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Review article:

CIRCULATING CELL-FREE DNA METHYLATION SIGNATURES IN METABOLIC DYSFUNCTION-ASSOCIATED STEATOTIC LIVER DISEASE: EMERGING BIOMARKERS FOR DISEASE DIAGNOSIS, PROGRESSION, AND NON-INVASIVE MONITORING

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ABSTRACT

Metabolic dysfunction-associated steatotic liver disease (MASLD) is an umbrella term for a chronic liver condition characterized by hepatic fat accumulation with at least one cardiometabolic risk factor and no other known predominant cause of hepatic steatosis. Driven by the steadily increasing prevalence of obesity and insulin resistance, the global burden of MASLD continues to rise. Often undiagnosed and therefore left untreated, this condition can progress to metabolic dysfunction–associated steatohepatitis (MASH), cirrhosis, and ultimately to liver failure, decompensated cirrhosis, or hepatocellular carcinoma. For this reason, early diagnosis of active disease is essential for timely therapeutic intervention. Despite several shortcomings, liver biopsy remains the reference method for the histological assessment of steatohepatitis and fibrosis, and for monitoring therapeutic response. This review discusses the rationale for non-invasive diagnostic approaches for detecting and monitoring early-stage MASLD. Circulating cell-free DNA (cfDNA) methylation profiling is emerging as a near-real-time analytical method that reveals dynamic tissue-specific epigenetic alterations during MASLD progression. Considering the complexity of MASLD pathogenesis, this review also proposes integrating multi-omics modalities with cfDNA methylation to improve diagnostic accuracy.

Keywords: Metabolic dysfunction-associated steatotic liver disease (MASLD), cell-free DNA (cfDNA), biomarkers, diagnosis, liquid biopsy

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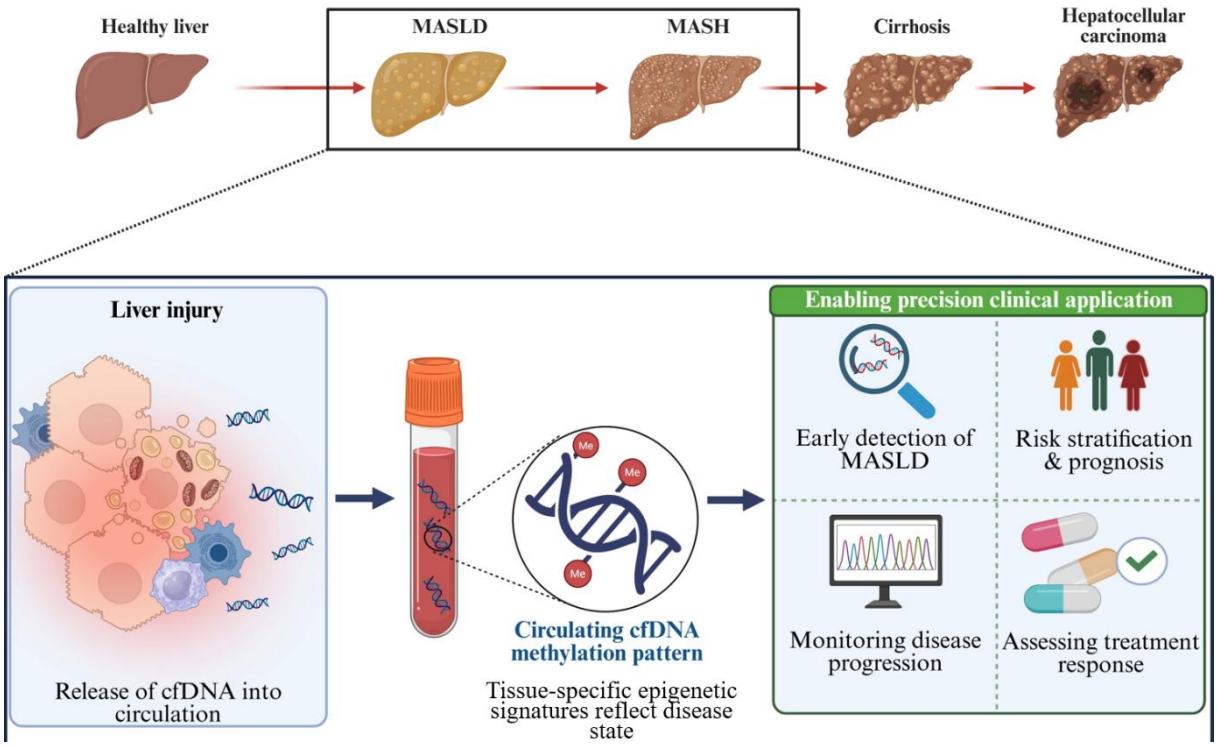

