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Circulating LncRNA biomarker variability in early cancer detection: implications of standardization and SUMOylation-associated Extracellular Vesicle packaging

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Abstract
Early cancer detection remains challenging due to the limited sensitivity and specificity of current diagnostics. Liquid biopsy offers a minimally invasive alternative, with circulating long non-coding RNAs (lncRNAs) emerging as promising biomarkers because of their stability in biofluids and roles in tumor progression. However, clinical translation is hindered by substantial pre-analytical and analytical variability, as well as biological heterogeneity that compromises reproducibility. This review examines factors affecting circulating lncRNA stability, detection, and interpretation, including sample handling, analytical bias, and inter-individual variability, and summarizes ongoing standardization efforts such as MIQE and MISEV2023 guidelines, consortium protocols, and emerging bioinformatic strategies, while highlighting persistent gaps. We extrapolate recent findings that selective lncRNA packaging into Extracellular Vesicles (EVs) may be regulated by RNA-Binding Proteins (RBPs), to propose that this mechanism may be potentially modulated by SUMOylation, leading to structured yet variable circulating lncRNA patterns. Since any single biomarker is unlikely to differentially translate these patterns, we suggest that analyzing individual exosomes and combinatorial lncRNA signatures—conceptualized as a multidimensional "SUMO-barcode"—using a combination of chip-based single-exosome profiling and AI-driven pattern recognition could improve reproducibility and enable clinically robust circulating lncRNA biomarkers for early cancer detection.

Keywords
Liquid biopsy, Cancer, Biomarker, Extracellular Vesicles, Long non-coding RNA, lncRNA, SUMOylation, RNA Binding Proteins, Single exosome profiling, Bioinformatics

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1. Introduction

Early cancer detection is fundamental to improving patient survival, reducing treatment burden, and enabling precision therapy. Despite advances in molecular oncology, traditional diagnostic techniques such as tissue biopsy and imaging remain limited by their invasiveness, sampling bias, and inability to capture tumour heterogeneity (1). In contrast, liquid biopsy, the analysis of tumour-derived components circulating in body fluids, has revolutionized early cancer diagnosis and monitoring. It provides a minimally invasive platform to detect genetic and epigenetic alterations, offering real-time insights into tumor dynamics (2). Among various biomolecules detectable in circulation, including circulating tumor DNA, microRNAs, and extracellular vesicle cargo, long non-coding RNAs (lncRNAs) have emerged as promising candidates due to their biological stability and cancer-specific expression patterns.

lncRNAs have only recently been investigated as potential biomarkers for the early identification of cancer. Various lncRNAs have been associated with cancer development and progression, serving to either promote or repress cancer associated genes (3). LncRNAs are a large group of ncRNAs over 200 nucleotides in length. As regulatory factors, they play a crucial role in complex cellular processes, such as apoptosis, growth, differentiation, and proliferation (4). Dysregulated lncRNA expression contributes to tumor initiation, progression, metastasis, and therapy resistance (5). Notably, oncogenic lncRNAs such as HOTAIR, MALAT1, ANRIL, and NEAT1 are overexpressed across multiple cancers and are detectable in biofluids, highlighting their potential as diagnostic and prognostic biomarkers (6, 7). Tumor cells actively secrete endogenous lncRNAs into circulation in the form of microvesicles, exosomes, or protein complexes, resulting in the formation of lncRNAs that are not degraded by RNA and are in a stable state (4). This stability distinguishes circulating lncRNAs from many other RNA species, supporting their application in non-invasive cancer detection. However, cancer is a complicated illness that spreads indefinitely due to genomic alterations. As a result, creating effective therapies requires a better knowledge of the mechanisms driving cancer development, progression, and resistance to medications (3). Despite the capabilities of lncRNAs, their use as biomarkers is impeded by their analytical and pre-analytical challenges. Factors like biofluid type, sample collection, extraction method, processing, storage conditions, and the choice of plasma versus serum can markedly influence the RNA yield and quality (8, 9). Additionally, analytical biases, including differences in RNA extraction chemistry, normalization strategies, and reverse transcription efficiency, further complicate cross-study reproducibility (10). These challenges collectively obscure true biological variation, making inter-laboratory comparisons and clinical validation difficult. Growing international efforts—such as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) and the Minimal Information for Studies of Extracellular Vesicles (MISEV2023), emphasize standardization across experimental workflows (11, 12). However, despite these frameworks, significant gaps persist in harmonising lncRNA detection pipelines, from pre-analytical processing to

computational interpretation. Addressing these inconsistencies is crucial to unlock the diagnostic potential of circulating lncRNAs and transition them from research settings to clinical laboratories.

Therefore, this review aims to systematically and precisely examine the pre-analytical and analytical challenges in circulating lncRNA detection and quantification of circulating lncRNAs in liquid biopsies for early cancer diagnosis. The objective is to identify key sources of technical variability, compare available detection and normalization approaches, and highlight practical strategies to improve reproducibility. The review also builds on recent findings that selective lncRNA packaging into Extracellular Vesicles (EVs) may be regulated by RNA-Binding Proteins (RBPs) and proposes SUMOylation as a possible modulator for this process. A multidimensional "SUMO-barcode" is hence suggested to form a pattern for circulating exosomes carrying cancer signature specfic SUMOylated lncRNA cargo, which can be detected using a combination of chip-based single-exosome profiling and AI-driven pattern recognition. This testable hypothesis; upon validation; can enable clinically robust circulating lncRNA biomarkers for early cancer detection. Overall, the review seeks to provide a clear understanding of challenges and guide future research toward the reliable use of circulating lncRNAs in cancer diagnosis.

2. Biological underpinnings of circulating lncRNAs

2.1. Mechanism of lncRNA release into circulation

Circulating lncRNAs are released from the cells into body fluids, such as blood, lymph, cerebrospinal fluid and urine through both active and passive secretion mechanisms. The active secretion of lncRNA is a tightly controlled and regulated process that enables cells to export these molecules into extracellular body fluids in a protective manner, thereby preventing their degradation. One of the most common methods of active secretion is encapsulation within extracellular vesicles, especially in exosomes (13). These vesicles range in size from 30 to 150 nm and form from multivesicular bodies, carrying nucleic acids, proteins, and lipids. Certain lncRNAs are selectively sorted and packaged into these exosomes via RNA-binding proteins. Examples include hnRNPA2B1, YBX1, and HuR (14). These proteins recognize specific sequences in lncRNAs and guide their packaging into the vesicles for export. This not only protects lncRNAs from enzymatic degradation by extracellular RNases but also helps transport them to distant cells (15). Additionally, lncRNAs can be stabilized and exported by forming complexes with RNA-binding proteins (RBPs), which extend the RNA's half-life even in biofluids with high nuclease activity (16). Moreover, lncRNAs may associate with lipoproteins, especially high-density lipoproteins, which serve as carriers of RNA in plasma. This lipoprotein-mediated transport enables lncRNAs to cross hydrophobic environments easily and protects them from enzymatic degradation in the body. In contrast, passive release happens due to uncontrolled cellular breakdown (Figure 1). For example, apoptosis (programmed cell death) triggers the release of cellular content,

including lncRNAs, into the extracellular environment (17).

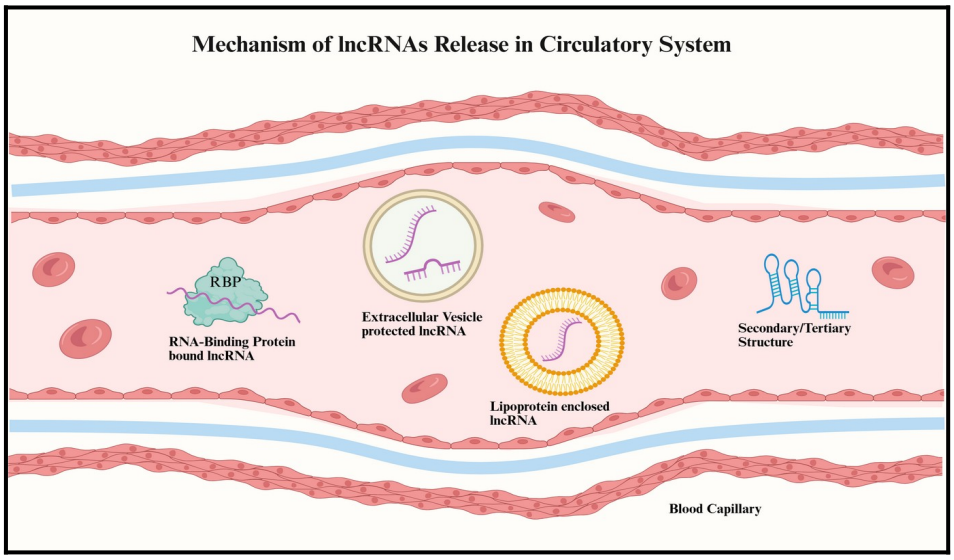

Figure 1. Illustration of lncRNA release in the circulatory System. The lncRNAs may be bound to RNA-binding proteins (RBPs), enclosed within extracellular vesicles (EVs), packaged into lipoproteins, or stabilized by secondary/tertiary structure. These mechanisms help to protect the lncRNAs from degradation in the blood capillary environment

2.2 Stability and half-life in various biofluids

The stability of lncRNA depends on multiple factors. The transcript's base sequence itself can either promote or reduce stability (such as AU-rich, GC-rich regions, poly A tail length, and RNase-sensitive sequences). The lncRNA may fold into secondary or tertiary structures (such as hairpins or stem-loops) that are stable and resistant to RNase digestion, while linear unstructured regions are more prone to degradation. Some lncRNAs bind to RNA-binding proteins, which helps stabilize them and prevents the RNA decay mechanism. Epitranscriptomic modifications such as N6-methyladenosine (m6A) also influence degradation pathways, stability, and recognition by RNA-binding proteins (18). Additionally, the biochemical environment where the target lncRNA is located affects its stability and determines its persistence. The exRNA Atlas and Human Biofluid RNA Atlas maps differences in RNA abundance across biofluids such as plasma, serum, urine, cerebrospinal fluid, and saliva (19, 20). Generally, lncRNAs exhibit a broad range of half-lives, from less than 2 hours to over 12 hours**.** This range indicates that some lncRNAs serve as brief signals for physiological processes or changes, while others persist longer, making them reliable for prognostic biomarker use (18). Encapsulation in EVs, lipoprotein particles, or binding with ribonucleoproteins (RNPs) protects these lncRNAs and significantly extends their circulatory half-life (21). Conversely, naked or unprotected circulating lncRNAs degrade rapidly, limiting their detectability (22). Experimental data have shown that RNA

stability in different matrices (biofluids) is not constant. For example, in blood samples, stability varies depending on the constituting fluid (plasma or serum) and on handling conditions. Data indicate that plasma is preferable to serum for circulating RNA studies (23). During clotting to form serum, some cells may rupture and release additional RNAs, increasing heterogeneity and altering abundance, which can result in false RNA profiling (23). Therefore, a transcript-specific assessment of stability is necessary to avoid overlooking degradation-sensitive lncRNAs. Furthermore, selecting the appropriate matrix based on the disease under study is essential; for instance, plasma is ideal for systemic conditions such as cancer, which can occur anywhere in the body, whereas urine is preferred for genitourinary malignancies (24, 25). Proper sample handling, such as prompt centrifugation, double-spinning to remove residual cells and platelets, and minimizing thaw cycles, can prevent RNA degradation and maintain integrity (26, 27). Since cell-free circulating lncRNAs are often partially degraded, using qPCR primers that can amplify short regions can help detect fragmented RNA (18). Assays should be designed to favor short amplicons, and fragment-aware sequencing should be encouraged in RNA-seq to account for degradation patterns, focusing on small fragments rather than full-length lncRNAs (28, 29).

2.3 Influence of physiological and pathological conditions

It is well established that the expression and function of lncRNAs is affected by various physiological and pathological conditions. Physiological factors include age, sex, circadian rhythms, and exercise. Ageing is characterized as a gradual decline in physiological function, often involving cellular and molecular instability (29). Age significantly influences circulating lncRNAs in body fluids. A 2020 study emphasized that "it has been extensively characterized, but less attention has been paid to the non-coding portion of the human genome, especially to lncRNAs" (30). Ageing also correlates with increased inflammation, which Schlosser et al. has shown to cause elevated lncRNA release via apoptosis (31). Biological sex also significantly affects lncRNA expression because of differences in sex chromosome-linked genes (32). Many lncRNAs exhibit oscillatory expression patterns in tissues such as the liver, pineal gland, and retina. In a genome-wide study of the mouse liver, 112 differentially expressed lncRNAs were identified, with eight showing variations between night and day (33, 34).

