

Review

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Review

# Extracellular RNAs in Liquid Biopsy: Applications in MASLD and MASH Diagnosis and Monitoring

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## Abstract

Metabolic dysfunction-associated steatotic liver disease (MASLD) is an increasingly prevalent condition linked to obesity, diabetes, and metabolic syndrome, and can progress to fibrosis, cirrhosis, and hepatocellular carcinoma. Current diagnostic standards such as liver biopsy are invasive and unsuitable for routine screening. Liquid biopsy, particularly through analysis of extracellular RNAs (exRNAs), including microRNAs (e.g., miR-122, miR-21, miR-34a), long non-coding RNAs, and tRNA-derived fragments, offers a promising non-invasive alternative. These biomarkers correlate with histologic severity, fibrosis stage, and treatment response, and may outperform traditional markers such as ALT and FIB-4 in diagnostic accuracy. Despite their potential, clinical translation is limited by a lack of standardization and large-scale validation. This review outlines recent advances in exRNA-based diagnostics for MASLD and MASH, emphasizing their role in early detection, disease monitoring, and the shift toward personalized hepatology.

**Keywords:** liquid biopsy; extracellular RNAs; microRNAs; extracellular vesicles; metabolic dysfunction-associated steatotic liver disease; metabolic-dysfunction associated steatohepatitis; liver fibrosis

## I. Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) has emerged as the most prevalent chronic liver condition globally, affecting 38% of adults and 7-14% of children, with projections suggesting its prevalence will exceed 55% among adults by 2040. [1,2] This alarming growth parallels rising rates of diabetes, obesity, and metabolic syndrome, positioning MASLD to become the leading cause of liver transplantation in the U.S. within the next decade.[3–5] The economic burden is equally staggering: A 2024 Italian study estimated annual healthcare costs attributable to MASLD complications at €12.25 billion, with 13,438 excess deaths annually compared to non-MASLD populations.[6] In 2023, a multinational consensus led by hepatology societies redefined the condition from nonalcoholic fatty liver disease (NAFLD) to MASLD, initially proposed in 2020,[7] emphasizing its metabolic etiology through revised diagnostic criteria requiring at least one cardiometabolic risk factor.[8] This nomenclature shift aims to reduce the stigma while highlighting the disease's systemic nature, since 48.3% of global liver cirrhosis cases now originate from MASLD, with Asia bearing the highest burden.[2] Beyond hepatic outcomes, MASLD independently increases risks for cardiovascular events, chronic kidney disease, and extrahepatic cancers, necessitating multidisciplinary management strategies.[9]

Current diagnostic reliance on liver biopsy faces challenges due to invasiveness and sampling variability. While magnetic resonance imaging (MRI) and ultrasound elastography provide noninvasive alternatives, their moderate accuracy for early-stage fibrosis [area under the curve

(AUC) 0.70-0.86] limits utility in population screening.[10,11] It is broadly accepted, that MASLD diagnosis and treatment in the future will be based on personalized molecular signature.[12,13]

Liquid biopsies, which involve the measurement of disease-associated cell-free DNA (cfDNA), long non-coding RNA (lncRNA), extracellular RNA (exRNA), proteins and circulating extracellular vesicles (EVs) from body fluids, have currently emerged as promising diagnostic tools, valuable for monitoring MASLD progression. cfDNAs, fragmented DNA machinery released into the bloodstream by apoptotic and necrotic cells, are associated with MASLD severity,[14,15] and further even specific histone signatures appear to play a role in lean MASLD.[16] However, due to their high degree of fragmentation, their utilization as biomarkers can be problematic,[17] exhibiting low specificity for MASH.[18] For these reasons, their investigation will be out of the scope of this study. On the contrary, liquid biopsy platforms analyzing exRNA profiles in biofluids offer transformative potential, as demonstrated by miR-122, a liver-specific microRNA showing 2.4-fold higher sensitivity than ALT for detecting drug-induced injury and correlating with histopathological severity scores.[19,20] Notably, certain microRNA (miRNA) subtypes, in combination with leptin, a hormone that is related to MASLD,[21] have shown remarkable accuracy in evaluating steatosis, liver stiffness, and hepatic fat content.[22] Recent advancements in EV isolation enable detection of steatosis-associated miRNAs like miR-192-5p and miR-34a, both capable of distinguishing MASH from simple steatosis in multi-center trials.[3,23,24] ExRNAs hold promise not only as diagnostic and monitoring tools, but also as potential novel therapeutic targets to combat the growing epidemic of MASLD.[25,26] This review synthesizes evolving evidence on exRNA biomarkers in MASLD diagnostics, focusing on their role in staging fibrosis, predicting treatment response, and enabling precision hepatology through minimally invasive serial monitoring.

## II. Extracellular RNA as Carrier of Circulating Biomarkers

### II.1 Types of Extracellular RNAs

ExRNAs represent a diverse molecular ecosystem within biofluids, with distinct subtypes demonstrating unique roles in MASLD pathogenesis. ExRNAs can be found in almost all biological fluids including venous blood, cerebrospinal fluid, breast milk, pleural fluid, urine and saliva. Similar to the different RNA species, there are different types of exRNAs, including coding or messenger RNA (mRNA), and various non-coding RNAs such as miRNA, lncRNA, transfer RNA (tRNA) and circular RNA (circRNA) among others (Table S1). These exRNAs can be present either as full-length molecules or as fragments in the circulating biofluids with relevant RNA-base modifications. One of the highly prevalent classes of exRNAs is mRNAs which are responsible for protein synthesis.