Figure 1: Graphical abstract

1. INTRODUCTION

Globally, metabolic dysfunction-associated steatotic liver disease (MASLD), formerly known as non-alcoholic fatty liver disease (NAFLD), currently affects 38 % of the adult population and is projected to exceed 55 % by 2040 (Younossi et al., 2024). It is characterized by excessive hepatic fat accumulation, which, if left undiagnosed or untreated, can progressively advance to a spectrum of clinical conditions ranging from MASH (metabolic dysfunction-associated steatohepatitis) to life-threatening cirrhosis, organ failure, and hepatocellular carcinoma (Chan et al., 2023). With advances in pharmacotherapy for MASLD/MASH, it is imperative to diagnose individuals at an early stage and prevent disease progression while the disease may still be reversible. Liver biopsy remains the histological reference standard for assessing steatohepatitis and fibrosis, but is not required for the diagnosis of MASLD in most clinical settings. Moreover, it is invasive, prone to sampling error, may cause pain at the puncture site, carries a risk of bleeding, is subject to interobserver variability, is expensive, and is not suitable for all patients (Hsu and Alazawi, 2025).

Several non-invasive diagnostic approaches based on serum analysis or imaging techniques have been developed to stratify patients according to fibrosis severity and identify those with an advanced-stage disease (Wang et al., 2025). However, evidence remains limited for distinguishing early-stage MASLD. To date, studies have primarily focused on cellular or molecular biomarkers that deviate from reference values as the disease progresses (Wang et al., 2025). Routine clinical practice includes measuring serum aminotransferase levels; however, the clinical utility of these biomarkers is limited by their low specificity for MASLD/MASH. Standard serum markers, such as the Fibrosis-4-Index (FIB-4) and the Enhanced Liver Fibrosis (ELF) test, have limited sensitivity for early-stage disease (Wang et al., 2025).

Imaging techniques are widely employed alongside serum biochemistry to assess liver morphology and pathology. However, several confounding factors can interfere with diagnostic

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imaging accuracy, including body mass index (BMI), the composition of a recent meal, and other causes of acute inflammation (Cathcart et al., 2025). These issues highlight the urgent need to explore novel, minimally invasive diagnostic tools capable of detecting the disease in real time to enable continuous monitoring throughout its progression.

Liquid biopsy has introduced a significant paradigm shift. This approach analyzes circulating cell-free nucleic acids, which can be used for monitoring the entire spectrum of the disease, from simple steatosis to severe fibrosis. Studies have investigated different cfDNA features in liver disease, including fragmentation patterns in cirrhosis and chromosomal instability in hepatocellular carcinoma (Figure 2) (Annapragada et al., 2026; Shu et al., 2025). The presence of circulating cfDNA in plasma was first reported in the 1940s. However, it took more than two decades to uncover its link to pathological states. Early studies demonstrated a remarkable positive correlation between cfDNA and cancer diagnosis, prognosis, and therapeutic response (Du et al., 2024; Tran et al., 2021). However, recent studies have increasingly recognized its role in determining the severity of MASLD. For instance, cfDNA fragments of approximately 90 bp and 222 bp, in particular, were reported to be significantly higher in patients with MASLD showing signs of hepatic inflammation (Karlas et al., 2017). Importantly, this study correlated circulating cfDNA fragments with established non-invasive markers of NAFLD/MASLD severity, which may increase in response to cellular injury, apoptosis, necrosis, or inflammation. In contrast, cfDNA methylation patterns reflect epigenetic information retained in circulating DNA and can facilitate the inference of cell-type origin and the identification of severity-associated methylation changes. Hardy et al. showed that differential methylation of the PPARγ promoter can be detected in plasma DNA and has potential for stratification by fibrosis severity (Hardy et al., 2017). Together, these different molecular features of cfDNA, including abundance, tissue-specific methylation, and disease-associated methylation patterns, may provide complementary information regarding MASLD progression.