Although less explored, research on cfRNA levels in human plasma found no significant diurnal variation overall, though notable differences between individuals were observed. This suggests that individual differences in circadian rhythms coupled with non-standardized temporal collection methods may influence the levels of specific circulating RNA transcripts (35). Pathological conditions such as diabetes, atherosclerosis, high glucose, and inflammatory cytokines modulate the level of endothelial lncRNAs, including LINC00607, which is upregulated in high (glucose and TNF-α) environments, causing vascular inflammation and thrombosis by stimulating *SERPINE1* expression. Similarly, when IL-1β is stimulated, lncRNA CCL2 is upregulated in

endothelial cells and enhances the chemokine expression, which, in turn, promotes immune cell recruitment and vascular inflammation (36). Hence, pathological conditions such as diabetes and vascular inflammation may increase the expression of lncRNA in blood vessel cells (37). Therefore, several lncRNAs exhibit disease-specific alterations and are identifiable in circulation, for example, MIAT and Mhrt, which are increased in heart failure and myocardial infarction (38).

3. Pre-analytical variables affecting LncRNA integrity

3.1 Sample collection: blood, plasma, serum, and urine

A significant part of the reliability of lncRNAs as a biomarker depends on sample selection, handling, and processing (5). For circulating lncRNAs, the choice of biological fluid can influence sample preparation and integrity for subsequent testing, ultimately affecting the results. Serum, plasma, and urine have been the most commonly used bodily fluids. Whole blood is generally not recommended for sample collection due to its high content of cell-derived RNAs from white blood cells, red blood cells, or platelets, which can compromise the accuracy of extracellular lncRNA quantification. Urine has been identified as the most suitable option because of its constant contact with circulating lncRNA as it passes through the tract, serving as a non-invasive alternative biofluid, especially for urological cancers. However, sample collection protocols, such as prior prostate massages, the use of preservative reagents, and collection methods, can significantly affect lncRNA yield and stability. When stabilization reagents such as EDTA or other preservatives are used, urine lncRNAs can remain intact for an extended period at room temperature (39).

3.2 Impact of hemolysis and cellular contamination

Hemolysis may occcur while blood drawing (venipuncture) or when stored – even in heparinized tubes - due to mechanical stress from pneumatic tube systems (PTS), underfilling the tube, rough handling, or specific interactions between heparin and the red blood cell membrane, resulting in intracellular RNA, such as miRNAs, other confounding biomarkers, and cellular content being released in the blood (40). Hence, it is a pre-analytical confounder in studies focused on circulatory RNA studies since some lncRNAs are also expressed at high levels in blood cells, and they might be falsely detected as circulating organ or tumor signals when hemolysis is not controlled and/or not assessed properly (41). Validated standardized metrics to measure hemolysis, such as the spectrophotometric absorbance at OD 414 nm to detect free haemoglobin, automated hemolysis index, and molecular ratios (e.g., miR-451/miR-23a), are available and are being used, with appropriate analytical corrections if these read beyond threshold values (40-42). Moreover, different downstream analytical approaches like RT-PCR and sequencing are variably sensitive to hemolysis, causing bias (43). Therefore, selected lncRNA reference transcripts for analytical purposes should be stable in plasma and, in addition, should not be enriched or highly expressed in blood cells (43). Contamination is not just limited to hemolysis; incomplete removal of platelets or leukocytes also releases cellular RNAs that can

interfere with the low level of extracellular signals (44). This indicates the need for standard centrifugation SOPs to ensure reproducibility and matrix non-interference. The inability to control hemolysis or other contamination can produce false positives, intra-study variability, and may overlook small, significant biological differences (26, 45, 46). Appropriate and standardized pre-analytical quality control filters and protocols are essential to detect low abundance and tissue-specific lncRNAs without false positives.

3.3 Time between collection and processing

Cell RNA contamination can also be caused by delays (~ > 2 hours) between blood draw and isolation of plasma/serum. Spontaneous cell lysis and activation of platelets can result in the release of intracellular RNAs. Serum is more prone to artificial contamination of intracellular RNAs and therefore plasma is a better option for detecting true circulating lncRNAs biomarkers, since it is collected using anticoagulants such as EDTA or citrate; before coagulation. Regardless of the development of tubes which permit longer delays without contamination, the best practice is to process quickly. Hence, immediate plasma processing and double-spin protocols can remove leftover cells and platelets to prevent contamination. Small amounts of contamination can compromise results in case-sensitive lncRNA assays (24, 44).

3.4 Storage conditions and freeze-thaw cycles

Wylezinski et al. found that storage of whole blood in PAXgene tubes, total RNA and cDNA for up to 1 year at − 80 °C or up to ten total RNA or cDNA freeze–thaw cycles did not significantly alter lncRNA expression profiles compared to baseline (47). miRNA standardization guidelines discourage multiple freeze-thaw cycles, and instead recommend aliquoting plasma/serum upon initial processing, immediately transferring it to -80°C, and subjecting it to minimal temperature fluctuations (48). The use of RNA stabilization tubes (such as PAXgene) can preserve RNA content for days at ambient temperature before it is required for processing (49).

4. Analytical challenges in lncRNA quantification

The lncRNAs are structurally and biologically distinct from the mRNAs. Moreover, they exhibit high tissue specificity, likely due to their complex regulatory patterns, and are typically expressed at low levels (50). These factors, along with a lack of string sequence conservation between species, annotation gaps in the reference genome, overlapping coding sequences, alternate splicing, and RNA instability can create challenges in their analytical quantification. Other technical challenges—including the choice of quantification method, library preparation, mapping discrepancies, normalization, signal mimicry, pseudogenes, and unspliced mRNAs —further hinder accurate lncRNA quantification.

4.1 RNA extraction methods and comparison

The different chemistries and enrichment approaches affect the purity and yield of the lncRNAs, especially if they are non-polyadenylated ones. The TRIzol kit (phenol–chloroform extraction) can be used across various sample types and yields high amounts of total RNA. However, it carries a high risk of

contamination, which can lead to false-positive or low RT-PCR signals. This issue is especially pronounced when lncRNA levels or plasma/serum sample volumes are low. In contrast, Silica Column-Based Kits offer less contamination and cleaner RNAs with greater reproducibility than TRIzol; however, they are expensive and may not accurately quantify small (non-polyadenylated) or highly structured lncRNAs of less than 200 nucleotides in size (51-53). Similarly, magnetic-based purification also provides better RNA purity, but a higher chance of overlooking certain lncRNA subsets (Figure 2) (53). rRNA depletion preserves both polyadenylated and non-polyadenylated lncRNAs, whereas poly(A) selection enriches only mature polyadenylated RNAs and fails to capture many lncRNAs, particularly those localized in the nucleus. Consequently, the choice of method influences which lncRNAs—and their lengths—can be studied (54, 55). Plasma/Serum-specific cfRNAs kits are bead-based, optimised for dilute RNA pools and fragmented RNAs, but suffer from low yields which require sensitive downstream quantification.

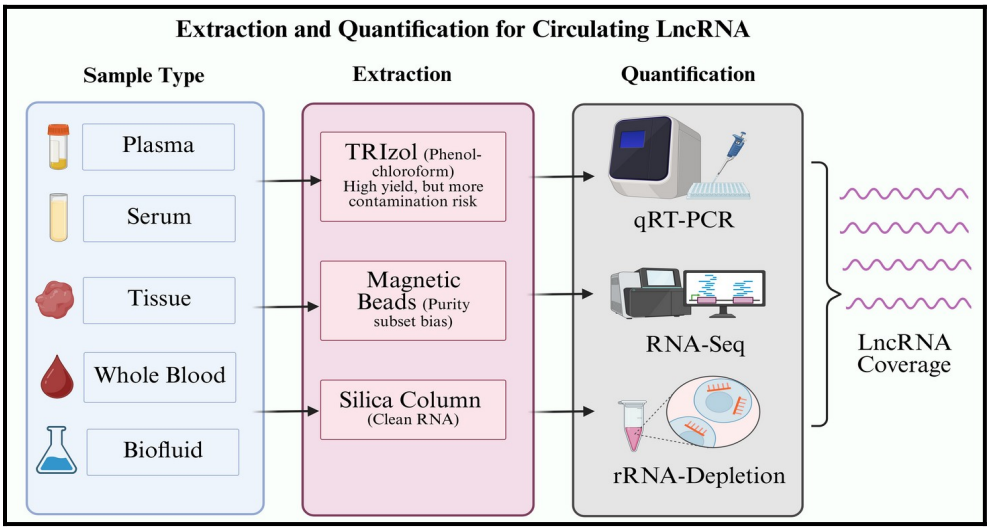

Figure 2. The figure outlines the workflow for lncRNA extraction from different sample types (plasma, serum, tissue, whole blood, or other biofluids like saliva, urine, and CSF) and its quantification. Different extraction methods include phenol-chloroform, TRIzol method, magnetic beads, and silica column. Quantification is performed by qRT-PCR, RNA sequencing (RNA-Seq), and rRNA-depletion, enabling the comprehensive lncRNA coverage.

The circulating lncRNAs in biofluids are usually present as small fragments either bound to protein complexes or enclosed within EVs. Here, standard cellular kits fail (total RNA) because they cannot lyse the EV or decomplex the RNA from protein complexes (53, 56). This causes a selective profiling of the lncRNA landscape. Although magnetic bead chemistries can improve recovery in the case of low plasma/serum volume, size-selective biases arise due to bead size (52). The composition of the biofluid is another important factor that

influences the extraction method. For instance, co-purified anticoagulants (heparin) and other intracellular RNAs are released by hemolysis. These intracellular components can mask the low-abundance circulating lncRNAs (57). This challenge can be solved by comparative analyses in which samples undergo pretreatments such as double centrifugation, hemolysis evaluation, and platelet removal. Regardless of the extraction kit, pretreatment can significantly improve lncRNA yield and attenuate noise (58). RNA depletion has been shown to provide broader coverage of the lncRNA landscape; however, its effectiveness depends on having a higher amount of input RNA (55). Moreover, manufacturer-specific chemistry—such as silica membrane pore size, bead surface coatings, lysis buffer composition, and wash buffer stringency—can influence which RNAs bind more or less efficiently. Because lncRNAs differ in size, structure, and polyadenylation status, even small methodological variations can affect their relative recovery. Differences in RNA binding and elution efficiency can also occur between production lots of kits from the same manufacturer. As a result, datasets generated using the same method may capture different lncRNA landscapes, reducing reproducibility and complicating cross-study comparisons (56). During RNA extraction, total RNA yield (ng/μL) and RNA integrity number (RIN) are commonly used to assess sample quality. However, even when yield and RIN scores are high, recovery of the target lncRNA may still be poor (56). Therefore, the use of spike-in RNAs (of known sequence) and appropriate recovery efficiency assays is crucial for evaluating true extraction performance. Hence, standard protocols and inter-laboratory calibration or metrics for validation are needed for circulating lncRNA biomarker analytical development.

4.2 Challenge of pre-isolation variation not corrected by spike-in controls

The clinical potential of circulating lncRNAs as diagnostic and prognostic cancer biomarkers is becoming increasingly evident, as recent studies have shown that plasma lncRNAs—such as HOTAIR, ANRIL, MEG3, and NEAT1—can sensitively and specifically distinguish cancer patients from healthy controls. However, accurate measurement of these molecules faces substantial technical barriers related to pre-analytical variation—differences introduced before RNA is even isolated from biofluid samples (6-8). Standard practice employs synthetic RNA spike-ins, added during or after RNA isolation, as internal controls to assess extraction and sequencing efficiency. Nonetheless, recent experimental evidence underscores that such spike-ins cannot account for variation introduced earlier in the workflow—such as differences in blood draw technique, blood/plasma/serum storge tube type, time to plasma separation, storage temperature, and the number of freeze-thaw cycles. Satake et al. (2024) showed that pre-analytical factors such as processing delays and improper storage measurably alter mRNA yield and integrity, with changes undetectable by spike-in control recovery assays. This limitation is directly translatable to lncRNA analyses, as these molecules are similarly vulnerable to degradation and loss at each pre-analytical step (9, 59). Recent studies on lncRNA biomarker development highlight the persistence of these technical vulnerabilities. For example, pan-cancer evaluations and

disease-specific studies on circulating lncRNA panels stress the need for optimal and standardized sample handling protocols to ensure reproducibility and biological validity of results. It is now widely recognized that while spike-ins correct for downstream losses, they cannot retrospectively fix variability resulting from pre-isolation conditions, which can unpredictably impact lncRNA quantification and thus confound disease- or treatment-related inferences (7-9, 59). Circulating lncRNAs are challenging to study because they are usually low in abundance, large, and often fragmented. This makes them sensitive to sample handling, so careful collection and processing are essential. Researchers recommend standardizing how samples are collected, processing them quickly, keeping them at consistent temperatures, and avoiding repeated freeze-thaw cycles. Using spike-in controls helps, but it cannot replace these careful handling practices (59). Therefore, robust clinical application of circulating lncRNA biomarkers requires that biobanking, processing, and analytical protocols be rigorously applied to minimise pre-isolation variability. Reliance on internal spike-in controls alone cannot ensure accuracy and consistency; instead, comprehensive pre-analytical standardization is indispensable for trustworthy lncRNA-based liquid biopsy diagnostics.