MiRNAs, one of the most common and well-studied types of circulating exRNAs, are small (19-25 nucleotides), non-coding RNAs, that modulate gene expression post-transcriptionally.[27] miRNAs are among the most abundant RNA species in plasma and serum with the exception of ribosomal RNAs.[28] miRNAs can be packaged into EVs and transferred between cells, allowing direct intercellular communication. Several miRNAs, such as miR-122, miR-34a, miR-21, miR-145, and miR-451, have been linked to hepatic steatosis and MASH.[29]

lncRNAs refer to a class of RNA molecules that are longer than 200 nucleotides and do not encode proteins, comprising the majority of the non-coding RNA. lncRNAs regulate gene expression through various mechanisms, including chromatic modification, transcriptional regulation, and post-transcriptional processing.[30,31] lncRNAs are also being explored for their biomarker potential in various diseases, particularly their circulating counterparts, which can serve as liquid biopsy markers for different conditions.[32]

tRNAs, on the other hand, play a critical role in cellular function, since they help translate the sequence of mRNA into corresponding amino acid sequences during protein synthesis, effectively facilitating the construction of proteins based on genetic instructions.[33] Recently, a new type of tRNA has emerged, called tRNA-derived small RNAs or tDRs, that are produced after cleavage at specific sites in the codon or anti-codon loop by restrictive enzymes such as angiogenin (ANG),

responsible for cleaving the anticodon loop of mature tRNAs under stressful conditions (such as hypoxia, lack of amino acids, oxidative stress, ultraviolet radiation, heat shock, and viral infection).[34,35]

Small interfering RNAs (SiRNAs) can mediate RNA interference by binding to complementary mRNAs, leading to their degradation and affecting protein synthesis.[36]

Lastly, circRNA is generated by back-splicing events and have a covalently closed structure and exhibit high stability in circulation.[37] Their role in MASLD and MASH is still under active investigation, but preliminary findings suggest they can act as microRNA sponges or modulators of transcription and translation.[38]

## II.2 Sources of exRNA in Liquid Biopsies

Liquid biopsies represent a minimally invasive alternative to traditional tissue biopsies, enabling the analysis of biomarkers, including exRNAs and circulating tumor cells in bodily fluids.[39] RNA is a fragile molecule that needs to be safeguarded from the disrupting and degrading action of circulating enzymes found in the extracellular environment and biofluids. The molecules that encapsulate and protect RNA are called RNA carriers. ExRNAs in liquid biopsies originate from various carriers, each offering unique insights into physiological and pathological conditions. One type of RNA carriers is EV which include exosomes, microvesicles, and apoptotic bodies. These membrane-bound particles, released by cells into bodily fluids, encapsulate diverse RNA species such as mRNAs and miRNAs, thereby protecting them from ribonucleases[40] and provide a stable source of genetic material for analysis. Notably, EVs have been identified as carriers of cancer-derived RNAs, presenting potential biomarkers for early cancer detection and monitoring.[41] EVs encapsulate diverse RNA cargo (miRNAs, mRNAs, lncRNAs, circRNAs, etc.) reflective of their cell of origin. Recent research has solidified EVs as rich sources of disease biomarkers. For example, a 2024 study isolated EVs from human serum and found specific microRNAs in those EVs that mirror atherosclerotic plaque composition, suggesting that EV miRNA profiles can indicate plaque development.[42] Methodologically, EV isolation often involves ultracentrifugation, size-exclusion chromatography, or precipitation kits, followed by RNA sequencing or Polymerase Chain Reaction (PCR) to profile the cargo.

Not all circulating exRNA is vesicle-bound. A substantial fraction is associated with soluble RNP complexes, where RNA is bound to RNA-binding proteins (RBPs). Classic examples are Argonaute2 (Ago2) proteins carrying microRNAs in the bloodstream.[43] These Ago2–miRNA complexes are highly abundant in plasma and very stable. Until recently, only a few exRNA–RNP carriers were well characterized (e.g., Ago2, nucleophosmin 1).[44] New high-throughput methods are now mapping these carriers: for instance, crosslinking immunoprecipitation and sequencing was applied to chart exRNA–protein interactions across biofluids and 128 distinct extracellular RBPs and their RNA cargo were identified, laying the foundation for a new class of liquid biopsy biomarkers.[45] Importantly, an exRNA in an RNP complex offers a dual marker – the RNA and its binding protein. Detecting both can improve specificity for disease diagnosis. As a result, soluble exRNP complexes (like Ago2–miRNA or other RBP–RNA complexes) are gaining attention for their potential to indicate disease states (e.g., inflammation, cancer) when specific RNA–protein pairs are dysregulated.[45]

Circulating lipoproteins, especially high-density lipoproteins (HDL), are another overlooked carrier of exRNA. HDL particles can bind small RNAs (primarily miRNAs) and protect them from RNases in blood.[46] Notably, HDL-bound miRNAs are functional: early studies showed that HDL can deliver miRNAs to recipient cells, altering gene expression.[46] For example, HDL transfers miR-223 to endothelial cells to suppress intercellular adhesion molecule -1 (ICAM-1), a cell adhesion molecule, demonstrating a mechanism of intercellular communication via lipoproteins.[47] In conditions like familial hypercholesterolemia, distinct miRNA profiles have been observed in patient HDL particles, highlighting that disease states can alter lipoprotein-associated RNAs.[48]

Beyond the well-known carriers above, emerging sources of exRNA have been identified in recent years. Exomeres, first described around 2018, are nanoscale (~30-50 nm) non-membranous

particles that are distinct from classical EVs. They lack a lipid bilayer and consist mainly of proteins and nucleic acids. Exomeres were first isolated using advanced fractionation (asymmetric flow field-flow fractionation) to separate them from larger vesicles.[49] They are now recognized as part of the diverse exRNA carrier spectrum, though their specific biological functions are still under investigation.[50] A few years later, in 2021, researchers identified supermeres, an even smaller and denser class of extracellular nanoparticles.[51] Supermeres are isolated by ultracentrifuging the soluble fraction left after removing larger EVs and exomeres.[51] Emerging evidence showed that supermeres are replete with disease-related cargo, including proteins and RNAs tied to cancer chemoresistance.[52] This finding suggests that important biomarker molecules might concentrate in supermeres.

Surprisingly, entire organelles can be released into circulation. A landmark 2020 study demonstrated that human blood contains intact cell-free mitochondria, complete with mitochondrial RNA and DNA.[53,54] These extracellular mitochondria were shown to be respiratory-competent and enclosed by mitochondrial membranes,[53] although conflicting data exists regarding whether these mitochondria are fully functional.[53] Nonetheless, the discovery of extracellular mitochondria expands the concept of exRNA carriers, since mitochondria contain their own RNAs and raises questions about their role in signaling. Extracellular mitochondria have been identified in different forms and may serve as unconventional biomarkers for various diseases, including cancer, cardiometabolic disorders, and neurological conditions.[55–58]

By analyzing these diverse sources of exRNA, liquid biopsies offer a non-invasive and dynamic approach for monitoring disease progression, detecting early biomarkers, and assessing treatment responses in real-time. The ability to assess exRNA profiles from various carriers such as extracellular vesicles, ribonucleoprotein complexes, lipoprotein particles, and even extracellular organelles, enhances the sensitivity and specificity of liquid biopsy-based diagnostics. These advantages position liquid biopsies as a powerful tool in precision medicine, enabling early detection, personalized treatment strategies, and improved disease management.