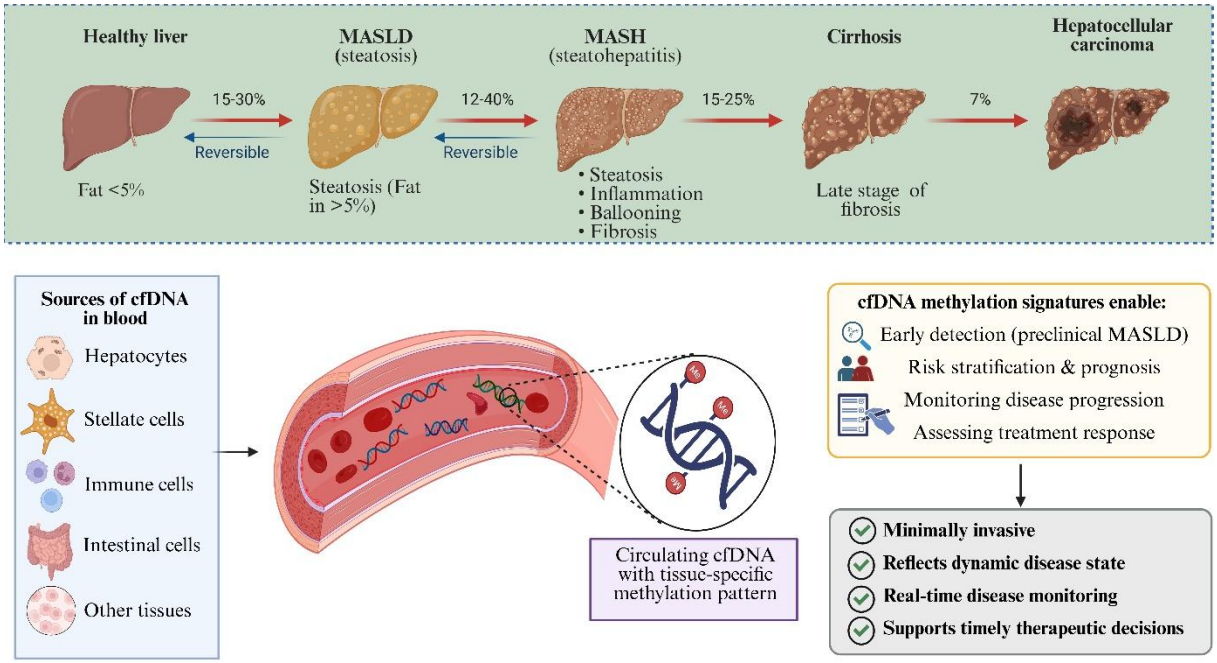

Figure 2: MASLD disease continuum and the role of cfDNA methylation. MASLD progresses from simple steatosis to MASH, fibrosis, and cirrhosis, with an increasing risk of hepatocellular carcinoma. During disease progression, hepatocytes and other liver-associated cell types release cell-free DNA (cfDNA) fragments into the circulation through apoptosis, necrosis, and active secretion. These cfDNA fragments retain tissue-specific DNA methylation signatures that reflect ongoing pathological processes in the liver. Analysis of cfDNA methylation profiles through liquid biopsy enables non-invasive early detection of

disease, risk stratification, disease monitoring, and assessment of therapeutic response. The relatively short circulating half-life of cfDNA makes serial sampling plausible, but its value for monitoring MASLD activity and treatment response requires prospective validation. Created using *BioRender.com*.

The present review primarily summarizes recently reported studies on cfDNA, particularly in the field of MASLD/MASH, emphasizing the principal technologies for cfDNA methylation profiling and their clinical utility. The review also highlights the transition from conventional invasive liver biopsy to liquid biopsy-based diagnostic approaches in MASLD/MASH.

2. BIOLOGICAL BASIS OF CFDNA METHYLATION IN MASLD

cfDNA refers to extracellular DNA fragments circulating in body fluids, often enriched at a mononucleosomal length of approximately 160-180 bp. It consists largely of double-stranded DNA fragments associated with nucleosomes, released into the circulation during apoptosis, necrosis, or active secretion by cells (Sanchez et al., 2021). The concentration of circulating cfDNA tends to be higher in pathological states than in healthy states, in which it is often present at low levels and difficult to quantify (Sorbini et al., 2024).

