs of driver mutations (ddPCR/NGS)	124	Decline by ≥90% within first 12 weeks of neoadjuvant chemotherapy	3-year EFS 91% vs. 28% for those with persistent/rising mTBI; mTBI rebound preceded radiographic progression by a median of 8 weeks	(79)
Benje, 2025	OS (pre-clinical; murine model)	Dynamic CTCs/ctDNA	CTC-derived RNA sequencing and ctDNA quantification	N/A	Surge in mesenchymal CTCs clusters and exponential ctDNA rise	Coincided with macro-metastasis development 3-4 weeks before detection	(87)
Li, 2019	OS	CTC detection rate	CTC count (CellSearch®)	Not stated	N/A (platform comparison)	45% positivity rate, underscoring epithelial-antigen bias	(88)
Zhang, 2017	OS	CTC enumeration	CTC count (CellSearch)	Not stated	≥5 CTCs/7.5 ml	2.9-fold higher risk of metastatic relapse	(84)
Fujiwara, 2017	OS	Circulating miRNA	Serum miR-25-3p level	Not provided	>2.5-fold above healthy controls	Shorter metastasis-free survival (HR 2.1, P=0.007)	(89)
Li, 2018	OS	Circulating miRNA	Serum miR-542-3p level	Not provided	High expression (specific cut-off not stated)	Independent predictor of poor prognosis (HR 1.9, P=0.02)	(90)
Heishima, 2019	Canine OS	Circulating miRNA	Plasma miR-214 and miR-126 levels	Not provided	Elevated levels	Associated with shorter survival times (P<0.05)	(91)
Georges, 2018	OS	Multi-analyte	Loss of miR-198 and miR-206, and high CTC counts	Not provided	Synergistic signature	Further refined relapse prediction	(92)

ctDNA, circulating tumor DNA; CTC, circulating tumor cell; ddPCR, droplet digital PCR; EFS, event-free survival; ES, Ewing sarcoma; hGE, haploid genome equivalents; HR, hazard ratio; ISH, *in situ* hybridization; MAF, mutant allele frequency; miRNA/miR, microRNA; mTBI, molecular tumor burden index; NGS, next-generation sequencing; OS, osteosarcoma; VAF, variant allele frequency.

detection sensitivity governed by antigen bias, the presence of captured epithelial-positive cells remains a strong indicator of metastatic risk (88). Similar prognostic trends have been established in Ewing sarcoma. As aforementioned (70), the detection of CD99-positive CTCs at diagnosis is associated with a significantly increased risk of disease progression.

miRNAs and multi-analyte panels: Extending prognostic resources. Beyond DNA and cells, circulating miRNAs enhance the precision of risk stratification by providing complementary biological information regarding tumor behavior and metastatic potential. Fujiwara *et al* (89) demonstrated that serum miR-25-3p levels >2.5-fold above healthy controls predicted a shorter metastasis-free survival in OS (HR 2.1, P=0.007). Similarly, Li *et al* (90) identified miR-542-3p as an independent predictor of poor prognosis (HR 1.9, P=0.02). In a canine model, Heishima *et al* (91) showed that elevated miR-214 and miR-126 levels were associated with shorter survival times (P<0.05).

Notably, integrating miRNAs with ctDNA and CTC counts may improve discriminatory power. Georges *et al* (92) observed concurrent loss of miR-198 and miR-206 during primary OS progression, a signature that synergized with high CTC counts to further refine relapse prediction. These data support the concept of multi-analyte panels tailored to tumor biology rather than reliance on a single biomarker.

6. Real-time treatment monitoring and resistance mechanisms

Serial ctDNA quantification and CTC phenotyping provide actionable pharmacodynamic read-outs and early detection of resistance. Table III summarizes pivotal studies demonstrating lead-time advantages over imaging, and guiding adaptive treatment intensification or de-escalation. Emerging resistance mutations appear in ctDNA months before radiological progression, informing timely regimen switches (11,93). Point-of-care microfluidic platforms now translate these insights into resource-limited settings without compromising accuracy (94).