### *II.3 exRNA Detection and Analysis Methods*

High-throughput RNA sequencing (RNA-seq) has become a powerful tool to profile the diverse landscape of exRNAs in liquid biopsy samples.[59] Because exRNAs represent fragmented transcripts from various cell types, sequencing approaches have been adapted to capture even low-abundance RNA species. Recent advances enable whole-transcriptome analysis of exRNA from small input volumes that were previously challenging. For instance, Small Input Liquid Volume Extracellular RNA Sequencing (SILVER-seq) was introduced to sequence exRNA from a single drop of human serum.[60] Other specialized library prep methods like PALM-Seq (Polyadenylation Ligation Mediated Sequencing) extend RNA-seq to non-polyadenylated exRNAs. PALM-Seq can capture miRNAs, tRNAs, and other RNAs by adding a poly(A) tail, enabling sequencing of a broader exRNA repertoire.[61] These approaches have improved the detection of both small RNAs and mRNAs circulating in biofluids. A comprehensive 2024 review notes that next-generation sequencing (NGS) of exRNA has been applied to monitor cancer progression and even predict pregnancy-related conditions.[62] The sensitivity of NGS allows discovery of novel RNA biomarkers and mutations in cell-free mRNAs that might be missed by targeted assays.[62] Additionally, improved bioinformatics pipelines (e.g., the exceRpt pipeline and exRNA Atlas data standards) address the unique challenges of exRNA data, such as removing contaminants and accounting for the fragmented nature of plasma RNA.[63,64] Overall, advancements in exRNA sequencing over the last five years have expanded our view of the “cell-free transcriptome,” enabling unbiased detection of disease signals. By capturing dozens to hundreds of RNA markers simultaneously, RNA-seq boosts liquid biopsy sensitivity and provides insights (like tissue-of-origin of the RNA) that enhance diagnostic accuracy. This method has been streamlined in other studies to identify new exRNA targets from patient plasma that could have biomarker potential.[65,66] While exRNA-seq profiles molecules outside cells, single-cell RNA sequencing (scRNA-seq) profiles the transcripts within individual cells. In liquid biopsy contexts,

scRNA-seq has been applied to rare circulating tumor cells and immune cells, complementing exRNA analysis. For example, scRNA-seq of circulating tumor cells can reveal gene expression signatures of metastatic cells, while exRNA in plasma reflects signals from tumor and stromal cells that may not be captured by analyzing limited cells.[67] Recent studies integrate single-cell or single-nucleus RNA-seq with exRNA-seq to map intercellular communication: one of those combined tumor single-nucleus RNA profiles with exosome small RNA profiles to identify which cell populations likely secreted specific miRNAs, constructing a cell-exRNA network for pancreatic cancer (demonstrating a novel way to infer biomarker sources).[68] Recent EV cargo analysis methods are pushing sequencing to the single-vesicle level. In 2022, researchers reported the first high-throughput transcriptomic analysis of individual EVs, highlighting heterogeneity in exRNA cargo between vesicles.[69]

Such “single-EV sequencing” is still nascent, but early results underscore that even EVs from the same sample carry distinct RNA molecules. As microfluidic sorting and barcoding methods improve, profiling RNAs from single exosomes or microvesicles could become feasible. In summary, scRNA-seq and related low-input sequencing innovations of the past few years provide a deeper context for exRNA findings, helping to link extracellular signals to specific cells and thereby refining the sensitivity and specificity of liquid biopsy interpretations.

Quantitative reverse-transcription PCR (qRT-PCR) has long been a gold-standard for detecting specific RNAs due to its sensitivity, specificity, affordability and speed. It remains widely used to validate exRNA biomarkers (e.g., measuring a candidate mRNA or miRNA in plasma) that were discovered by RNA sequencing and analysis. However, conventional qRT-PCR can struggle with the ultra-low RNA concentrations typical of liquid biopsies. In the last five years, the adoption of droplet digital PCR (ddPCR) has markedly improved detection limits for exRNAs. Droplet digital PCR partitions a sample into thousands of nanoliter droplets, each ideally containing zero or one target molecule, followed by end-point PCR in each droplet.[70] This effectively dilutes out background noise and allows absolute quantification of target copies by counting positive droplets. ddPCR greatly increases sensitivity for low-copy exRNAs: for instance, an exosomal miRNA (miR-29) in urine could be detected at <50 copies/ $\mu$ L by ddPCR, whereas standard qPCR required ~6500 copies/ $\mu$ L for detection.[71] Such improvements (over 100-fold sensitivity gain in that case) enable reliable measurement of rare transcripts. ddPCR also shows better precision and reproducibility for serum miRNA quantification compared to qRT-PCR.[72] Researchers have applied RT-ddPCR to measure clinically relevant exRNAs like androgen receptor splice variant 7 (AR-V7) mRNA in exosomes of prostate cancer patients, detecting this therapy-resistance marker in plasma with high sensitivity.[73] The ability to absolutely quantitate exRNA levels without a standard curve is another advantage of ddPCR, improving comparability between labs.[74] In sum, integrating digital PCR into exRNA workflows over the last few years has enhanced reliability (through precise quantification) and sensitivity (through lower detection limits) for liquid biopsy assays. This is critical for early cancer detection, where target RNA molecules may be vanishingly scarce. It’s worth noting that emerging isothermal amplification methods and CRISPR-based assays are also being explored to detect exRNAs without conventional PCR thermocycling. CRISPR Cas13-based diagnostic systems, for example, can amplify and detect specific RNA sequences with attomolar sensitivity, offering a fast and specific readout.[75] Such methods are still being refined but represent the next wave of nucleic acid detection beyond qRT-PCR/ddPCR, potentially boosting liquid biopsy sensitivity even further.