4.3 Input volume vs RNA amount in circulating lncRNA biomarker analysis

A key technical challenge in circulating RNA biomarker analysis is whether to normalize input samples by equal RNA amount or by equal plasma/serum volumes. Conventional assays typically use equal RNA mass to standardize quantification; however, this can be problematic in cancer, where circulating RNA levels vary widely between patients and controls. Cancer patients often have elevated circulating RNA levels due to increased release of tumor-derived nucleic acids, significantly affecting total RNA yield from plasma or serum (10, 60). Normalizing by equal RNA amount can mask true biological differences since samples with diverse RNA burdens are adjusted to the same RNA mass, skewing downstream biomarker measurements. Northrop-Albrecht et al. showed that most circulating RNA is fragmented and dominated by ribosomal and mitochondrial RNAs, while tumor-specific RNAs such as lncRNAs constitute a minor, variable fraction (10). This complexity challenges normalization purely based on RNA quantity. Volume-based normalization better preserves physiological differences in circulating RNA reflective of tumor burden and disease. Recent studies, including prostate cancer biomarker validation by Brokāne et al., support equal plasma volume as input to mitigate bias introduced by disparate RNA yields (1, 2). Moreover, the exRNAQC Consortium emphasized the necessity of standardized pre-analytical protocols to minimize variability when using volume normalization (61). For circulating lncRNAs, often in low-abundance and heterogeneous, equal RNA input normalization further risks diluting or obscuring tumor-related signals (1). Thus, volume normalization combined with lncRNA-specific sensitive detection and rigorous quality controls has emerged as the preferred approach for accurate biomarker quantification and clinical relevance.

4.4 Detection techniques (PCR, Microarray, Next Generation Sequencing)

The most widely used method for lncRNA detection is quantitative PCR (qPCR) and real-time PCR (RT-PCR) in clinical and translational research. Both are cost-effective, have acceptable sensitivities and reproducibility. Low abundance, complex structure, and susceptibility to degradation pose the most critical challenges in the diagnosis of circulating lncRNAs. Therefore, to ensure reliable quantification in clinical translation, highly sensitive and specific analytical approaches are needed. qPCR is commonly used to quantify exosomal lncRNAs. For instance, in prostate cancer, PCA3 and MALAT1 levels measured by qPCR have shown better diagnostic potential than PSA-based tests (62). In breast cancer, RT-PCR has been applied to profile exosomal lncRNAs. These examples demonstrate that RT-PCR applied to non-invasive liquid biopsies, such as blood or urine, can not only detect and quantify lncRNAs but also reveal their roles in cancer biology and clinical outcomes, including tumor growth, tumor microenvironment interactions, intercellular signaling, and drug resistance. In contrast, qPCR-based assays require prior sequence information and can be biased by primer design, secondary structures, or RNA abundance (63). Microarray offers a parallelized approach to quantify multiple lncRNAs simultaneously. However, microarrays are limited in use due to challenges such as low abundance, cross-hybridization, and lower sensitivity for novel lncRNA isoforms (64). Next-generation sequencing (NGS) technologies have transformed lncRNA detection through transcriptome profiling. RNA sequencing (RNA-Seq) discovers novel lncRNAs, their isoforms, and fusion transcripts along with quantitative information and expression dynamics (65). Although the strand-specific protocols, library preparation, and benchmarked computational pipelines have increased the sensitivity for low-abundance lncRNA, the high cost of NGS, complex bioinformatic analyses, and biases in reverse transcription and amplification have limited their use (20). Recently, direct RNA sequencing with nanopore technology has enabled the detection of full-length lncRNAs, their post-transcriptional modifications, and secondary structures without converting them to cDNA, thereby reducing amplification-related challenges. (20). To overcome the limitations of thermal cycling and improve sensitivity, isothermal amplification strategies are increasingly being used. For example, a recently developed fluorescent biosensor based on multi-probing and rolling circle amplification (RCA) enables multi-primer binding to long sequences and signal amplification. This method was designed to detect the oncogenic lncRNA HULC in hepatocellular carcinoma and demonstrates high specificity, speed, and simplicity, showing promise in both cell lines and in plasma samples. Other isothermal approaches include loop-mediated isothermal amplification (LAMP) and strand-displacement amplification (SDA), but their use is limited to analyzing lncRNA length and secondary structures (64). Another emerging approach is biosensor-based detection, which is highly sensitive and can serve as an alternative to PCR. It uses nanoparticles for signal amplification to detect lncRNAs directly from serum at the point of care, without specialized laboratories. These nanoparticles are small, highly specific,

portable, and easy to use, making the method promising for rapid diagnosis, early cancer detection, and routine monitoring with minimally invasive samples. (64). Thus, while qRT-PCR and NGS remain the gold standards for lncRNA identification, other emerging approaches are advancing rapidly by providing ultra-sensitive, clinically adaptable, and cost-effective platforms. In the future, the integration of these approaches with more robust normalization strategies and workflows will ensure reproducibility and clinical translation.

4.5 Effect of microarray expiration and background noise on circulating lncRNA detection

Unlike circulating lncRNAs, which are diluted in plasma or serum, tissue lncRNAs are more concentrated in their local environment, producing stronger microarray signals. (5). Accurate detection of circulating lncRNAs by microarrays is limited by their low abundance in biofluids and rising nonspecific background noise, which worsens as arrays age and disproportionately affects low-abundance plasma lncRNAs (5, 66, 67). Gao et al. (2025) systematically compared gene expression measurements between RNA-seq and microarray platforms and noticed that the microarrays underperformed at detecting low-expression genes. The number of detectable targets dropped sharply at lower concentrations due to background interference. They demonstrated that the fluctuations in differentially-expressed gene numbers were most pronounced at the lowest input concentrations, coinciding with decreases in microarray sensitivity and specificity as background increased - an effect that paralleled clinical observations in circulating lncRNA analyses (66). Further, Qi et al. (2016) and subsequent platform evaluations confirmed that the microarray-based detection of circulating lncRNAs was particularly sensitive to chip background; not only did background noise obscure lncRNA signals, but its effect increased as chips aged or degraded, significantly reducing the number of reliably detectable lncRNAs in patient samples (5, 68, 69). Tyagi et al. (2025) highlighted in a multi-omics integration study that the detection and predictive accuracy for weakly expressed, copy number variation (CNV)-affected lncRNAs significantly worsened when using older or expired microarray chips, thereby undermining biomarker discovery and model reproducibility. Methodological comparisons further reveal that while microarrays can effectively detect high-abundance, well-characterized lncRNAs, the proportion of the lncRNA transcriptome that can be confidently quantified decreases sharply with declining chip quality or upon approaching expiration (69). These technical limitations are exacerbated by the inherent characteristics of circulating lncRNAs, which are typically present at low concentrations, tend to be large molecules, and exist as fragmented species in circulation. As chip expiration nears, nonspecific hybridization and degradation of probe performance become significant, transforming previously detectable lncRNAs into false negatives within biomarker discovery pipelines (5). Recent microarray studies focused on peripheral blood mononuclear cell transcriptome analysis emphasize the necessity of chip quality control and the date tracking as detection rates of lncRNAs and quantifiable transcripts decrease measurably on older or expired platforms, indicating that timely

replacement and calibration of arrays is essential for reproducible liquid biopsy diagnostics (67). Recent studies consistently recommend carefully checking microarray chip quality and keeping track of their expiration dates. Whenever possible, switching to RNA sequencing (RNA-seq) or digital PCR for analyzing circulating lncRNAs is advised. While newer microarray designs with better probes offer some improvements, they cannot fully prevent the loss of sensitivity caused by increasing background noise over time. RNA-seq, on the other hand, provides a wider range of detection, is more sensitive to low-abundance lncRNAs, and does not suffer from this age degradation that plagues microarrays. Because of this, RNA-seq is generally preferred for detailed and large-scale liquid biopsy studies (66, 67, 70).

4.6 Data interpretation and bioinformatic analyses

Interpreting circulating lncRNA data requires frameworks that can model low-abundance, fragmented lncRNA, various carriers, composition (including physiological impacts, other disease influences, diet, etc.), batch effects (lab-to-lab differences), and incomplete lncRNA annotations. Since the composition of carriers and biofluid origins can reshape RNA classes and their distributions, rigorous batch correction is necessary for cross-study comparisons (19, 71). Normalization strategies can also directly influence downstream statistics and biomarker grading. Analyzing lncRNAs involves processing raw reads through a standard pipeline, which includes adapter removal, handling unique molecular identifiers (UMIs), and filtering out other rRNA, among other steps; mapping to lncRNA catalogs like GENCODE and NONCODE or the whole reference genome to identify new lncRNAs. The DESeq2 pipeline is used to quantify counts and reduce noise. Because lncRNAs are present at lower levels, each step from library preparation to normalization significantly affects the results and their reproducibility across batches (72). Many publicly available databases suffer from incomplete annotations, particularly for lncRNAs derived from biofluids (extracellular lncRNAs) (73). As a result, pipelines that rely only on known references may miss unknown signals that could serve as potential biomarkers. Rather than relying only on reference alignment, read-cluster or peak-based quantification collects all genomic clusters or peaks and analyzes them regardless of annotation. This allows detection of novel lncRNA signals and opens the possibility for hybrid approaches, where peaks overlapping known loci are assigned to specific lncRNAs, while non-overlapping peaks may represent novel extracellular lncRNAs, selectively released RNA fragments, or uncharacterized isoforms (74). Such hybrid models can capture both annotated and novel lncRNAs with high sensitivity, improve annotations (flexibility), and facilitate the discovery of new biomarkers. Additionally, RNA-seq sometimes produces false positives due to ultra-low counts, but statistical adjustments like FDR correction can reduce false discoveries. Recently, new circulating RNA and lncRNA-specific computational tools have been proposed, focusing on peak-centric approaches to enhance reliability. These models search for protected fragments, which appear as peaks within EVs, thereby improving the signal-to-noise ratio and accurately identifying

circulating lncRNAs. These developments are especially valuable in liquid biopsy applications where lncRNA input is minimal. Quality checks for EV-protected lncRNAs—such as insert size, strand orientation, and 5'/3' coverage—along with controlling confounders like hemolysis, age, and other diseases, and validating results with RT-qPCR and independent studies, are recommended to support robust biomarker claims (75).

5. Inter-individual and biological variability

Due to the inter-individual and biological variabilities in lncRNA expression profiles, translating circulating lncRNAs into reliable clinical biomarkers becomes all the more nontrivial. The heterogeneity in expression profiles originates from intrinsic factors such as gender, age, genetic polymorphisms, and ethnicity. Lifestyle choices and comorbidities add further confounding. These variables can be addressed with the careful design of rigorous cohort studies (see Section 10)

5.1 Influence of age, gender, and ethnicity

Few studies have examined age- or sex-specific patterns of circulating lncRNAs; however, anecdotal evidence suggests that lncRNA mutations (SNPs) may have different effects in men and women or across ethnic groups. A meta-analysis study on lncRNA H19 polymorphisms showed ethnicity-dependent differences in cancer susceptibilities, such as rs2107425 C>T being a protective mutation in Caucasian cohorts, while rs2839698 G>A being linked to increased cancer risk in the Asian population (76, 77). These allele-specific effects on lncRNA expression or function in different populations, may require validation cohort studies. A case–control study in Chinese women with breast cancer found that the *H19* SNP rs2071095 was associated with reduced risk. In ER-positive patients, the AA genotype showed lower *H19* expression than the CC genotype (78), highlighting genotype-dependent expression influenced by hormonal status. These findings underscore the need for age-, sex-, and hormone-stratified studies of circulating lncRNAs.