In MASLD, metabolic disturbances, including lipotoxicity, glucotoxicity, oxidative stress, and mitochondrial dysfunction, promote hepatocyte apoptosis, triggering the release of hepatocyte-derived cfDNA fragments (Kuchay et al., 2025). Generally, cfDNA preserves the molecular signatures of its tissue of origin, including methylation patterns (Oberhofer et al., 2022). DNA methylation, a fundamental epigenetic modification, involves the methylation of cytosine residues within CpG islands and other genomic regions that regulate transcription. The progression of MASLD involves several cascading events driven by a network of inflammatory mediators. Interestingly, inflammation may alter cfDNA methylation patterns in a locus- and cell-type-specific manner, potentially reflecting changes in the cells releasing cfDNA as well as epigenetic changes within these cells that can be triggered by activation of DNA methyltransferases (DNMTs) and TET enzymes (Zhang and Li, 2023). Hepatocyte-derived cfDNA fragments preserve these tissue-specific methylation marks and correlate well with established MASLD biomarkers (Lehmann-Werman et al., 2018). Unlike conventional serum liver enzyme assays, cfDNA methylation profiles can provide tissue-of-origin information. Therefore, the cfDNA methylation pattern can offer valuable epigenetic information about the pathological transition from MASLD to MASH.

3. TECHNOLOGIES FOR CFDNA METHYLATION PROFILING

The detection and characterization of circulating nucleic acids in biological fluids, referred to as liquid biopsy, are increasingly being recognized as minimally invasive tools in biomedical research and clinical diagnostics. Here, we summarize the principal technologies for cfDNA methylation profiling and emphasize their applicability to liquid biopsy-based diagnostics.

3.1 Bisulfite-based approaches

In principle, sodium bisulfite treatment selectively converts unmethylated cytosines in denatured DNA to uracils, while methylated cytosines remain unchanged, thereby enabling methylation detection. However, this conversion results in a considerable loss of DNA, posing challenges when the DNA input is limited (Fisher et al., 2022). For low-input plasma DNA, methods optimized for fragmented DNA, such as targeted bisulfite sequencing or adapted reduced-representation bisulfite sequencing (RRBS) workflows, may improve recovery, although bisulfite-associated DNA loss remains a challenge (Zhang et al., 2027). Additionally, when coupled with next-generation sequencing (NGS), RRBS provides a more cost-effective option (Meissner et al., 2005). A single-tube workflow for RRBS eliminates intermediate purification steps

before bisulfite conversion, thereby maximizing DNA recovery from low-input samples. Leveraging this optimized workflow, RRBS has enabled the successful methylation profiling of low-input cfDNA. Because methylated CpG islands (CGIs) are preferentially amplified owing to their higher methylated CpG density compared with unmethylated CGIs and non-CGI regions, methylated CpG Tandem Amplification and Sequencing (MCTA-seq) has enabled the identification of multiple highly sensitive hypermethylated DNA markers for early detection of hepatocellular carcinoma (Wen et al., 2015). Targeted PCR- or hybrid-capture assays concentrate sequencing on selected regions and may offer greater depth or lower cost than broad profiling, depending on panel design. However, bisulfite-converted DNA can complicate primer and probe design (Neefs et al., 2025).

3.2 Targeted methylation assays

In addition to sequencing-based approaches, array- and PCR-based methods are valuable, robust tools for cfDNA methylation profiling. The Infinium HumanMethylation450 BeadChip (HM450K) is a microarray platform with growing data repositories in The Cancer Genome Atlas (TCGA) and the Gene Expression Omnibus (GEO), facilitating the development of new methylation-based diagnostic assays (Bibikova et al., 2011). A major limitation of array-based methods is their limited genome-wide coverage, as they are confined to predefined CpG sites, which may result in the loss of crucial information. One study suggested that conventional methylation-specific PCR (MSP) can detect methylated DNA sequences at proportions as low as 0.1 %, making it an effective approach for liquid biopsy-based clinical diagnosis (Kristensen et al., 2008). However, this analytical sensitivity of MSP-based assays is influenced by primer design, target abundance, background unmethylated DNA, amplification conditions, and characteristics of the biological specimen. Methylation-specific PCR and its advanced quantitative derivatives, including quantitative MSP, which provides real-time quantification of methylated DNA; methylation-sensitive high-resolution melting, which enables semi-quantitative assessment of methylation heterogeneity; and droplet digital MSP, which provides absolute quantification of methylated cfDNA without the need for standard curves, have contributed significantly to the identification and validation of biomarkers for early diagnosis and monitoring of disease (Dugast-Darzacq and Grange, 2009; Wojdacz and Dobrovic, 2007).