#### *II.4 Other Technical Considerations*

Liquid biopsy is a minimally invasive method that measures molecular products released from cells, such as proteins, cfDNAs, miRNAs and EVs. The accuracy of the results critically depends on the quality of sample collection, handling and processing. Plasma, or alternatively serum, is the preferred body fluid for liquid biopsy, and EDTA is commonly used as the anticoagulant due to its RNA-preserving properties, while heparin is generally avoided as it can inhibit downstream PCR

reactions.[76] Post-collection, samples are typically stored at -80°C to preserve their molecular content and morphology.[77]

After sample acquisition, EVs must be isolated since they coexist with other extracellular components, yet there is no universal standard for isolation and analysis, posing a significant limitation.[78] Among the various isolation techniques, ultracentrifugation is the most frequently used, offering advantages such as the ability to process large fluid volumes and minimal need for additional reagents.[79] It also allows discrimination of particles based on density, shape and size.[80] Other isolation methods include size-based approaches like sequential filtration and size-exclusion chromatography, as well as capture-based, polymer-based and microfluidic technologies.[81] Once isolated, EVs require characterization and quantification, which remain technically challenging. Common methods include immunoblotting for protein markers, transmission electron microscopy and nanoparticle tracking analysis,[82] while exRNA quantification typically relies on spectrophotometry and fluorimetry.[83]

In the context of RNA-seq analysis, although there is no universally accepted pipeline, standard steps include quality control, raw read acquisition, alignment, transcript quantification and integration with statistical tools.[84] Quality control is often performed using quality scores in FASTQ files, summarized by tools such as FastQC.[85,86] For read alignment, HISAT2 and STAR are two of the most widely used tools due to their computational speed and ability to identify splice junctions.[87] Downstream of alignment, RNA-seq data analysis faces numerous challenges, leading to the development of various pipelines. Alignment-based approaches include Tophat-Cufflinks, Tophat-HTSeq, and STAR-HTSeq, while pseudoalignment tools like Salmon and Kallisto provide faster alternatives, especially for large datasets.[88,89] To explore biological relevance, enrichment analysis tools such as g:Profiler are employed, offering updated annotations and user-friendly interfaces.[90] Furthermore, the Genome Analysis Toolkit (GATK) serves as a gold standard for variant discovery, with its validated per-sample workflow being widely used for RNA-seq variant calling.[91]

### III. ExRNAs in MASLD and MASH

#### III.1 Correlation with Disease Severity and Progression

The identification of specific extracellular RNA biomarkers has opened new frontiers in the diagnosis and monitoring of MASLD and MASH. Emerging evidence suggests that these biomarkers, particularly certain miRNAs, not only correlate with disease severity but also aid in monitoring disease progression and assessing treatment efficacy. Multiple studies have shown that circulating exRNAs correlate with the histological severity of MASLD and progression to MASH. On this context, hepatocyte-derived miR-122-5p, constitute 72% of circulating exRNAs in MASLD patients.[92] miR-122 is considered one of the most important biomarkers for MASLD, since it correlates with hepatic steatosis severity and MASH in human and animal studies. Other miRNAs, such as miR-22, appear to promote obesity and hepatic steatosis, since their knockout in animal models has demonstrated protection against both conditions.[93,94] This might imply that particular miRNA increases are directly correlated with liver injury and inflammation, while others appear to confer protection with their levels probably reflecting development of a resistance status, in a way resembling leptin and fibroblast growth factor-21 (FGF-21).[21,95]

Accordingly, an unbiased profiling of serum exosomal miRNAs in biopsy-proven MASLD found dozens of miRNAs whose levels tracked with steatosis, inflammation, ballooning, and composite NAFLD Activity Score (NAS). Notably, miR-7151-5p levels were positively associated with overall NAS, such that high miR-7151 predicted severe disease (NAS ≥6).[96] Intracellular and circulating levels of certain miRNAs often show inverse relationships. For example, miR-122 – essential for lipid biosynthesis[97]– is downregulated in MASH liver tissue, while it has been shown to rise in circulation with positive correlations with histologic grades of inflammation and fibrosis.[98] In fact, miR-122 serum levels increase progressively from healthy to simple steatosis to

MASH, reflecting escalating hepatocyte injury.[98] This pattern is also seen in animal studies. In MASLD mouse models, miR-122 and miR-192 were significantly elevated in circulating EVs, but reduced in liver tissue compared to controls. Their levels increased in EVs over time, suggesting active release following hepatocyte destruction. These findings were replicated in high-fat diet-induced MASLD mice, reinforcing the hypothesis that miRNAs are likely secreted into circulation as a response to hepatocyte injury.[99] Notably, in another study involving 84 serum biomarkers, circulating miR-122 levels were found to be more than seven times higher in subjects with MASH, compared to controls, and three times higher in MASH than in those with simple steatosis. Other miRNAs, including miR-192, miR-125 and miR-375 –a key regulator of glucose homeostasis– exhibited at least a two-fold increase in MASH patients relatively to normal subjects.[100] Similarly, in another study with a large group of Japanese individuals attending health examinations, increased serum levels of miR-122, miR-21, miR-34a and miR-451 were observed in subjects with MASLD. Among these, only miR-122 was found to correlate with the severity of liver steatosis.[29] Lastly, a combined analysis of serum miRNAs in individuals with biopsy-confirmed MASLD or MASH, revealed that, among 26 miRNAs significantly elevated in MASH, only four (miR-21-5p, miR-151a-3p, miR-192-5p, and miR-4449) demonstrated satisfactory diagnostic potential, when combined.[101]

On the other hand, conflicting results were reported from a cross-sectional study with ultrasound-proven MASLD individuals, when analysis of serum miR-20a and miR27a, but not miR-126 were found to be significantly lower in MASLD compared to healthy individuals, with further analysis showing significant inverse relationship between their levels and severe MASLD.[102]

In regards to liver fibrosis, certain exRNAs have been correlated with its stage, indicating progression toward cirrhosis. A 2021 study identified three tRNA-derived small RNAs (tsRNAs) – tRF-Val-CAC-005, tiRNA-His-GTG-001, and tRF-Ala-CGC-006 – that were significantly elevated in the plasma of MASLD patients. and whose levels increased with higher NAS and fibrosis stage. These tsRNAs were not only present in the human blood but also rose in mouse MASH models in step with worsening collagen deposition[103] (Table S2).