5.2 Comorbidities and lifestyle factors

Comorbidities and disease-specific signaling pathways can alter lncRNA expression. For example, chronic inflammatory conditions such as diabetes and cardiovascular disease are often associated with dysregulated lncRNA levels. Polymorphisms in the lncRNA ANRIL (located at the CDKN2A/B locus) have been linked not only to cancer susceptibility but also to confounding conditions such as coronary artery disease and systemic inflammation. For example, ANRIL variants have been associated with elevated levels of high-sensitivity C-reactive protein (hs-CRP) (78). These findings suggest regulatory crosstalk between inflammatory and oncogenic pathways. ANRIL SNPs (rs1333048, rs4977574, and rs10757278), which affect transcript splicing and stability, are associated with both cardiovascular disease and cancer. This overlap indicates that comorbidities and genetic variation can mimic cancer-related signals by altering lncRNA expression, complicating the identification of truly cancer-specific lncRNA changes. In addition, lifestyle factors such as smoking, alcohol consumption, physical activity, and diet—known to influence transcriptional regulation and epigenetic states—can further modulate circulating lncRNA levels (78). Despite this, relatively few studies

address these effects. Therefore, careful phenotyping and adjustment for comorbidities and lifestyle covariates are essential in validation studies.

5.3 Genetic polymorphisms affecting lncRNA expression

Inter-individual variability in lncRNA profiles, their altering transcripts, secondary structures, or interactions with other biomolecules arises from SNPs in lncRNA loci or their regulatory regions. For example, in the case of H19, multiple SNPs are linked to different associations with cancer risk. The variation rs2839698 is associated with higher susceptibility in Asians with digestive system tumors, whereas rs2107425 may provide a protective effect in Caucasians (77). Additionally, some SNPs are related to susceptibility to pediatric cancer in the Chinese population, and others are associated with breast cancer risk along with variations linked to estrogen receptor status. Hence, genetic polymorphisms can modify lncRNA function and biomarker utility across populations. Therefore, the relationship between genotype and expression highlights the importance of combining genomic data with circulating lncRNA profiling to improve risk stratification and biomarker models (75, 78-80). In addition to *H19*, polymorphisms in *ANRIL* influence transcript processing and disease susceptibility. The SNP rs1333048 has been associated with breast cancer risk and functional alterations in the tumor suppressor INK4/ARF locus (79). Another variant, rs1333049, has been linked to improved disease-free survival, with the CC genotype showing longer survival than the GG or GC genotypes (1). These findings indicate that SNPs can affect both lncRNA expression and treatment outcomes, adding complexity to the interpretation of lncRNA biomarkers.

5.4 Intra-individual temporal variability

A critical challenge in circulating lncRNA biomarker research is the significant intraindividual variability in lncRNA levels over time. Emerging evidence suggests that measured circulating lncRNA concentrations may reflect a cumulative record of individual behaviours and environmental exposures, such as diet, physical activity, and stress rather than exclusively indicating a specific disease state (5, 25). Analyses by Roy et al. (2024) emphasized the necessity of frequent sampling to monitor the dynamic fluctuations in circulating lncRNAs, highlighting that lifestyle factors can induce significant temporal variation. Similarly, Sandau et al. (2024) demonstrated persistent but variable microRNA stability patterns in plasma samples from healthy adults over extended periods, which shows inter and intra-individual fluctuations that likely extend to lncRNAs given their shared regulatory pathways (25, 81). Correia et al. (2021) showed that aerobic exercise modulates expression of cardiac-related lncRNAs such as MALAT1, H19, and MIAT, which are involved in cardiomyocyte function and fibrosis (82). The degree and duration of exercise correlate with rapid shifts in circulating lncRNA levels, suggesting lifestyle greatly influences lncRNA biomarker signals. Plaza-Florido et al. (2022) reviewed how sedentary behaviour and varying intensities of physical activity epigenetically alter gene expression profiles associated with metabolic health, likely impacting circulating lncRNA profiles. Dietary patterns further affect circulating lncRNAs. High-fat diets and

hypercholesterolemia have been shown to modify extracellular vesicle-associated lncRNA cargo, affecting measured circulating levels independently of disease (83). Together, these studies show that the intra-individual behavioural and metabolic factors modulate circulating lncRNA abundance dynamically, representing composite signals rather than static disease markers (84, 85). From a clinical biomarker perspective, the temporal fluctuation and behaviour-driven variability necessitate designing studies with longitudinal sampling and integrating detailed lifestyle metadata. Such approaches are vital to distinguish disease-specific lncRNA signatures from background noise driven by changing behaviours, thereby enhancing biomarker robustness and translational potential (25, 81).

5.5 Polymorphisms at lncRNA Loci: impact on differential expression and biomarker potential

The differential expression of lncRNAs is increasingly attributed to genetic polymorphisms within their chromosomal loci, with substantial implications for disease susceptibility, progression, and biomarker development. Recent studies clarify both the prevalence and the mechanistic basis of these effects across human tissues and cancers. Senousy et al. (2024) demonstrated that the rs941576 SNP in the lncRNA MEG3 is significantly associated with colorectal cancer (CRC) susceptibility and prognosis, with certain genotypes correlating with altered circulating MEG3 levels in patient plasma. Their findings establish MEG3 polymorphism as an independent risk factor and potential early diagnostic marker for CRC, which provides direct support to genotype-driven lncRNA expression changes with clinical relevance (86). Similarly, a large cohort study by Chen et al. (2021) characterized the RNF144A-AS1 lncRNA locus in non-small cell lung cancer (NSCLC), showing that the rs3806609 TT genotype is associated with increased RNF144A-AS1 expression and a greater risk of NSCLC (87, 88). This genetic variant enhances cell proliferation and migration via the IFN-γ/JAK2/STAT1 signaling axis, supporting the assertion that lncRNA expression and cancer risk are often modulated by inherited polymorphisms (88). Krishna et al. (2024) provided a comprehensive investigation of HOTAIR locus polymorphisms. They reported that the specific SNPs are linked to both expression modulation and cancer risk, including in breast and other solid tumors. Other Genome-wide eQTL mapping studies showed, in multiple human tissues, including the brain, further reinforce that common genetic variants have local (cis) and distal (trans) effects on lncRNA transcription levels, facilitating systematic prediction of disease-associated lncRNA expression profiles (89, 90). These findings are strongly supported by the large-scale meta-analyses in human cancer cohorts where lncRNA SNPs (e.g., in MALAT1, HOTTIP, HULC, and PRNCR1) have been shown to associate with overall cancer risk and to influence patterns of lncRNA abundance and function. The biological mechanisms underlying these effects include altered RNA transcription, splicing, stability, and transcript modification, as well as effects on chromatin states (78, 91). These recent discoveries have shown that the genetic variation at lncRNA loci is a fundamental driver of lncRNA expression heterogeneity in human populations. For biomarker research, underlying genetic backgrounds must be

considered in the clinical interpretation of lncRNA signatures, the design of vigorous diagnostic assays, and the stratification of patient populations for personalized medicine. Future research should systematically map lncRNA variants and their regulatory networks to refine lncRNA-based diagnostics and therapeutics.

5.6 Copy number variation at lncRNA loci: expression heterogeneity and its disease versus non-disease specificity

Copy number variations (CNVs), either gains or losses of genomic segments at lncRNA loci, are now recognized as significant contributors to interindividual differences in lncRNA expression, with mounting evidence from human disease and population genetics. Recent research indicates that while CNV-driven lncRNA expression changes are frequently observed in cancer and complex diseases, these polymorphic events can arise independently of any disease association and may merely reflect neutral genetic diversity. Tyagi et al. (2025) advanced this field with LncRNACNVIntegrateR, a pan-cancer framework that systematically correlates lncRNA expression with CNVs and measures clinical impact. Their analyses in multiple cancer cohorts found that numerous lncRNAs subjected to CNV-driven deregulation are also present as "neutral" polymorphisms in non-tumor tissues and across healthy control populations. While certain CNV-lncRNAs were identified as associated with disease progression or poor prognosis, a large fraction was found independently of clinical phenotype, suggesting that CNV dependency is not always disease-specific (69). A notable cross-disease investigation by Lu et al. (2023) found that up to 68% of CNVs associated with congenital heart disease harboured lncRNAs with tissue-specific, but not disease-specific expression patterns. Their experimental validation underscored that some CNV-lncRNAs are strongly co-expressed with cardiac genes only in the developing heart, whereas others are ubiquitously expressed regardless of disease (142). Similarly, Ning et al. (2021) demonstrated that, in lung squamous cell carcinoma, while global amplification of lncRNA-copy number correlates with overexpression of select lncRNAs (e.g., SOX2-OT), the same CNV events are seen in non-neoplastic lung tissues, indicating their contribution to both baseline and pathological expression heterogeneity (141). Further, genome-wide studies further elucidate that CNVs can drive both up-regulation and down-regulation of lncRNAs in a highly polymorphic manner, with the same variant manifesting as either a benign polymorphism or a pathogenic lesion depending on allele frequency, epistatic interaction, and genomic context. For example, Lu et al. (2023) integrated CNV and transcriptomic data in tumors, observing that while a subset of CNV-driven lncRNAs showed disease association, most were part of a larger backdrop of CNV polymorphism across the population (76, 142).

5.7 Diet as a critical confounder in circulating lncRNA biomarker research

Diet has emerged as a significant confounding factor in the analysis and interpretation of circulating lncRNA biomarkers. Recent primary research has highlighted that many lncRNAs contained in food sources can be absorbed or can interact with biological systems in a manner that renders their

circulating forms indistinguishable from endogenous lncRNAs at the sequence or functional level (85, 92). Brandt et al. (2024) found that diet-induced metabolic changes modulate lncRNA expression profiles, particularly via lncRNAs being involved in lipid metabolism and inflammatory signaling in metabolic disorders (85). These lncRNAs may be present both endogenously and/or originate from dietary intake, complicating the attribution of changes in circulating lncRNA levels solely to pathological states. Tian et al. (2025) used weighted gene co-expression network analysis to associate dysregulated lncRNAs with hepatic steatosis linked to environmental exposures; their findings implicitly suggest that external factors, such as diet, influence lncRNA networks and circulating levels (85, 93). Additional work from Kovácsházi et al. (2024) demonstrated that diet-associated hypercholesterolemia altered circulating extracellular vesicle (EV) RNA composition, including lncRNAs, independent of disease progression (84). Diet-induced changes in EV-associated lncRNAs highlight the dynamic nature of circulating RNA pools, which are regulated by homeostatic mechanisms controlling vesicle packaging and release. Dietary intake during pregnancy can alter extracellular RNA profiles, including vesicle-associated lncRNAs, potentially confounding biomarker studies (84, 94).

Emerging nutrigenomic research shows that food-derived RNAs and diet-induced changes in host RNA expression can converge in circulation, creating a complex molecular environment. This complexity underscores the need for strict dietary control and detailed metadata collection in biomarker studies, as variations in circulating lncRNAs may reflect dietary effects as well as disease biology (95). Hence, diet profoundly influences circulating lncRNA levels through multiple pathways, including direct RNA uptake and modulation of vesicle biogenesis. Future biomarker discovery and validation efforts must incorporate strategies to account for dietary confounders to ensure clinical accuracy and biological relevance of circulating lncRNA signatures.

5.8 Influence of pharmacological treatments and chemotherapy on circulating lncRNA levels

Pharmacological interventions, including chemotherapy, significantly affect circulating lncRNA profiles, presenting a key challenge for biomarker development and interpretation. Recent studies show that drug treatments can alter lncRNA expression, release, and stability, reflecting both direct drug effects and cellular adaptation, which complicates distinguishing treatment-induced changes from disease-related signatures (97, 98). Siahestalkhi et al. (2025) found increased levels of specific lncRNAs in the serum of drug-resistant cancer patients after chemotherapy, indicating that pharmacological pressure reshapes circulating RNA landscapes. LncRNAs like ERIC and PCGEM1 are linked to resistance mechanisms by suppressing apoptosis and promoting survival pathways. Mechanistic studies demonstrate that lncRNAs mediate chemoresistance by regulating apoptosis, autophagy, drug efflux, and the epithelial-mesenchymal transition, as seen in breast and gastric cancers. LncRNAs such as XIST, MEG3, and UCA1 influence key pathways affecting responses to drugs like 5-FU and cisplatin (97-100). Additionally,

circulating lncRNA levels vary dynamically in response to chemotherapy, reflecting tumor adaptation and treatment effectiveness. Liu et al. (2022) developed lncRNA-based risk scores to predict chemotherapy sensitivity, demonstrating the translational potential of lncRNAs as biomarkers, while emphasizing the need to account for treatment-related expression changes (100, 101). Beyond chemotherapy, emerging evidence suggests broader pharmacological effects on circulating lncRNAs. Zhang et al. (2023) reviewed how doxorubicin resistance-associated lncRNAs influence pathways involved in drug transport and tumor microenvironment remodeling (102). Studies in addiction biology have also shown that the commonly used psychoactive drugs regulate lncRNAs, suggesting pharmacotherapy impacts circulating lncRNA profiles beyond cancer (103). These studies highlight the dual role of drugs as modifiers of circulating lncRNAs while complicating biomarker interpretation. They also present opportunities for targeting lncRNAs therapeutically to overcome drug resistance. Therefore, to achieve accurate biomarker results, it is important to collect samples over time, incorporate clinical data, and consider patients' treatment histories.