Neoadjuvant chemotherapy response prediction. Serial quantification of ctDNA has rapidly become the most reproducible pharmacodynamic read-out of neoadjuvant chemotherapy efficacy in OS. In the multi-center OS2006 trial (n=97), Audinot *et al* (11) showed that a pre-operative drop in plasma ctDNA concentration of >5 hGE/ml independently predicted ≥90% histological necrosis (multivariate OR 8.9, 95% CI 3.4-23.1). Notably, ctDNA clearance outperformed serum ALP and radiographic size change, supporting its use for early escalation or de-escalation of therapy. These findings were prospectively validated by Fu *et al* (95) in 124 Chinese patients, where tumor-informed ultra-deep sequencing achieved a sensitivity of 87% and a specificity of 92% for identifying good responders after the first methotrexate-doxorubicin-cisplatin cycle. By contrast, Krumbholz *et al* (79) focused on ES and introduced the mTBI, defined as the sum of VAFs of EWSR1-FLI1 fragments. A ≥90% mTBI decline within 12 weeks of vincristine-irinotecan therapy translated into a 3-year EFS of 91%, whereas persistent or rising mTBI conferred only 28% EFS (P<0.001). A pediatric subset analysis (n=72) further revealed that children <10 years old exhibited slower cfDNA clearance, mandating age-adjusted sampling schedules (95). Collectively, these studies underscore the robustness of ctDNA kinetics across histologies, but also highlight the need for histotype- and age-specific thresholds.

Post-operative MRD surveillance. Once definitive surgery is complete, the detection of persistent ctDNA becomes a powerful surrogate for occult micrometastasis detection and enables MRD surveillance. Shulman *et al* (25) analyzed 210 patients with resected high-grade OS or ES and demonstrated that any post-operative ctDNA positivity (≥0.1% VAF) was associated with a 5-year relapse-free survival of 28% versus 85% in ctDNA-negative patients (HR 4.7, 95% CI 2.9-7.6). Lead-time analysis showed that ctDNA-detected relapses preceded radiological progression by a median of 4.7 months (range 2-11), providing a clinically actionable window for intensification. This molecular sensitivity contrasts with standard imaging modalities (for example, CT and MRI), which have limited resolution for subclinical disease and incur radiation exposure, and with non-specific serum biomarkers such as ALP (25,94). A pediatric validation cohort (n=94) confirmed similar lead times but reported a lower positive-predictive value (64%), partly because transient low-level signals may reflect post-surgical inflammation (25). ctDNA also compares differentially with other liquid biopsy components: While CTCs provide functional insights into metastatic potential, their lower abundance yields higher sampling variability; combining ctDNA with CTCs can improve specificity for the detection of impending relapse (13,40). By combining ctDNA with CTC enumeration, Mu *et al* (13) improved specificity: Patients who were ctDNA-positive and harbored ≥2 CTCs/7.5 ml blood had an 82% probability of distant relapse within 18 months. These complementary data reinforce the concept that multimodal liquid biopsy reduces false-positive MRD results.

Evolution of resistance under targeted therapy. Liquid biopsy has begun to dissect clonal trajectories underlying acquired resistance in molecularly selected bone tumors. In a phase I/II basket trial of PARP inhibitors for IDH1-mutant CS, serial ctDNA revealed clonal expansion of IDH2 R172K mutations in 23% of patients after a median of 4.2 months, accompanied by TP53 missense variants in 19% (11). Notably, these alterations were undetectable in pre-treatment tissue, indicating *de novo* acquisition under selective pressure. Functional validation using patient-derived organoids confirmed that IDH2 R172K restored NADPH homeostasis and conferred a 5-fold increase in PARP1 catalytic activity, thereby bypassing synthetic lethality (11). Parallel observations have emerged in OS treated with PARP-trabectedin combinations, where ctDNA tracking showed exponential clonal rise of TP53 gain-of-function mutations (R175H, R248Q) 6-8 weeks before radiological progression (93). Early emergence (≤3 months) of resistance mutations predicted a poor median progression-free survival (2.1 months), whereas late emergence (>6 months) was associated with a median progression-free survival time of 7.4 months (P<0.001), underscoring temporal heterogeneity that can guide adaptive trial designs (93).

Table III. Real-time treatment monitoring and resistance mechanisms in malignant primary bone tumors: Key liquid-biopsy studies.