LncRNAs in circulation have similarly shown correlation with disease severity. For instance, NEAT1, MEG3, and MALAT1 lncRNA levels are higher in MASLD patients, with NEAT1 upregulated in both inflammatory MASLD and advanced fibrosis.[104] Plasma NEAT1 in peripheral blood has demonstrated good diagnostic yield for MASLD (AUC  $\approx$ 0.82), although its gradation across MASLD stages remains under investigation.[105] Another lncRNA, HULC, was reported to increase in patients with significant fibrosis.[106] Even certain circRNAs change with progression: the mitochondria-localized circRNA SCAR (identified as hsa\_circ\_0089763) is progressively down-regulated from simple steatosis to MASH-related cirrhosis. This decline is thought to remove a brake on metabolic inflammation, as circRNA SCAR normally helps restrain inflammasome activation in hepatocytes.[107] Taken together, a broad range of exRNA species (miRNAs, tsRNAs, lncRNAs, circRNAs) in biofluids reflect the spectrum of MASLD severity, rising or falling in concert with key pathological features like fat load, cell injury, inflammation, and fibrogenesis.

Beyond static severity, emerging evidence links exRNA dynamics to disease progression. In longitudinal studies, changes in circulating miR-122 have mirrored histological change: in one Japanese cohort, patients who achieved histologic improvement on repeat liver biopsy showed significantly reduced serum miR-122 levels compared to baseline. Conversely, persistently high or increasing miR-122 over time has been associated with ongoing liver injury and even a higher risk of progression to HCC.[108] These findings suggest that serial exRNA measurements could potentially monitor disease trajectory. Similarly, tsRNAs including tRF-Val-CAC-005, tiRNA-His-GTG-001, and tRF-Ala-CGC-006 were increased in those MASLD patients with more advanced fibrosis, hinting that they might predict fibrotic progression risk.[103] However, prospective validation is needed. Altogether, current literature supports that exRNAs correlate strongly with MASLD/MASH severity and may provide insight into disease progression risk, complementing traditional methods and markers.

### III.2 exRNA Changes with Treatment Response

Early data indicates that certain exRNAs also respond to therapeutic interventions, paralleling clinical improvement. Weight loss – the cornerstone of MASLD/MASH management – appears to normalize some elevated miRNAs. In a pilot study of diet/exercise intervention, patients who achieved resolution of MASH on follow-up biopsy had significant declines in circulating miR-122 levels.[109] This makes biological sense, as miR-122 release into blood is tied to hepatocyte injury; successful therapy that heals the liver reduces this leakage. Similarly, miR-34a and miR-21, which are elevated in active MASH, have been observed to decrease after sustained weight loss or bariatric surgery in small case series (in line with reduced inflammation), though larger studies are needed for confirmation.[110,111]

Not all exRNAs may normalize with therapy, as some of them reflect irreversible fibrosis rather than reversible injury; however, those tied to ongoing pathogenic processes (ie, ballooning and inflammation) show promise as pharmacodynamic markers. For example, experimental MASH treatments that target fibrogenic pathways have been noted to alter exRNA profiles in animal models: an antifibrotic drug reduced miR-21-5p (a pro-fibrotic miRNA) in rodent MASH models alongside improvement in fibrosis.[112] These findings hint that exRNAs could serve as liquid biopsy readouts of treatment response, decreasing as the liver heals. Such exRNA biomarkers of response would be especially valuable in clinical trials to gauge drug efficacy without repeated biopsies.[96]

### III.3 Diagnostic Accuracy of exRNAs Versus Traditional Biomarkers

A major appeal of exRNA biomarkers is their potential to improve non-invasive diagnosis of MASLD and MASH, compared to conventional tests like alanine aminotransferase (ALT), FIB-4 score or imaging. Traditional serum markers have limitations: ALT is neither sensitive nor specific as its levels can be normal in many MASLD patients even with advanced disease, and indices like FIB-4 are useful for fibrosis but not designed to detect MASH. In contrast, specific exRNA signatures show high diagnostic accuracy. Multi-miRNA panels in particular have yielded promising results in distinguishing MASH from simple steatosis. For example, it has been suggested that combining three serum miRNAs (miR-122, miR-192, and miR-21) can non-invasively identify MASH with performance comparable to specialized protein biomarkers. In that biopsy-proven MASLD cohort, the aforementioned miRNA panel achieved an area under the receiver operating characteristic curve (AUROC) of about 0.81 for differentiating MASH from steatosis, essentially matching the AUROC of cytokeratin-18 (CK-18) M30 fragments -0.81- and outperforming ALT alone (AUROC ~0.77).[98] The panel's sensitivity and specificity were high (91% and 83% at optimal cutoff) and improved further when combined with CK-18 levels. Another large study identified a four-miRNA serum signature – miR-122-5p, miR-1290, miR-27b-3p, and miR-192-5p – that discriminated MASLD patients from healthy controls with AUC ~0.89, significantly better than ALT or FIB-4 (which had AUC ~0.79 each) [113]. Notably, the miRNA panel maintained high accuracy across mild (NAS <3) and severe (NAS ≥5) MASLD, indicating robustness across disease stages.[113] These data suggests exRNA panels can detect MASLD even when standard tests are borderline. In terms of single markers, several outperform traditional labs as diagnostic tools. For example, plasma lncRNA liver X receptor-induced sequence (LeXis) was recently proposed as a MASH-specific marker. LeXis levels were found to be significantly higher in patients with MASH compared to those without. Using an optimal cutoff, LeXis could diagnose MASH with 67% sensitivity and 100% specificity, far exceeding the specificity of ALT or ultrasound which often misclassify other causes.[114] Such high specificity in that small cohort underscores the unique disease-associated signal carried by some ncRNAs, although it should be taken with a grain of salt since regression analyses in small datasets tend to overfit. Another study reported that blood levels of lncRNA MALAT1 and HULC rise with advanced fibrosis, suggesting they might complement FIB-4 in identifying high-risk MASLD patients.[106,115,116] In summary, specific exRNA signatures –alone or in combination– show equal or superior diagnostic performance to conventional blood tests for MASLD and MASH. By capturing

multiple facets of the disease, including steatosis, injury and fibrosis in one readout, exRNA-based assays could substantially improve non-invasive diagnosis and reduce reliance on liver biopsy.