6. Disentangling circulating lncRNA signals: tumor-derived versus host response

Another challenge in interpreting circulating lncRNA biomarkers in cancer lies in discerning whether observed expression changes originate primarily from tumor cells or from the host's systemic response to tumor growth. Emerging evidence indicates that because cancerous cells constitute a minority of total body cells and tumor-derived circulating material is often diluted. Many alterations in specific circulating lncRNAs reflect indirect effects of the body's physiological and immune response rather than of the tumor burden alone (4, 8). Studies have also reported increased circulating ribonuclease (RNase) activity in cancer patients, which contributes to the degradation of circulating lncRNAs, potentially confounding circulating RNA-based biomarker quantification. Elevated RNase levels are thought to be secreted by immune cells as part of cytotoxic anti-tumor defence, promoting apoptosis of cancer cells and modifying extracellular RNA profiles. For instance, MALAT1 shows paradoxical decreased stability in circulation despite tumor overexpression, attributable to enhanced RNase degradation in plasma (8). Additionally, tumor–host cross-talk via the tumor microenvironment (TME) involves lncRNAs secreted not only by cancer cells but also by stromal cells, immune cells, and endothelial cells. These lncRNAs regulate immune cell differentiation, angiogenesis, extracellular matrix remodeling, and immunosuppression, profoundly shaping the circulating lncRNA milieu. For instance, lncRNAs such as HOTAIR and NKILA modulate tumor immunity and the survival of cytotoxic T lymphocytes, influencing systemic RNA profiles beyond direct tumor secretion (4, 104, 105). Further, recent biomarker studies highlighted that the circulating lncRNA patterns reflect not only the number of cancer cells, but also often reflect the body's immune response, inflammation, and how the body reacts to treatment (106, 107). This means that their levels can change because of the body's reaction, not only because of the tumor size or activity. This complexity necessitates integrated approaches combining tumor-specific assays with immune and systemic

response profiling to accurately interpret circulating lncRNAs (see Section 10 for a cohort design).

7. Current efforts towards standardization

Even though research in liquid biopsy has expanded and the potential of circulating lncRNA as non-invasive biomarkers has grown, its clinical application remains limited due to variability in analysis methods. Current efforts focus on first recognizing these limitations and establishing baseline guidelines, then encouraging collaboration among multiple institutions to develop consistent protocols/SOPs to ensure reproducibility (108). It is clear, there is no universal SOP or standardized workflow from blood collection to result analysis. RNAs are very fragile and can quickly degrade due to RNase activity; therefore, rapid processing, maintaining colder temperatures, and using RNase-free environments are crucial (108). This concern is heightened for lncRNAs, which are more prone to degradation. Additionally, inter-lab experiments lack concordance, with no consistency in pre-analytical steps such as selecting tube types, temperature, processing times, extraction, and QC procedures. This variation in pre-analytical protocols is common across all cell-free RNA, ncRNA, and circulating lncRNAs (108). Similarly, EV-carrying lncRNAs are a promising but still an emerging field, facing standardization challenges from isolation to analysis (109). As a result, there is no universal method for isolating and measuring biomarkers from circulating tumor material or EVs. Moreover, labs often use different reference genes or controls, making results difficult to compare and reducing reproducibility. Large projects like CANCER-ID and global efforts in sample preservation are working to simplify and standardize sample handling and analysis across labs (110). Achieving this requires collaboration among all stakeholders, including research labs, regulatory agencies, and clinicians, to develop standardized protocols/SOPs with common quality assurance measures, which must then be validated before clinical use. While liquid biopsies are now a key part of modern cancer diagnostics, technical challenges remain; tests may miss true positives due to low sensitivity or produce false positives due to low specificity. The FDA has issued special approvals and guidelines, such as those from NCCN and ESMO, for circulating DNA tests; however, no lncRNA-based assays have received FDA approval or recognition to date (Figure 3). This highlights a significant gap, as no regulatory framework or guidelines currently exist to support clinical translation. A multi-omics approach combining data types such as genetic alterations in circulating DNA and proteomics may improve detection accuracy compared to studying circulating lncRNA alone (111, 112).

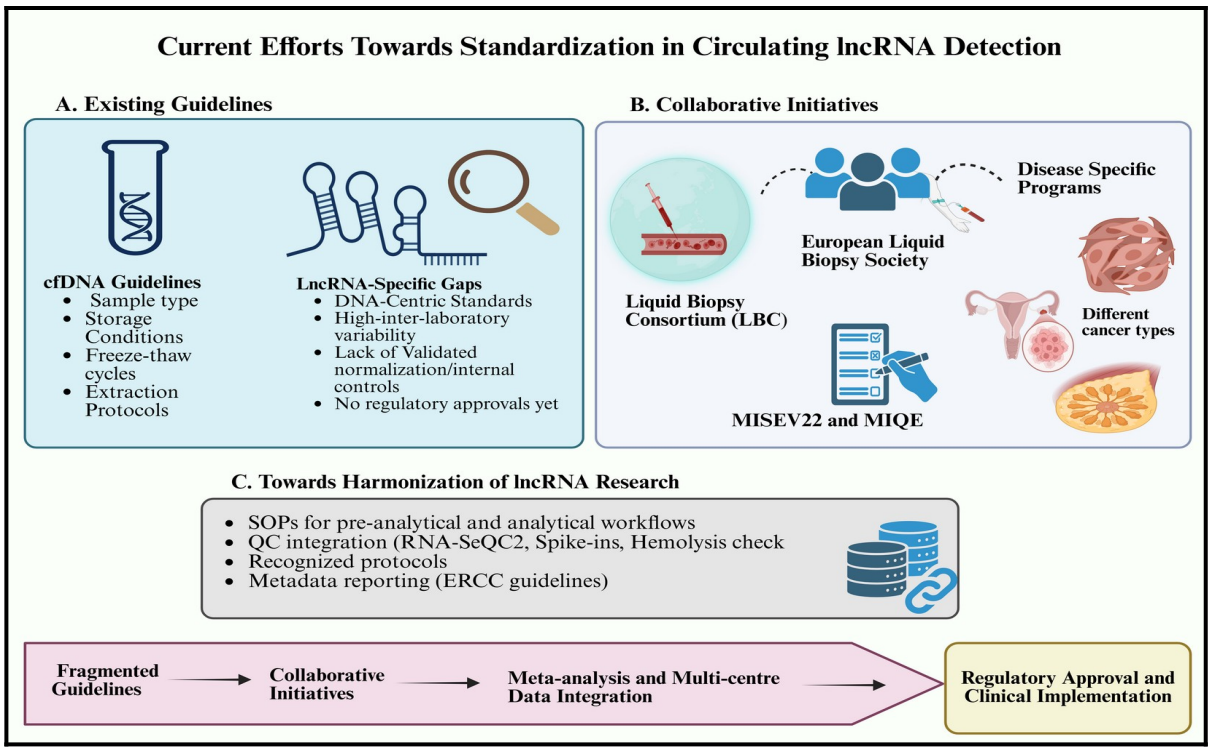

Figure 3. The figure summarizes the ongoing efforts to establish standard practices for circulating lncRNA detection. **(A)** Existing guidelines are DNA-centric and highlight gaps in lncRNA-specific guidelines. **(B)** Collaborative initiatives by worldwide consortia and societies to unify the protocols. **(C)** Harmonization strategies that emphasize SOP standardization, QC integration, recognized protocols, and metadata reporting, moving from fragmented guidelines to regulatory approval and clinical implementation

8. Recommendations for harmonizing lncRNA research

The lack of harmonized SOPs, which track pre-analytical and analytical workflows, poses a major challenge in circulating lncRNA research. Variability in sample collection, storage conditions, and isolation kits often leads to false positives and poor reproducibility in batch analysis. Therefore, SOPs should adhere to consistent standards to minimize variability (113). Recently, the international standardization bodies CEN in Europe and ISO globally have issued technical specifications to guide researchers in handling blood samples before analyzing for cell-free RNAs (ccfRNAs) or EV-protected RNAs, which carry circulating lncRNAs. Studies by the exRNAQC consortium also highlighted that exRNA quality is affected by the type of collection tubes and processing delays. They recommended standardizing SOPs to specify tube type, limit room-temperature delays, and use validated extraction methods tailored to each tube's chemistry and additives, rather than mixing incompatible methods (114). This ensures consistent RNA yield and quality, reduces bias, and enhances reproducibility. It is advised to follow the reliable guidelines MISEV2023 when studying lncRNAs within EVs. This protocol considers EV source, isolation method (ultracentrifugation, size-exclusion, etc.), and the use of multiple

independent markers to confirm EV identity. Maintaining a balance between yield and purity during EV isolation is encouraged (12, 115). For detection, if the approach is targeted qPCR/dPCR, MIQE/dMIQE guidelines should be followed, and results should be reported in the standard format provided by Real-time PCR Data Markup Language (RDML). For RNA-seq assays, SOPs must detail library preparation steps, including kit selection, RNA input quantity, and UMI or adapter use, to prevent biases related to lncRNA length or GC content that could hinder accurate detection (116). Bioinformatic pipelines should be based on appropriate references with spike-ins (reference controls) and incorporate multi-level QC (such as RNA-SeQC2) to compare and correct sample-to-sample variation. Additionally, study design and metadata, including inclusion/exclusion criteria, sample handling, sample type, data normalization, and batch effects, should be recorded and registered per guidelines from the Extracellular Communication Consortium (ERCC) (117). Including these details in SOP templates can improve data interpretation and facilitate cross-lab data integration, helping to better account for pre-analytical variables. To address challenges such as hemolysis, it is recommended to assess hemolysis using spectrophotometry or sequencing-based metrics, establish exclusion criteria for heavily hemolysed samples, and perform hemolysis-sensitive analyses to evaluate their impact on results. It is also important to record but not ignore poor-quality pre-analytical data; such samples should be retested or processed with new samples or leftovers. If QC failure persists, the sample should be removed with proper justification. In cases of low hemolysis, adjustments can be made and reported accordingly. These practices will support reproducibility and trust in the data among researchers (117).

Hence, the different studies performed for quantitative synthesis in oncology have demonstrated that pre-analytical and analytical heterogeneity can obscure the interpretive analytical biology of circulatory lncRNAs. The clinical relevance (diagnostic or prognostic performance of circulating lncRNAs is already well established in several meta-analyses, but certain factors such as sample type, transcript intrinsic characteristics, can also result in poor reproducibility. Therefore, future studies aiming to develop circulating lncRNAs as biomarkers should not stop at independent discovery within a single patient cohort. Instead, investigators should lock in the identified lncRNAs as a fixed panel, define threshold values (e.g., expression cut-offs), and apply standardized algorithms to generate risk scores (such as high vs. low risk). Freezing the selected lncRNA panel enables unbiased external validation and allows assessment of whether the biomarker performs consistently in independent patient populations. Such methodological rigor is essential for translating discovered lncRNA biomarkers into reliable tools for clinical diagnosis or prognosis.

9. Integrated approach for lncRNA discovery and validation

Building upon the evidence summarized in earlier sections, a unified framework is proposed to improving reproducibility and clinical applicability of circulating lncRNA biomarkers **(**Figure 4).