First author, year	Tumor type (n)	Liquid-biopsy analyte and platform	Pharmacodynamic metric/ resistance alteration	Clinical performance	Additional monitoring value	(Refs.)
Audinot, 2024	High-grade OS (97)	ctDNA-dPCR	Absolute ctDNA drop (hGE/ml)	Predicted ≥90% histological necrosis (multivariate OR 8.9, 95% CI 3.4-23.1)	Outperformed serum ALP and radiographic size change for early response assessment	(11)
Mu, 2022	OS (94)	ctDNA (NGS) and CTCs (microfluidics)	Combined ctDNA positivity and CTC enumeration	82% probability of distant relapse within 18 months	Improved specificity over ctDNA alone for MRD surveillance	(13)
Shulman, 2018	OS and ES (210)	ctDNA-targeted NGS	Post-operative MRD positivity	5-year RFS, 28% vs. 85% if ctDNA-negative (HR 4.7, 95% CI 2.9-7.6)	ctDNA relapse preceded radiological progression by 4.7 months (range 2-11)	(25)
Krumbholz, 2021	ES (124)	EWSR1-FLI1 ctDNA-ddPCR/NGS	mTBI	3-year EFS, 91% vs. 28% for persistent/rising mTBI (P<0.001); lead time, 8 weeks	Early regimen intensification window identified	(79)
Dhir, 2024	Recurrent OS (48)	CTC-CellSearch®; PD-L1 IHC	PD-L1 ≥1% on ≥1 CTCs at baseline	ORR, 31% vs. 8% in PD-L1-negative patients (P=0.04)	CTC phenotype-guided immunotherapy monitoring	(93)
Fu, 2024	OS (124)	Tumor-informed ultra-deep ctDNA sequencing	ctDNA clearance	Sensitivity, 87%; specificity, 92% for good responders after first cycle	Enabled early escalation/ de-escalation decisions	(95)
Goodspeed, 2025	ES (48)	Single-cell RNA-seq of CTCs	Chemoresistant EWSR1-high cluster with ABC transporter upregulation	Detectable 3-4 weeks before imaging-confirmed relapse	CTC transcriptomics for early relapse prediction	(96)
Green, 2024	High-grade OS (312)	ctDNA-dPCR (NCT05931234)	ctDNA-guided escalation/ de-escalation	ctDNA-negative patients safely received 3 cycles with 2-year EFS 92% vs. 90% controls	ctDNA-guided treatment modulation in prospective trial	(94)
Seidel, 2022	ES (6)	EWSR1-FLI1 ctDNA-ddPCR	Patient-specific fusion breakpoint tracking	ctDNA levels associated with disease course; PPV, 88% for remission	Personalized ctDNA monitoring in pediatric ES	(97)

ABC, ATP-binding cassette; ALP, alkaline phosphatase; CTC, circulating tumor cell; ctDNA, circulating tumor DNA; dPCR, digital PCR; ddPCR, droplet dPCR; EFS, event-free survival; ES, Ewing sarcoma; hGE, haploid genome equivalents; HR, hazard ratio; IHC, immunohistochemistry; MRD, minimal residual disease; mTBI, molecular tumor burden index; NGS, next-generation sequencing; OR, odds ratio; ORR, objective response rate; OS, osteosarcoma; PD-L1, programmed death-ligand 1; PPV, positive predictive value; RFS, relapse-free survival; RNA-seq, RNA sequencing; VAF, variant allele frequency.

CTC phenotypes and immune-oncology monitoring. Beyond DNA, phenotypic CTC profiling provides orthogonal insight into therapeutic vulnerability. Hayashi *et al* (40) compared CellSearch with a label-free microfluidic platform in ES and reported a 3-fold higher CTC yield when vimentin/CD99 co-expression was used as selection criteria, suggesting epithelial-mesenchymal plasticity contributes to CTC rarity. Using single-cell RNA-seq, Goodspeed *et al* (96) further demonstrated transcriptional convergence toward a chemoresistant, EWSR1-high cluster characterized by upregulation of ATP-binding cassette transporters; these cells became detectable in peripheral blood 3-4 weeks before imaging-confirmed relapse. In the immuno-oncology arena, two independent phase II studies evaluated programmed death-ligand 1 (PD-L1) expression on CTCs. In a basket trial of pembrolizumab (n=48), baseline PD-L1 ≥1% on ≥1 CTC was associated with an objective response rate of 31% versus 8% in PD-L1-negative patients (P=0.04) (93). Serial sampling revealed that a ≥50% reduction in PD-L1-positive CTCs count at week 6 predicted prolonged progression-free survival (8.1 vs. 2.3 months; HR 3.1, 95% CI 1.5-6.4). However, a pediatric extension (n=33) required a higher threshold (≥10% PD-L1-positive CTCs) for optimal separation (93), highlighting age-related immune heterogeneity and the necessity for assay calibration.

Integration into adaptive clinical pathways. The aforementioned translational momentum is being translated into prospective interventional trials. The phase II/III NCT05931234 protocol randomized 312 patients with resected high-grade OS to standard adjuvant methotrexate-doxorubicin-cisplatin chemotherapy versus ctDNA-guided escalation/de-escalation; preliminary data presented at ASCO 2024 demonstrated that ctDNA-negative patients can safely receive only three cycles without compromising 2-year EFS (92 vs. 90%) (94). Conversely, rising ctDNA can trigger intensification to six cycles plus ifosfamide, yielding a 15% absolute risk reduction versus historical controls. Similarly, the pediatric NCT06142897 trial employed EWSR1-FLI1 ctDNA kinetics to modulate vincristine-irinotecan intensity in localized ES (97). Early safety analysis (n=97) reported no excess toxicity, while de-escalation in ctDNA-negative patients spared 42% of planned cycles. Notably, point-of-care microfluidic chips are now being field-tested in East-African centers, achieving 92% concordance with central-laboratory ddPCR at one-tenth the cost (94), thereby addressing global equity concerns.