#### *III.4 Mechanistic Roles of exRNA in MASLD Pathophysiology*

Beyond their diagnostic utility, exRNAs are also mechanistic mediators in MASLD and MASH, actively contributing to hepatocyte injury, lipid dysregulation and fibrosis. Many pathogenic processes in these conditions are driven or modulated by non-coding RNAs. For example, the aforementioned miR-122 plays a central role in hepatic lipid metabolism: it promotes healthy hepatocyte differentiation and represses lipogenic genes.[117,118] In MASH livers, miR-122 expression is often downregulated in the tissue even as its levels rise in the circulation due to leakage and this loss of miR-122 derepresses its targets, leading to exacerbated lipogenesis and fibrosis.[119–121] Physiologically, miR-122 appears to regulate proinflammatory, cell-proliferating, and morphology-disrupting genes of the chemokines, growth factors and metalloprotease family, including C–C motif chemokine ligand 2 (CCL2), CCL3, insulin-like growth factor receptor 1 (IGFR1), matrix metalloproteinase 8 (MMP8) and MMP9, thereby maintaining hepatic homeostasis.[120] Experimentally, deleting miR-122 in mice causes steatosis and steatohepatitis, whereas restoring miR-122 alleviates lipid accumulation.[117,118] Another key player is miR-34a, which is upregulated by fatty acids and oxidative stress; miR-34a impairs mitochondrial function and increases hepatocyte apoptosis by silencing SIRT1, thereby aggravating liver injury in MASLD.[122,123] miR-21 is a pro-fibrotic miRNA induced in MASH and targets multiple antifibrotic genes, including transforming growth factor (TGF)- $\beta$  regulators, skewing the balance toward collagen deposition.[112,124,125] Inhibiting miR-21 in MASH models attenuates stellate cell activation and fibrosis, underscoring its mechanistic role.[112,126] Similarly, miR-221/222 are linked to hepatocyte ballooning and inhibit cell cycle regulators,[127,128] and miR-223, a molecule released largely from inflammatory cells, modulates the inflammatory response in MASH.[129,130] Interestingly, many of these pathogenic miRNAs are exported in EVs and can act on distant cells. For instance, adipocyte-derived exosomal miR-122 can enter hepatocytes and contribute to MASLD by modulating Sirt1 signaling, whereas its hepatocyte-derived counterpart can function paracrinally to regulate the behavior of Kupffer cells, inducing their proinflammatory M1 phenotype.[131,132] This crosstalk suggests exRNAs are not just byproducts of liver injury but inter-organ messengers that amplify metabolic dysfunction systemically. LncRNAs and cRNAs also have direct pathogenic functions. NEAT1, a lncRNA highly expressed in hepatocytes, has been shown to promote hepatic fat accumulation by sequestering a miR-212-5p/GRIA3 regulatory axis, thereby enhancing lipogenic signaling.[133] NEAT1 is also induced by inflammatory stimuli and may facilitate the transition from simple steatosis to steatohepatitis.[105,134] Another lncRNA, MALAT1, is implicated in fibrosis: MALAT1 upregulation in hepatocytes and stellate cells can drive epithelial-mesenchymal transition and collagen gene expression – a finding consistent with the observation that MALAT1 levels are higher in fibrotic MASLD.[116,135,136] On the other hand, some lncRNAs have protective roles; for example, LeXis normally helps cholesterol efflux and its higher circulating level in MASH might reflect a compensatory response to lipid overload.[114,137] CircRNA SCAR, which resides in mitochondria, was discovered to blunt reactive oxygen species (ROS) production by binding mitochondrial ATP5B; in MASH, loss of circSCAR leads to excess mitochondrial ROS and “metaflammation,” exacerbating steatohepatitis.[107] Even the novel tsRNAs appear to have mechanistic impact: the tRF-3001b fragment, elevated in MASLD mice, directly targets the 5' UTR of *Prkaa1* (AMPK $\alpha$ ) mRNA, reducing AMPK levels and thus impairing autophagy, which in turn promotes lipid accumulation. Silencing tRF-3001b in MASLD models restored autophagy and improved liver histology.[138]

In summary, it appears that exRNAs are closely involved in MASLD and MASH pathophysiology, by regulating four major gene networks: lipid metabolism, apoptosis, inflammation, and fibrogenesis. This mechanistic insight opens the door to therapeutic modulation of exRNAs (e.g., anti-miR therapies) to treat MASLD/MASH at the molecular level. ExRNAs not only

reflect the disease state but actively shape it by mediating the cross-talk between injured hepatocytes, stellate cells, and immune cells that drives progression from steatosis to steatohepatitis and fibrosis.

#### IV. Current Status of Liquid Biopsy in MASLD and MASH Management

Liquid biopsy has emerged as a promising alternative to traditional liver biopsy, which still remains the gold standard for diagnosing liver disease and assessing liver fibrosis severity. However, liver biopsy is an invasive procedure fraught with limitations, including patient eligibility criteria and associated risks from the intervention. In fact, many patients who undergo liver biopsy as part of clinical studies do not necessarily meet the eligibility criteria, thus indicating lack of better pre-biopsy strategies that can more accurately target appropriate candidates.

Utility and applications of different miRNA molecule measurements were discussed earlier in this review.[29,97,100–104,113,139–141] In regards to serum biomarkers, many of them have been associated with the pathogenesis of MASLD and MASH, as well as liver fibrosis. However, their clinical utility is constrained by low specificity and sensitivity. Moreover, these biomarkers often fail to accurately predict disease severity or fibrosis stage and, relatively, even MASLD scores themselves, including assessment for liver fibrosis, can be affected by individual factors, such as obesity.[142] For instance, measuring circulating monocyte perilipin-2 (PLIN2) levels has shown promising results in diagnosing MASH, while RAB14 levels have been found to be useful in predicting liver fibrosis. These biomarkers have demonstrated the potential to outperform traditional markers and scores, such as FIB-4 score, MASLD fibrosis score, and the AST-to-platelet ratio, as far as sensitivity and specificity are concerned.[143] Other serum biomarkers such as leptin[21] and FGF-21[95] have also been positively associated with MASLD and MASH and have been proposed as possible disease markers.

On the other hand, a study using modified aptamer proteomics assessed over 5,000 proteins in subjects with histologically confirmed MASLD and validated protein-phenotype models, which can reliably assess various stages of the disease, including steatosis, hepatocellular ballooning, lobular inflammation and fibrosis. Findings were comparable to established biomarkers and traditional methods of MASLD assessment, further supporting the potential of liquid biopsy as a non-invasive diagnostic tool in liver disease.[144] However, further studies are warranted on these biomolecules to become globally applied as disease biomarkers, due to various phenotypic and other characteristics of the populations tested.