9.1 Synthetic RNA standards for assay calibration

Recent literature highlights that synthetic RNA oligonucleotides and transcripts serve as critical internal standards in the quantification of lncRNA, significantly reducing assay variability and enhancing detection accuracy (8). Their implementation as spike-in controls in qRT-PCR and RNA sequencing workflows effectively calibrates for biases encountered during RNA extraction, amplification, and sequencing stages (post-RNA extraction) (118, 119). This approach enables improved specificity by distinguishing target lncRNAs from homologous transcripts and overlapping genomic regions, a major analytical challenge in the field (120). Commercial platforms (e.g., Qiagen RT2 lncRNA qPCR assays) rely heavily on *in silico* design combined with synthetic controls to ensure primer specificity against overlapping and homologous transcripts, including coding genes (121). The use of synthetic RNA standards to mimic splice variants and overlapping genomic regions to overcome confounding signals is emerging, though not yet widespread, which represents a promising area for assay refinement (119). Overall, incorporating synthetic RNA standards can standardize lncRNA quantification, mitigating variability due to sample quality or methodological differences, and would enhance the clinical applicability of lncRNA biomarkers (8, 118-120). Nonetheless, synthetic spike-ins cannot compensate for pre-analytical variations, which include differences in sample collection, processing delays, storage conditions, and freeze-thaw cycles, factors known to significantly affect circulating lncRNA integrity, abundance, and fragmentation (5). Recent studies underscore that such pre-analytical variables are the primary source of irreproducibility in circulating RNA measurements and cannot be solely addressed through internal controls added after sample collection (9). Therefore, while synthetic RNA spike-ins help ensure accurate and reproducible quantification once RNA has been extracted, they must be used alongside rigorous, standardized sample collection and processing protocols (e.g., timely plasma separation, temperature control, minimal freeze-thaws) to minimize variability at the source. Combining well-controlled pre-analytical handling with synthetic RNA quality controls can achieve reliable, high-integrity circulating lncRNA measurements suitable for clinical and research applications. Therefore, developing a real-time monitoring system for RNA stability or microfluidic-based on-chip stabilization and sorting for circulating RNA molecules may help in pre-analytical variability. This could reduce the root source of variability, reduce the time and complexity of downstream processing and maintain specificity and analytical robustness.

9.2 Isolation of tumor-specific exosomes

Tumor-derived exosomes (TEXs) carry oncogenic lncRNAs that reflect parental tumor cells and possess membrane markers (e.g., EpCAM, CD9, CD63), facilitating immunoaffinity-based isolation, which enhances signal-to-noise ratio in liquid biopsies (122, 123). Advances in microfluidic and electrochemical biosensor platforms have shown acceptable sensitivity and specificity in isolating and quantifying exosomes, enabling multiplexed detection of RNA cargo, including lncRNAs (124). Clinical applications increasingly use exosome-enriched lncRNA

profiles to monitor tumor progression and treatment response with superior stability compared to free RNA (8, 123). Some clinical trials and translational studies use tumor exosome RNA (use exosome-enriched lncRNA profiles) to diagnose or monitor tumor progression and treatment response with superior stability compared to free RNA (125, 126). Such technologies not only refine exosome enrichment but also facilitate rapid, automated processing suitable for clinical applications. Commercial solutions like the exoRNeasy Serum/Plasma Kit apply membrane-based affinity binding to purify exosome-derived RNA, reinforcing the clinical translation of these methods. Overall, immunoaffinity capture combined with emerging microfluidic biosensors addresses key challenges in pre-analytical sample heterogeneity and contamination, enabling robust detection of tumor-specific lncRNAs for cancer diagnosis and monitoring (122-124).

9.3 Machine learning

Advanced machine learning (ML) models trained on extensive, multi-source datasets, including healthy controls, cancer patients, and individuals with inflammatory or benign conditions, can computationally distinguish true cancer-associated lncRNA expression patterns from irrelevant co-expressions, background ribonucleoprotein complexes, or inflammatory or co-morbid confounders. Recent reports have demonstrated ML's efficacy in enhancing biomarker specificity and processing complex co-expression signatures (8, 127). Integrating sequence features, RNA abundance, RNA fragment profiles, and patient metadata, ML algorithms enable nuanced noise filtering indispensable for liquid biopsy accuracy. Noise-filtering tools like noisyR reduce false positives, especially for low-abundance lncRNAs (128). The multi-modal models that integrate metadata, co-expression networks, and sequence features can refine classification or pattern recognition accuracy in complex datasets (129). This computational sophistication is critical for overcoming limitations of single-marker approaches and assay variability, leading to more reproducible and accurate liquid biopsy diagnostics. Overall, ML-guided biomarker discovery addresses key challenges in liquid biopsy by increasing diagnostic precision, reducing variability, and enabling robust interpretation of complex transcriptomic data in oncological research and clinical applications (127, 129-131)

9.4 CRISPR-based functional validation

Transcriptome-wide CRISPR-Cas13 has emerged as a powerful tool for precise and transcript-specific knockdown of lncRNAs, offering superior specificity and reduced off-target effects compared to traditional RNAi and CRISPRi methods (132, 133). This technology enables rigorous functional validation that confirms the biological relevance of candidate biomarkers, increasing confidence in their specificity and clinical utility, a step often overlooked in lncRNA studies (134). Recent research has profiled over 6,000 lncRNAs across various cancer cell lines, helping identify key candidates for CRISPR-Cas13-based lncRNA perturbations. This enables validation of the causal roles of these biomarkers, strengthening clinical confidence in their involvement in cell cycle regulation and cancer cell survival, and allowing for their effective inhibition and induction of apoptosis (135-138). These studies show that many

lncRNAs function independently of nearby protein-coding genes, with essential lncRNAs exhibiting cell-type specificity and dynamic expression during development. Overall, CRISPR-Cas13 RNA-targeting platforms provide a vital link between molecular detection and functional validation of lncRNAs, boosting their potential as reliable cancer biomarkers and therapeutic targets (132, 133).

9.5 Multi-omic integration for biomarker contextualization

Combine circulating lncRNA profiles with orthogonal datasets; genomic CNVs, single nucleotide polymorphisms (SNPs), immune transcriptomes, and proteomic markers to delineate tumor vs. host response origin (139, 140).

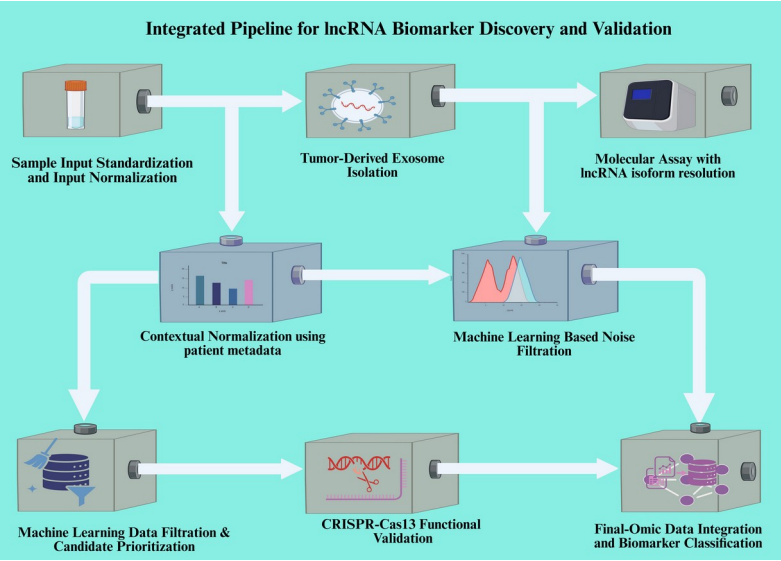

Figure 4. The figure depicts an integrated pipeline designed to identify reliable circulating long non-coding RNA (lncRNA) cancer biomarkers with high specificity and reproducibility. The workflow is represented metaphorically as a factory pipeline, where each distinct processing unit (machine) refines the biological sample and data toward final biomarker qualification. The process begins with sample input standardization and normalization to control for pre-analytical variability, followed by enrichment of tumor-derived exosomes for increased tumor specificity. Using immunoaffinity capture techniques targeting tumor-associated surface markers, tumor-derived exosomes are purified from biofluids. Sensitive molecular assays (such as strand-specific RNA sequencing or RT-qPCR) provide isoform-level resolution. Contextual statistical normalization leverages patient metadata (including diet, medication, infection status, and circadian rhythm, etc) to improve clinical generalizability. Machine learning-based noise filtration and data filtration, combined with candidate prioritization, remove technical and biological confounders, shortlisting robust candidates. Functional CRISPR-Cas13 screening validates the causal role of prioritized lncRNAs. The workflow concludes with integrative multi-omic data packaging and classification, delivering clinically actionable biomarker signatures.

10. Targeting SUMO-modified exosomal cargo-loading machinery for accurate identification of tumor-derived circulating lncRNA pattern

Multiple recent studies offer converging but incomplete evidence that specific RBPs actively sort lncRNAs into EVs. Chen et al. (2021) demonstrated a mechanistic example where the lncRNA ELNAT1 upregulates UBC9, which SUMOylates hnRNPA1 at K113, thereby promoting ESCRT-dependent recognition and packaging of ELNAT1 into EVs. Mutation or inhibition of the SUMO axis reduced EV loading and biological transfer, providing strong causal evidence for a post-translational modification (PTM)-enabled RBP-dependent pathway (143). Earlier mechanistic studies on small RNAs found that SUMOylated hnRNPA2B1 binds specific RNA motifs to drive exosomal sorting (144), a paradigm later extended to lncRNAs. Similar work involves YBX1 as a general loading factor for longer RNAs. Additionally, cancer cell studies show YBX1 binds specific lncRNAs (e.g., AC073352.1), and its depletion decreases their presence in exosomes, supporting a role for RBP–RNA condensates in cargo selection (145). More recently, IGF2BP family members (notably IGF2BP2/IMP2) were shown to reprogram EV cargo in cancer cells and influence recipient macrophage polarization, indicating RBPs can broadly alter EV RNA composition with functional consequences (146). Recent research also highlights deficiencies, such as different studies using different experimental methods, inconsistent EV isolation quality, and the scarcity of large-scale studies mapping how PTMs control RBP sorting into EVs (147). Overall, the literature supports that RBPs (hnRNP family, YBX1, IGF2BPs, FMR1/FXR) mediate lncRNA loading into EVs, and that PTMs (e.g., SUMOylation) modulate this process; however, larger-scale, orthogonal studies, including uniform EV purification, cross-cell-type validation, residue-level PTM mapping, and live-cell dynamics are still needed to determine how general and targetable these mechanisms are.

In normal cells, SUMOylation is a tightly regulated, low-stoichiometry, and often transient PTMs that modulate nuclear transport, transcriptional regulation, and selective RNA–protein interactions by temporally altering substrate activity or localization (148). In contrast, cancer cells frequently display dysregulated SUMO machinery, including upregulated UBC9 and altered SUMO E3 ligases, producing a net increase in SUMOylation (a “hyper-SUMOylation” state) that supports oncogenic programs such as proliferation, stress tolerance, and immune evasion (149, 150). Functionally, this shift changes the SUMOylation status of RNA-binding proteins (RBPs), stabilizing RBP–RNA complexes or changing their interactions with ESCRT and trafficking machinery. An example is SUMOylation of hnRNPA1/hnRNPA2B1 that promotes loading of specific RNAs into extracellular vesicles (EVs) and facilitates intercellular transfer in tumors (143). Mechanistically, cancer associated SUMOylation often converts transient regulatory marks into more persistent signals, either by elevated conjugating enzyme expression or altered deSUMOylases thereby biasing RBPs toward EV-sorting pathways and enhancing export of oncogenic lncRNAs (149, 151). Clinically, these differences make SUMO

pathway components attractive biomarkers and drug targets, and recent studies have shown that pharmacologic SUMO inhibition can reverse oncogenic SUMOylation patterns and modulate downstream RNA trafficking, although context dependence and off-target risk remain challenges (152, 153).

Despite its prominent role as mechanistic signal packaging of specific RNAs into EVs, translation of this mechanistic insight into a generalised "SUMO-based sorting" tool has been impeded for several reasons. First, SUMOylation is often low-stoichiometry and transient, making biochemical detection and proteome-wide mapping difficult without enrichment strategies or highly sensitive MS workflows (154). Second, evidence is currently contextual and cell-type specific; only a handful of cargos (e.g., ELNAT1, circTLCD4) and RBPs have been validated, so broad applicability across tissues and lncRNAs is unproven (143, 155). Third, methodological gaps persist such as heterogeneous EV isolation, lack of single-EV PTM assays, absence of validated antibodies or microfluidic sensors for SUMOylated RBP species, and limited live-cell SUMO reporters suited to capture transient modification at budding sites (154, 156). Finally, cancer-associated "hypersumoylation" offers promise as a differential marker, but it also raises specificity and toxicity concerns for global SUMO-manipulation strategies and demands single-vesicle resolution to link SUMOylated RBPs to specific oncogenic lncRNA cargo (154). In summary, SUMOylation is mechanistically validated in select cases, but broader application for EV sorting will require sensitive, single-vesicle PTM mapping, standardised EV preparation, and robust live/affinity sensors for SUMO-modified RBPs.