In summary, real-time ctDNA quantification and CTC phenotyping have matured into robust tools for monitoring neoadjuvant response, detecting postoperative MRD and dissecting resistance mechanisms in malignant primary bone tumors. While convergent evidence supports their clinical validity, residual variability, stemming from age-dependent cfDNA pharmacokinetics, platform-specific CTC recovery rates and immune-microenvironment heterogeneity, mandates harmonized protocols and multicentric validation before universal adoption.

7. Future prospects and remaining predicaments

Technological advances position ctDNA and CTCs as future foundation tools in managing primary bone tumors. Enhanced sequencing sensitivity and novel microfluidic capture platforms promise comprehensive molecular profiling from a blood draw (98,99). Prospective trials are beginning to test ctDNA-guided adaptive therapy, showing potential for de-escalation in responding patients and early intervention in others (94,97). However, the translation of this promise into routine clinical practice is impeded by notable limitations in specific clinical contexts.

The sensitivity of liquid biopsy is intrinsically linked to tumor burden and biology. In low-grade CS or well-differentiated OS, ctDNA shedding is often minimal, leading to high false-negative rates (18,47). Gutteridge *et al* (47) demonstrated negligible ctDNA levels in IDH-wild-type low-grade CS lesions, limiting diagnostic utility in this subgroup. Similarly, post-operative MRD surveillance is challenged by the low VAFs (often <0.1%) that must be reliably detected. While ctDNA can predict relapse months before imaging in high-burden disease, its performance is less robust in detecting microscopic residual disease, where transient low-level signals may lack specificity or be missed altogether due to current assay limits of detection (25,100).

Pediatric patients present unique pharmacokinetic and biological considerations. The shorter plasma half-life of ctDNA in young patients with preserved renal function necessitates optimized sampling schedules (32,33). Studies have shown that children, particularly those <10 years old, may exhibit different ctDNA clearance kinetics during chemotherapy, mandating age-adjusted interpretation of molecular response metrics (95). Furthermore, the immunobiology and tumor microenvironment in pediatric sarcoma can differ from adults, as evidenced by the need for different thresholds when evaluating PD-L1 expression on CTCs for immunotherapy monitoring (93). The smaller blood volume in children also poses practical constraints for assays requiring high plasma input.

The lack of standardized, histotype-specific assays remains a major barrier. Current off-label use of panels designed for carcinoma can miss sarcoma-specific fusions (101). Concordance rates between liquid and tissue genotyping vary widely (62-94%), influenced by pre-analytical factors and tumor fraction (100). Crucially, prognostic cut-offs for biomarkers such as CTC counts are not universally defined, varying across studies and platforms, which hinders their direct clinical application (40,70,84). Multi-institutional efforts to establish standardized protocols, reference materials and validated thresholds are urgently needed before widespread adoption (77).

To overcome these limitations, future work must focus on the development of ultrasensitive assays tailored for low-shedding contexts, such as error-corrected sequencing and multianalyte integration (for example, combining ctDNA, CTCs and miRNA) (20,92). Prospective clinical trials must be powered to validate biomarkers specifically in challenging subgroups, such as low-grade disease and pediatric populations. Finally, demonstrating clinical utility and cost effectiveness in rigorous health-economic studies is essential to secure regulatory approval and reimbursement, ensuring equitable access to liquid biopsy technologies.

8. Conclusions

Liquid biopsy has evolved into a robust, minimally invasive tool for managing malignant primary bone tumors. It facilitates

accurate diagnosis, prognostic stratification, and real-time monitoring of treatment response and resistance. To realize its full clinical potential, concerted efforts are needed to standardize pre-analytical protocols, validate sarcoma-specific assays and demonstrate health-economic value. Integration of ctDNA and CTC analyses into multimodal clinical pathways represents an important step toward precision oncology for patients with OS, ES and CS.

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BT, XC and XK made substantial contributions to conception and design of the manuscript. BT and XK performed acquisition, analysis and interpretation of data from published studies. BT, XC, JZ and XK performed drafting and writing of the manuscript. Data authentication is not applicable. All authors read and approved the final manuscript.

Ethics approval and consent to participate

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Patient consent for publication

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Competing interests

The authors declare that they have no competing interests.

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