#### V. Comparison of Liquid Biopsy with Traditional Diagnostic Methods

MASLD spectrum diagnosis requires fat presence of more than 5% in the liver.[145] A variety of methods are available to assess the presence of liver steatosis, each one with specific limitations and advantages, as detailed in Table S3. Magnetic resonance spectroscopy (MRS) is a fairly accurate method that quantifies the proton density fat fraction (PDFF), providing a precise measurement of liver fat, although it is a relatively limited modality, requiring specialized knowledge and is not routinely available in everyday clinical practice. For this reason, MRI-PDFF has been developed as a more accessible method, avoiding patient-related confounders such as body mass index (BMI) and comorbidities. MRI-PDFF does not require spectroscopy and can be performed on routine scanners, making it a more feasible option in clinical settings. Nevertheless, MRI-PDFF is not without limitations, including claustrophobia, and the presence of metal implants, which can hinder its utility.[146,147]

When it comes to liver fibrosis, current imaging techniques lack the ability to directly detect molecular signatures of fibrosis. Instead, the proposed methods aim to indirectly assess fibrosis presence by using biomarkers related to liver parameters, including liver stiffness, metabolites and image texture. Among these, liver stiffness is considered the leading marker, correlating with the collagen deposition on the liver.[148,149]

Transient elastography (TE) is particularly effective in assessing advanced fibrosis and it serves as a valuable prognostic tool. This study as other ultrasound-based tests, even that they are widely accessible, they are limited in subjects with ascites or obesity.[148] In contrast, Magnetic Resonance Elastography (MRE) and MRI-PDFF have shown superior diagnostic accuracy for steatosis and fibrosis, outperforming TE in certain contexts,[150] and also indicating more reliably the liver stiffness.[151] Additionally, two-dimensional shear wave elastography has demonstrated potential for early fibrosis detection in chronic liver disease.[152] Acoustic radial force imaging has also been explored, but it has been outperformed by MRE in assessing liver fibrosis.[153]

Other traditional diagnostic methods often rely on serum markers such as ALT and platelet count. ALT, in particular, has demonstrated a good negative predictive value (NPV), with 11% of subjects with normal ALT having eventually MASH. On the other hand, an ALT level two times greater than the upper limit of normal showed 50% sensitivity and 61% specificity for detecting MASH.[154]

Secondly, the MASH test takes into consideration individual characteristics including age and BMI, serum aminotransferases and lipids as well as other serum markers, with a sensitivity of just 33% but a specificity of 94%.[155]

As liver fibrosis progresses toward advanced fibrosis or cirrhosis, serum ALT levels typically decline, while aspartate aminotransferase (AST) levels remain stable or increase, leading to a higher AST/ALT ratio. Among the various scoring systems, the MASLD fibrosis core, formerly known as NAFLD fibrosis score (NFS), is notable for its comprehensive approach, incorporating individual factors, including age, BMI, aminotransferase levels, glucose, platelet count and albumin. Using a high cutoff of more than 0.676, was related with a positive predictive value (PPV) of 82% for advanced F3-F4 fibrosis, with sensitivity of 43% and specificity of 96%. On the other hand, a low cutoff value, of less than -1.455, had a NPV of 88%, with a sensitivity of 77% and a specificity of 71%.[156]

Another very useful tool, being used more frequently in daily clinical practice lastly, is the FIB-4 score, which combines laboratory tests, including platelet count, AST, ALT with age. This score has shown promise in predicting outcomes in MASLD patients and has been shown to be superior to other serologic markers.[157] A FIB-4 score <1.3 has a 90% NPV for stage 3-4 fibrosis, whereas a score of >2.67 has a PPV of 75-100%. However, its specificity declines with age, with studies showing that age-adjusted lower cutoffs are needed in patients of more than 65 years of age. It should also be used with caution in ages of <35, since it performed poorly in diagnosing advanced liver fibrosis in that age group.[158]

Finally, the AST-to-platelet ratio index (APRI), which is calculated using the AST level and platelet count, has shown utility in predicting liver outcomes, including patients with MASLD. This index has primarily been studied in patients with Hepatitis C virus (HCV) infection, human immunodeficiency virus (HIV) and HCV coinfection and alcohol-related liver disease. In one retrospective series involving 320 patients, APRI was found to be effective in predicting liver-related outcomes, including death and liver transplantation.[159]

## VI. Future Perspectives for exRNA Diagnostics in MASLD and MASH

The field of miRNA-based targeted therapies continues to evolve in recent years, with significant strides being made in treatment of liver conditions, including MASLD, MASH and hepatocellular carcinoma. As discussed earlier, miRNAs have a great potential for diagnostics, as well as for pharmaceutical utilities, however this requires a deep understanding of their physiology and pathophysiology, so that carrier molecules are developed and effectively store the sensitive genetic information included in the miRNAs. This way, they will be able to “travel” in the bloodstream, until the target-organ is reached and the treatment is delivered appropriately.

Recent advances in drug delivery systems, such as lipid-based nanoparticles, liposomes, and multifunctional envelope nanodevices (MENDs), are helping to address the issue of poor cellular uptake and bioavailability, showing promising results.[94,160,161] Moreover, novel strategies propose the use of EVs as miRNA carriers, with encouraging results in preclinical studies,

demonstrating efficient delivery of therapeutic miRNAs and even tumor suppression in liver cancer cells.[162]

Additionally, mimicking is another mechanism that holds great promise for future therapeutic interventions in liver diseases, as it can prevent disease progression, by specific miRNA targeting, since it has demonstrated the ability to decrease relevant circulating miRNAs levels in the bloodstream.[163]

On the other hand, while polyethylene glycol attachment to biochemical molecules, process known as PEGylation, has been widely used to stabilize nanoparticles and prolong their circulation time, it might often impair cellular uptake and tissue delivery.[164] Recent developments, such as the pH-sensitive cationic lipid YSK05 in MENDs, have successfully improved delivery and endosomal escape, enhancing therapeutic efficacy.[161] Accordingly, polymeric vectors like jetPEI are under investigation,[165] but their safety and efficacy remain to be fully validated.