Based on emerging evidence that RNA-binding proteins (RBPs) and PTMs influence RNA sorting into extracellular vesicles, we hypothesize that cancer-associated alterations in SUMOylation may contribute to patterned, rather than specific, differences in the packaging of lncRNAs into exosomes. In this conceptual model, dysregulated SUMOylation, reported in several cancers, could modify subsets of RBPs at specific amino-acid residues, thereby influencing their interactions with lncRNAs during vesicle biogenesis. We assume that such SUMOylation patterns are unique to all cancers or conserved across tumor types (like a molecular tag or barcode), and that they may -in addition- represent context-dependent molecular features shaped by tumor state, cellular stress, and microenvironmental cues. Because normal cells exhibit much lower SUMOylation levels and different SUMO patterns, their exosomes carry RBPs with little or no SUMO marking. We further propose that, in some cases, SUMO-modified RBPs may co-associate with lncRNAs during exosome formation, potentially persisting within vesicles long enough to serve as detectable molecular features. However, the stability of RBP–lncRNA complexes and the fidelity with which SUMO modifications are preserved within extracellular vesicles remain open questions that require direct experimental validation. Moreover, non-malignant conditions characterized by elevated cellular stress or inflammation may exhibit overlapping SUMOylation signatures, underscoring the need for appropriate disease and comorbidity

controls. From an analytical perspective, we suggest that these molecular features be interpreted as combinatorial patterns, as a "SUMO-barcode", rather than as deterministic cancer-specific individual markers. Accordingly, we envision chip-based nanoarray platforms capable of capturing and interrogating large numbers of individual exosomes using multiplexed SUMO- and RBP-sensitive probes, coupled with lncRNA detection and AI pattern recognition algorithms (Figure 5). Such platforms would enable single-exosome–resolved, pattern-based analyses rather than binary classification. If supported by appropriately powered and controlled studies, this approach may offer a complementary layer of biological information that, when integrated with standardized pre-analytical and analytical workflows, could enhance the interpretability of circulating lncRNA measurements for early cancer detection.

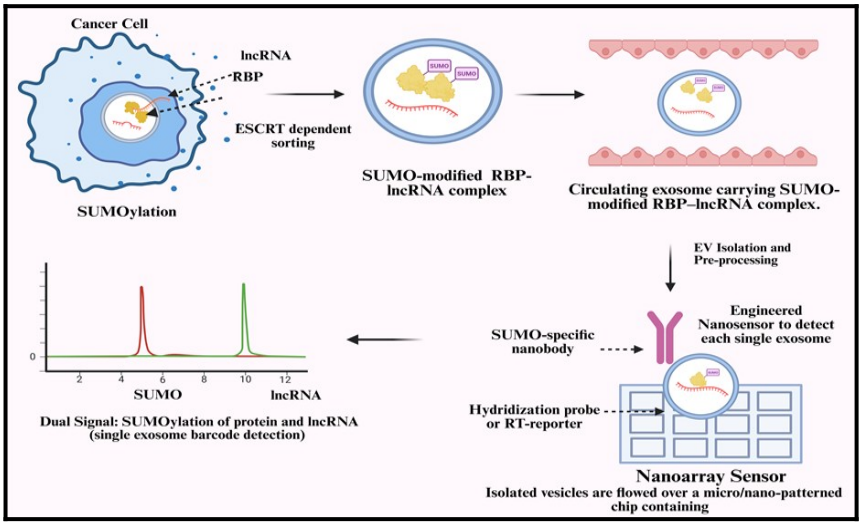

Figure 5. This schematic depicts the workflow from blood-borne exosomes to nanosensor-based molecular characterization. Circulating EVs are isolated and flowed across a nano-array chip that captures individual exosomes via tetraspanin-specific ligands. SUMO-specific nanobody or aptamer probes detect SUMO-modified RBPs bound to oncogenic lncRNAs, producing a quantifiable single-vesicle SUMO-barcode signal. Captured vesicles are then lysed on-chip, enabling direct measurement of associated lncRNAs through integrated molecular detection modules. Combined SUMO-barcode intensity and lncRNA identity allow precise discrimination of tumor-derived exosomes from background vesicles.

10.1 Proposed cohort framework for Sumo-RBP-lncRNA pattern classifier development

Despite rapid growth in extracellular vesicle (EV) research and increasing interest in their clinical utility, few EV-based biomarkers have matured into clinically validated tests. Nonetheless, comprehensive profiling approaches demonstrate the promise of circulating EVs as fluid-based indicators of disease state and subtype, particularly in oncology, where EV contents reflect tumor cell origin and progression-associated molecular features in plasma and other biofluids. For example, differential protein profiles in breast

cancer-derived EVs were shown to stratify molecular subtypes and correlate with cell-of-origin signatures, supporting the concept that EV cargo composition can carry disease-relevant information in a non-invasive context (157)**.** Circulating EVs, including exosomes, carry a diversity of macromolecules, proteins, non-coding RNAs, lipids, and metabolites that collectively reflect their cellular source and physiological context (158). Therefore, cohort design must account for these factors to isolate patterns attributable to malignancy. To translate the proposed SUMOylation-guided framework from a conceptual model into a testable strategy, a realistic and carefully controlled clinical study design is required. Importantly, any clinically useful "SUMO-barcode" pattern classifier cannot rely on binary thresholds or bulk measurements, but must instead learn multidimensional patterns from single-exosome–level data while accounting for biological and pathological confounders. We propose a minimal, hypothesis-testing cohort design suitable for an exploratory, medium-term study rather than a large consortia-scale effort. The primary objective of this design is not immediate clinical deployment, but to determine whether reproducible, cancer-associated SUMO-RBP–lncRNA patterns exist, and can be differentially identified, at the level of individual EVs.

10.1.1 *Cohort A - healthy controls*

Collection of ~ 10–20 healthy individuals without a history of malignancy, acute infection, chronic inflammatory disease, or systemic stress. This group establishes baseline distributions of SUMO isoforms, RBP modification frequencies, and lncRNA associations in circulating EVs under physiological conditions.

10.1.2 *Cohort B- cancer patients*

Collection of 10-20 patients diagnosed with a specific cancer type, matched for stage and clinical features, to minimize heterogeneity driven by tumor burden or treatment. Focusing on a single cancer subtype, for example, breast, ovarian, or colorectal cancer, enhances the ability to detect disease-associated deviations from baseline EV profiles. Such approaches are in line with targeted proteomics and classification efforts in EV biomarker research, where differential EV content provides diagnostic and prognostic information (159 - 161).

10.1.3 *Cohort C- cancer disease controls*

Collection of 10–15 individuals with documented non-cancer conditions that activate systemic stress or SUMO pathways, such as chronic inflammatory diseases (e.g., rheumatoid arthritis), neurodegenerative disorders (e.g., Alzheimer's disease), cardiovascular conditions, or viral infections. This cohort is critical for assessing the specificity of SUMO-RBP–lncRNA patterns to malignancy rather than non-specific stress responses.

10.1.4 *Measurable EV features for hypothesis testing*

Rather than relying on a single biomarker, this design emphasizes multidimensional pattern detection at the level of individual EVs. Features of interest for each sample may include: relative distribution of SUMO isoforms (e.g., SUMO1 vs. SUMO2/3) on EV-associated RBPs; frequency of SUMOylation at

specific RBP residues known to influence RNA binding and EV sorting; ratio of mono-SUMO vs. poly-SUMO modifications; co-occurrence of SUMOylated RBPs with specific lncRNAs, including those previously implicated in cancer biology; and subpopulation heterogeneity within EV pools. These measurements can support the construction of a patient-level SUMO-barcode profile, capturing combinatorial signatures of post-translational modification and cargo association. Such high-dimensional profiles align with emerging approaches in multi-omics and machine-learning-based classification of EVs, which have shown promise in cancer detection and prognosis when leveraging rich feature sets (162). The proposed sample sizes, while modest, are sufficient for initial signal exploration (preliminary study) when coupled with single-EV profiling and appropriate statistical methods. Previous studies leveraging multi-marker or machine-learning models for EV classification have demonstrated better capability of detecting differences between patient groups using small to moderate sample sets (160). Therefore, integrating SUMOylation features with conventional EV cargo analysis can allow us to reliably test our main hypothesis while maintaining feasibility within the scale of exploratory research.

10.2 Single-Exosome statistical profiling and pattern classifier construction

Despite advances in liquid biopsy, most studies of EVs have relied on bulk measurements, which average molecular signals across heterogeneous populations of vesicles. However, individual exosomes can differ markedly in cargo content, surface markers, and PTMs, reflecting the diverse cellular environments from which they originate. Single-vesicle studies in cancer have demonstrated that genomic and proteomic measurements at the level of individual circulating extracellular vesicles can reveal subpopulation structures and rare but biologically important features that are obscured in bulk analyses. For example, single-EV fluorescence and interferometric techniques have identified distinct subpopulations of tumor-derived EVs with diagnostic potential in pancreatic and lung cancers, highlighting the value of vesicle-resolved profiling when developing sensitive biomarkers (163, 164). In the context of the SUMOylation-guided extracellular RNA (exRNA) hypothesis, single-exosome profiling is particularly important due to the expected heterogeneity in both SUMO PTMs patterns and RNA cargo across vesicle subpopulations. SUMOylation itself is a dynamic and reversible PTM that can be differentially regulated across cell states, stress responses, and cancer progression, leading to variable modifications of RBPs involved in EV cargo selection. Moreover, lncRNA content within EVs exhibits selective enrichment patterns that are influenced by cell type, disease state, and stress pathways, including epitranscriptomic regulation such as N6-methyladenosine (m^6A) modifications (165-167).

10.2.1 *Frequency and distribution metrics at single-exosome resolution*

In practice, analytical strategies must capture the frequency and distribution of SUMOylated RBP–lncRNA associations at the level of individual vesicles. Key measurable parameters include proportion of SUMOylated versus non-SUMOylated RBP in individual EVs, which

reflects PTM heterogeneity within circulating vesicle populations; distribution of SUMO isoforms (e.g., SUMO1 vs SUMO2/3) and the prevalence of mono-SUMO vs poly-SUMO chains, indicative of distinct regulatory states of RBPs; co-occurrence patterns of specific RBPs with cancer-associated lncRNAs within single EVs; subpopulation heterogeneity, characterized by combinations of surface markers, SUMOylation status, and RNA cargo signatures. High-throughput single-EV capture platforms combined with multiplexed detection (e.g., fluorescence microscopy, nanofluidic devices) now enable the analysis of thousands of individual exosomes per patient sample, yielding statistically robust distributions of these features. Single EV analyses using techniques such as nano-flow cytometry or ExoView profiling have successfully decoded heterogeneity in EV surface proteomes and RNA content, establishing precedents for this level of resolution in biomarker studies (168, 169).

10.2.2 *Multidimensional profiles and machine language-assisted classification*

Given the high dimensionality of the parameters described above, single features are unlikely to provide sufficient discriminatory power between cancer and non-cancer states. Instead, a multidimensional profile, the "SUMO-barcode", comprising combinations of PTM distributions and cargo co-occurrence patterns, offers a more informative representation of disease-related perturbations. Statistical modeling and machine-learning approaches are well-suited to extract patterns from such high-dimensional data. In liquid biopsy research, multivariate classifiers built on proteomic, transcriptomic, and epigenomic features have significantly outperformed single markers in distinguishing cancer patients from controls, particularly when data are heterogeneous and noisy. For example, machine-learning models applied to multiplexed EV proteome and RNA data have shown improved classification accuracy in lung and pancreatic cancer cohorts relative to conventional univariate approaches (170, 171). In the SUMOylation context, classifiers may be trained on feature vectors representing: SUMO isoform and chain length distributions across vesicles; relative abundance of SUMO-modified RBPs; co-occurrence patterns of SUMOylated proteins with specific lncRNAs; and EV subpopulation structures derived from combination markers. Machine-assisted pattern recognition enables discrimination between states even when individual features overlap between groups due to biological variability, a situation that traditional threshold-based diagnostics cannot reliably address (171**).**

10.2.3 *Biological heterogeneity and sampling noise*

Even with single-EV profiling and pattern classifiers, biological heterogeneity remains a key challenge. Variability in tumor burden, genetic and epigenetic tumor landscapes, systemic stress responses, and comorbid conditions can all influence SUMOylation and EV cargo patterns. Consequently, statistical analysis of single-EV data should incorporate appropriate models of intra- and inter-patient variability, account for covariates such as age and inflammation, and use cross-validation and independent test sets to avoid overfitting in classifier development (172). In summary, moving beyond bulk measurements to single-exosome profiling combined with pattern recognition approaches provides the most

scientifically rigorous path to test the hypothesis that SUMOylation patterns of RBPs influence selective lncRNA packaging in EVs. This integrated analytical strategy is essential not only for discovery but also for eventual clinical translation of SUMO-based EV signatures as biomarkers.