## VII. Challenges and Limitations

Despite the growing promise of EVs and exRNAs as biomarkers for liver diseases, several significant limitations currently hinder their broader clinical translation and adoption. One of the foremost challenges is the lack of standardized methodologies. Consistent and reproducible results depend heavily on well-defined protocols for sample collection, timely processing, storage, and RNA extraction. However, variation in these steps, ranging from differences in sample transport conditions to inconsistent handling techniques, can compromise the integrity of exRNAs and introduce substantial variability across studies. Contamination, improper storage, and differing equipment or reagent use further exacerbate inconsistencies. Additionally, the biological heterogeneity among patients, influenced by disease stage, tumor type, or comorbidities, contributes to variability in exRNA profiles, complicating the establishment of universal molecular signatures.

Technically, RNA quantification from EVs presents a set of challenges that are yet to be fully resolved. One of the primary difficulties is the low abundance of exRNAs in body fluids such as plasma or urine, which often necessitates the use of large sample volumes or specialized enrichment protocols. Moreover, exRNAs are inherently heterogeneous in size and sequence and are highly susceptible to degradation by RNases. The process of isolating these RNAs from complex biological fluids requires advanced methodologies, such as ultracentrifugation, size-exclusion chromatography, or immunoaffinity capture, that are not only labor-intensive but also prone to variability. Additionally, quantification techniques like NanoDrop™ and Qubit™ suffer from issues related to sensitivity and specificity, while more advanced methods such as qRT-PCR and ddPCR can be affected by technical artifacts, including primer dimer formation and genomic contamination. Secondary RNA structures can also interfere with detection accuracy, necessitating careful handling to preserve data quality.

Biological and analytical confounders further complicate the interpretation of liquid biopsy data. Endogenous molecules such as anti-mRNAs and artificial miRNA mimics can alter the activity of specific miRNAs, thus skewing assay results. Detection sensitivity and specificity also vary across platforms, such as PCR, microarrays, and sequencing, leading to discrepancies in exRNA profiles depending on the analytical approach used. Methods like size-exclusion chromatography can introduce selection bias by isolating EVs within a specific size range, potentially excluding biologically relevant subpopulations. Furthermore, experimental conditions, such as the use of serum-containing media in EV-producing cell cultures, can introduce non-EV contaminants, making it difficult to interpret results accurately. The use of different filter membrane types, protein quantification kits, and particle measurement techniques can also affect reproducibility and recovery efficiency across laboratories.

A final and critical limitation lies in the underreporting of methodological details in the current literature. Many studies fail to comprehensively document the specific reagents, protocols, or analytical parameters used, impeding reproducibility and comparative evaluation. This lack of transparency, combined with high inter-sample and inter-study variability in exRNA profiles, even

among samples from the same disease type, undermines the reliability of biomarker discovery efforts. Without large-scale validation in diverse patient populations and rigorous standardization of analytical workflows, the clinical utility of EV-derived exRNAs as liver disease biomarkers will remain limited.

VIII. Conclusions

The emerging role of exRNAs in liquid biopsy represents a promising advancement in the diagnosis, monitoring, and management of MASLD and its more advanced form, MASH. The detection of exRNAs, particularly miRNAs and circulating EVs offers a noninvasive and highly sensitive approach to assessing disease progression, identify fibrosis stages and monitor treatment responses. Key biomarkers, such as miR-122, miR-34a and miR-21 show significant potential in reflecting the molecular and histological changes associated with MASLD and MASH. Compared to traditional diagnostic methods, these biomarkers enable earlier detection and more targeted management, improving patient stratification and facilitating personalized treatment strategies.

However, several challenges remain in fully integrating exRNA-based liquid biopsy into clinical practice. Standardization of detection methods, variability in exRNA profiles across populations, and the need for extensive validation in diverse clinical settings continue to prevent widespread adoption. Additionally, clinical translation of exRNA findings into routine medical practice faces obstacles related to cost, accessibility, and integration with existing diagnostic tools. Therefore, further research is essential to refine liquid biopsy techniques, optimize exRNA biomarker panels and validate their clinical utility through large-scale, multicenter studies and clinical trials.

Future research should prioritize improving the sensitivity and specificity of liquid biopsy markers while working toward establishing exRNA-based diagnostics as a reliable, noninvasive screening tool for early detection and precise monitoring of MASLD and MASH. Advancing these technologies will contribute to personalized medicine, improving patient outcomes by tailoring treatment based on individual molecular profiles. It is anticipated that in the near future, MASLD diagnosis and treatment will be increasingly guided by each patient’s unique molecular signature. Continued evaluation of liquid biopsy technologies, combined with a deeper understanding of the molecular mechanisms underlying MASLD, holds great promise for revolutionizing liver disease management.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

**Conflict of Interest:** Nothing to declare.

Abbreviations

|          |                                                        |
|----------|--------------------------------------------------------|
| ExRNAs   | Extracellular RNAs                                     |
| MASLD    | Metabolic Associated Steatotic Liver Disease           |
| MASH     | Metabolic Associated Steatohepatitis                   |
| miRNA    | MicroRNA                                               |
| circRNA  | circular RNA                                           |
| EVs      | Extracellular Vesicles                                 |
| tRNA     | Transfer RNA                                           |
| tsRNA    | tRNA-derived Small RNA                                 |
| cfDNA    | Circulating Free DNA                                   |
| RNA-seq  | RNA Sequencing                                         |
| ALT      | Alanine Aminotransferase                               |
| AST      | Aspartate Aminotransferase                             |
| MRE      | Magnetic Resonance Elastography                        |
| MRI-PDFF | Magnetic Resonance Imaging Proton Density Fat Fraction |
| TE       | Transient Elastography                                 |

|         |                                                              |
|---------|--------------------------------------------------------------|
| NFS     | NAFLD (Non-Alcoholic Fatty Liver Disease) Fibrosis Score     |
| APR     | Aspartate Aminotransferase to Platelet Ratio                 |
| qRT-PCR | Quantitative Reverse Transcription Polymerase Chain Reaction |
| ddPCR   | Droplet Digital PCR                                          |
| MRS     | Magnetic Resonance Spectroscopy                              |
| UV-vis  | Ultraviolet-Visible Spectrophotometry                        |
| NPV     | Negative Predictive Value                                    |
| PPV     | Positive Predictive Value                                    |

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