10.3 A microfluidic nanoarray platform for single–exosome SUMO-RBP-lncRNA profiling

A central challenge for translating the SUMOylation–guided hypothesis of selective lncRNA packaging into EVs into a testable framework is the development of an analytical platform capable of single-vesicle resolution, multiplexed molecular readouts, and quantitative detection of both PTMs and RNA cargo. Conventional EV analysis methods, such as bulk ultracentrifugation followed by Western blot or qPCR, yield averaged signals across large, heterogeneous vesicle populations and are therefore ill-suited for dissecting the co-occurrence patterns of SUMO-modified RNA binding proteins (RBPs) with specific lncRNAs at the individual vesicle level (173). Microfluidics and nano-sensing technologies have provided compelling alternatives that offer high sensitivity, minimal sample requirements, and the capacity for integrated multi-omics profiling, positioning them as promising platforms for hypothesis-driven EV analysis (174).

10.3.1 *Microfluidic immunocapture enables specific, high-throughput EV capture*

Microfluidic platforms using antibody-based capture have become widely used to isolate and profile exosomes at the microscale. For example, the ExoSearch chip integrates continuous-flow immunoaffinity capture with on-chip multiplexed detection of tumor markers (CA-125, EpCAM, CD24) from blood plasma, demonstrating quantitative exosome isolation and marker profiling for ovarian cancer diagnosis. This approach uses surface markers to specifically capture vesicles and achieve measurable disease signatures within microliter-scale samples (175). Early integrated platforms such as the nano-plasmonic exosome (nPLEX) chip, which exploited a periodic nanohole array with surface plasmon resonance (SPR) for label-free, parallel detection of up to 12 exosomal protein markers, have demonstrated diagnostic potential from clinical ascites samples with high accuracy (176, 177). Platforms such as ExoView™ (based on SP-IRIS technology) have demonstrated the ability to capture and image tens of thousands of single EVs per sample with high reproducibility, enabling multiplexed fluorescent detection of surface proteins and RNA-associated signals (178, 179).

10.3.2 *Nano-engineered chips offer enhanced capture and multiplex profiling*

Nano-engineered microarrays have further improved capture efficiency and detection sensitivity. The ExoProfile chip employs 3D porous serpentine nanostructures functionalized with antibodies (e.g., anti-CD81) to capture exosomes with both high efficiency and morphology confirmation via SEM (175, 177). This platform not only improves capture over flat surfaces but also supports multiplexed on-chip fluorescent detection of surface markers, enabling eight-marker profiling of exosome subpopulations relevant to cancer (e.g., CD9, CD63, EGFR, HER2, EpCAM) (180). Reviews of microfluidic EV technologies illustrate a

range of immunoaffinity platforms where antibodies against surface proteins (CD63, CD81, EpCAM) are conjugated to channel surfaces or micropost structures, resulting in substantial EV capture and downstream molecular analysis. Similarly, ExoSearch microfluidic chip combined immunoaffinity capture with *in-situ* multiplexed fluorescent detection of surface antigens, enabling discrimination of cancer versus control samples based on panels of tumor-associated proteins (181). These innovations establish [1] multiplexed profiling of multiple molecular targets in a single run, which enhances diagnostic discrimination relative to single biomarkers, [2] integrated capture and detection minimizes sample loss and processing time, and [3] chip-scale designs with parallel microchannels or nanostructured surfaces support scalable analysis from small sample volumes typical of clinical liquid biopsies.

10.3.3 *Towards single-exosome resolution and multi-modal profiling*

Microfluidics has enabled highly sensitive EV isolation based on size, immunoaffinity, and physical properties, combined with downstream detection of internal cargo such as nucleic acids. Reviews of chip-based EV technologies underscore the diversity of approaches, ranging from nanowire and membrane filtration for efficient isolation to integrated immunoaffinity systems capable of capturing small EV subsets with high purity (181, 182)**.** In the context of multiplexed functional profiling, platforms that capture individual vesicles on micro/nanoarrays and interrogate them with multiple probes are especially relevant. For example, 3D nanostructured microfluidic chips engineered through self-assembly techniques have achieved ultra-sensitive quantification of surface proteins on individual EVs down to tens of vesicles per microliter, enabling simultaneous phenotyping across multiple markers (180, 183)**.** Such structural innovations highlight the feasibility of expanding single-EV platforms to incorporate detection modalities beyond proteins, including PTMs and RNA content.

10.3.4 *Analytical considerations for SUMO-RBP–lncRNA profiling*

To operationalize the SUMOylation hypothesis, an analytical platform must integrate the following capabilities at the single-exosome level.

10.3.4.1 Multiplex antibody/nanobody detection of SUMO isoforms and RBPs

Once exosomes are immobilized, their molecular contents can be interrogated sequentially. The most widely used method to detect SUMOylation of specific proteins relies on antibodies against SUMO1, SUMO2/3, or SUMO-conjugated substrates. A recent systematic evaluation demonstrates considerable variability in the sensitivity and specificity of commercially available SUMO monoclonal antibodies across applications such as immunoblotting, immunoprecipitation, and immunofluorescence. Some antibodies can detect free SUMO and conjugated forms, while others preferentially recognize particular SUMO paralogs under specific conditions, underscoring the importance of reagent selection when probing SUMO modifications on RBPs. These antibody tools can be adapted for multiplexed fluorescence detection when

combined with RBP-specific antibodies (e.g., against hnRNPA2B1, YBX1, HuR) to assess co-occurrence of SUMOylation and RBP identity via multichannel imaging or flow-based platforms (183). In cellular systems, SUMO chain–sensitive probes, FRET-based reporters, and enzymatic strategies using SUMO proteases have enabled discrimination between mono- and poly-SUMOylation states. Although such approaches have not yet been extensively implemented in EV contexts, their established performance at single-cell resolution supports their conceptual transferability to single-exosome platforms (184).

10.3.4.2 In situ detection of lncRNA cargo

Following protein-level characterization, RNA cargo within the same immobilized exosomes can be detected *in situ*. Molecular beacons, aptamer probes, and SMFISH-inspired hybridization strategies have demonstrated direct, extraction-free detection of exosomal RNAs, particularly microRNAs, with sensitivities comparable to those of conventional amplification-based assays (185). The integration of these probes into immuno-capture chips enables the simultaneous capture of EVs and RNA detection within confined microfluidic environments, thereby reducing sample loss and analytical bias. While most existing studies focus on short RNAs, advances in probe design and enzymatic signal amplification suggest that extension to lncRNAs is technically feasible, albeit requiring careful optimization due to low copy number constraints (186-188). Advances in microfluidic architectures have facilitated on-chip nucleic acid detection using aptamer probes, molecular beacons, or enzyme-assisted amplification within capture chambers. Microchips with microcolumn arrays have enabled the simultaneous *in-situ* detection of exosomal proteins and microRNAs, achieving limits of detection comparable to those of conventional fluorescent assays (185). Expanding these strategies to include lncRNAs and, where feasible, modification-specific probes (e.g., motifs indicating epitranscriptomic markers) would support more comprehensive cargo profiling.

10.3.4.3 Co-localization and high-resolution imaging

A defining advantage of chip-based single-exosome platforms is the ability to resolve co-occurring molecular signals within the same vesicle. Multi-channel fluorescence microscopy allows simultaneous visualization of surface capture markers, SUMO isoforms, RBPs, and RNA cargo, while super-resolution techniques such as STORM and DNA-PAINT provide nanometer-scale spatial resolution. These approaches have already been used to map heterogeneous EV surface proteomes and identify clinically relevant vesicle subpopulations. Applied in combination, they enable the extraction of per-exosome molecular profiles, capturing co-localization patterns rather than isolated markers (189). This enables the construction of a high-dimensional "SUMO-barcode" by recording patterns of protein and RNA markers across thousands of vesicles.

10.3.4.4 Signal Amplification Strategies for Low-Abundance Targets

Given the inherently low abundance of many EV-associated molecules, optional amplification strategies can substantially

enhance analytical sensitivity. Proximity ligation assays (PLA) convert protein–protein or protein–modification proximity into amplifiable DNA signals and have been widely used to visualize SUMOylated proteins in situ (190, 191). Adaptations of PLA and nanoPLA have been applied to EVs, enabling sensitive detection of vesicle-associated protein complexes. Rolling circle amplification, either as part of PLA or as a standalone strategy, further enhances fluorescence signals for both protein and RNA detection, supporting reliable single-vesicle analysis (192, 193).

10.3.4.5 Alternative detection strategies: Resonance Energy Transfer (FRET/BRET) and biotinylation approaches

Beyond antibodies, resonance energy transfer methods such as TR-FRET and BRET have been used to detect SUMOylation *in vitro* by fusing SUMO and substrate proteins to donor/acceptor pairs, enabling dynamic observation of modification events. These approaches do not require separation steps and can provide site-specific interaction data when designed appropriately (194). The SUMO-ID technique utilizes proximity biotinylation (TurboID) to label proteins that are either covalently SUMOylated or interact via SUMO interaction motifs (SIMs), allowing identification of SUMO-dependent interactors in complex biological contexts. Although not yet applied to exosomes, it illustrates the potential for proximal labeling strategies that could be adapted to vesicle-based detection (195).

10.3.4.6 Limitations and translational considerations

Despite rapid progress, challenges remain in standardization, reproducibility, and clinical validation of EV-based chip assays. Heterogeneity in sample handling, device fabrication, and probe performance can introduce variability that complicates cross-laboratory comparisons and regulatory pathways. Moreover, distinguishing tumor-specific signals from the abundant background of normal EVs and other nanoparticles in biofluids requires both highly specific probes and robust analytical frameworks (196). Most traditional detection methodologies (antibodies, PLA) do not discriminate SUMO chain length or specific attachment sites. SUMOylation can involve polySUMO chains or multiple lysine acceptor sites, complicating interpretation because different chain topologies may affect protein function differently. Mass spectrometry approaches exist to map SUMO conjugation sites at high resolution, but they require peptide enrichment and specialized workflows (197). Engineered biosensors designed to report SUMO modification state (e.g., fluorescent SUMO traps) have been used to track global SUMO dynamics in cells but are not yet widely scaled for multiplexed or high-throughput detection of specific SUMO isoforms on individual proteins (198). Nevertheless, the trajectory of technology development, from early microfluidic capture devices to integrated, multiplexed, single-vesicle analysis platforms, supports the feasibility of developing an analytical chip capable of simultaneously detecting SUMOylation signatures, RBP identity, and lncRNA cargo with single-exosome resolution.

11. Conclusion

Circulating lncRNAs, despite their potential, have clinical utility that is hindered by challenges in sample sourcing, handling, low abundance, extraction chemistries, quantification platforms, and bioinformatic workflows. Therefore, standardizing each step is essential to achieve good yield, quality, and to ensure reproducibility and comparability across studies. Physiological conditions and genetic polymorphisms leading to inter-individual variation further emphasize the need for a rigorously designed and ethnically diverse validation cohort. Moreover, harmonizing analytical and bioinformatic workflows is essential to minimize variability introduced by extraction chemistries, data normalization strategies, and low-abundance transcript detection. This harmonization will improve comparability across studies and enable reproducible results suitable for clinical validation. Notably, the current analysis advances the field by highlighting that the developing lncRNA biomarkers should not stop at discovery and independent validation; instead, researchers should move toward a fixed biomarker panel with set transcript levels, cut-offs, and risk algorithm integration to ensure successful and reliable clinical utility. Further, adopting standard SOPs is only feasible through multi-centre collaboration among clinicians, researchers, and laboratories, recognizing the importance of integrating multi-omic data to achieve precision. The integrated approach proposed in this review by combining optimized pre-analytical control, analytical validation, and biological standardization, offers a structured pathway to strengthen reproducibility and ensure reliable biomarker interpretation. This framework can guide the establishment of uniform reference datasets, facilitate regulatory approval, and ultimately translate circulating lncRNAs into clinically actionable tools for early cancer detection and personalized oncology. After establishing a standardized way to detect circulating lncRNAs, we suggest that natural regulatory processes inside cells may help in explaining why circulating lncRNA patterns are organized but still variable. One such process is the chemical modification of lncRNA-binding proteins, which can influence how these RNAs behave. In particular, patterns of SUMOylation on these proteins can be considered as molecular “barcodes” which may help distinguish cancer-related lncRNA signals from normal background signals. This idea can be tested by analyzing individual exosomes and interpreting combinations of signals rather than single markers. Although this concept requires experimental validation, combining such biologically informed ideas with rigorous standardization could improve consistency between studies, support regulatory approval, and help translate circulating lncRNAs into reliable tools for early cancer detection and personalized cancer care. This represents a promising future layer of specificity that may further enhance the clinical accuracy and discriminatory power of lncRNA-based diagnostics.

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BioRender (https://www.biorender.com/), was used to create Figures in the manuscript.

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