3.3 Enrichment-assisted methylation profiling

These techniques use either antibodies against 5-methylcytosine, as in methylated DNA immunoprecipitation sequencing, or methyl-CpG binding proteins, as in methyl-CpG binding domain protein capture sequencing, prior to genomic sequencing, selectively enriching methylated regions. These techniques are particularly valuable for cfDNA profiling because they avoid bisulfite-induced DNA degradation and require only low DNA input (Chan et al., 2017). However, they are biased toward highly methylated regions and usually require optimization for low-input cfDNA samples (1-10 ng cfDNA). This limitation has been addressed by the development of cell-free methylated DNA immunoprecipitation sequencing (cfMeDIP-seq), which uses exogenous methylated and unmethylated lambda DNA controls/spike-ins to maintain a consistent antibody-DNA ratio (Nair et al., 2011; Shen et al., 2019). Nevertheless, these profiling methods require thorough calibration to prevent inefficient capture due to low cfDNA input.

3.4 Bioinformatics and machine-learning approaches

The computational pipeline for analyzing cfDNA methylation sequencing data begins with aligning bisulfite-based data using bisulfite-specific aligners, such as Bismark (Krueger and Andrews, 2011) and BS Seeker 2 (Chen et al., 2010), and aligning non-bisulfite or enrichment-

based data using standard aligners such as Bowtie and the Burrows-Wheeler Aligner (BWA). This is followed by data visualization and rigorous post-alignment quality control to assess alignment efficiency and bisulfite conversion efficiency, evaluate fragment size distribution, filter known single-nucleotide polymorphisms, and remove PCR duplicate reads from methylated genomic regions (Daca-Roszak et al., 2015). For base-resolution assays, methylation can be estimated from methylated and total reads at covered CpG sites. Enrichment-based assays instead measure signal across captured genomic regions and require assay-appropriate normalization and quality control. Normalization methods such as reads per kilobase per million mapped reads (RPKM) (Mortazavi et al., 2008) and DESeq2 normalization (Anders and Huber, 2010) are routinely used to account for differences in sequencing depth and library composition. Differentially methylated CpG sites or regions should be identified using methods appropriate to the assay and data structure, accounting for sequencing coverage or enrichment signal, variation between samples, relevant clinical and technical covariates, and multiple testing. Following their identification, differentially methylated regions are annotated with genomic datasets, facilitating novel biomarker discovery. All these currently available techniques for cfDNA methylation profiling may be adapted for liquid biopsy-based assessment of MASLD/MASH. Moreover, combining different techniques may yield complementary data.

4. CFDNA METHYLATION SIGNATURES FOR DISEASE DETECTION, MONITORING, AND THERAPEUTIC RESPONSE

MASLD is often a silent liver condition that may remain asymptomatic until it advances to serious tissue damage. However, detecting it early allows patients to prevent its progression through lifestyle changes. Unlike conventional diagnostic methods, cfDNA-based liquid biopsy profiling provides a non-invasive means of examining molecular signatures derived from injured hepatocytes and released into the circulation (Zhang and Li, 2023).

The length distribution and genome-wide coverage of cfDNA vary with disease severity. Characteristic changes in fragmentation patterns, particularly shifts in the abundance of short fragments, reflect alterations in tissue chromatin organization and, consequently, transcriptional activity (Annapragada et al., 2026). A recent study performed whole-genome methylation sequencing of cfDNA to explore the relationship between MASLD stages and tissue methylation profiles (Ahn et al., 2025). The findings revealed distinct methylation changes in cfDNA, depending on sample source, sex, and genomic location, that mirror hepatic epigenetic alterations associated with fibrosis risk and severity. Hypomethylation of TM4SF5, a gene of interest in MASLD, has been detected in cfDNA from patients with evolving fibrosis (Ahn et al., 2025). However, these findings should be considered exploratory rather than evidence of a clinically validated biomarker. The limited sample size, cohort design, lack of standardized clinical phenotyping, and absence of external validation constrain the ability to establish robust associations across fibrosis stages and clinical groups. Another study reported differential methylation of PPARγ in circulating cfDNA that may assist in stratifying patients with MASLD by disease severity (Hardy et al., 2017). Furthermore, these plasma cfDNA methylation marks distinctly reflect the histological changes in fibrotic liver tissue (Hardy et al., 2017). A pilot study has also investigated cfDNA methylation and histone signatures in pediatric NAFLD; the clinical significance of these findings requires further validation (Buzova et al., 2022).

Circulating mitochondrial DNA copy number (mtDNAcn) has recently emerged as a dynamic indicator of tissue oxidative stress, displaying a biphasic pattern with an initial increase followed by a decline in advanced fibrotic stages (Lim et al., 2026). Likewise, nuclear copy number variations (CNVs), such as XPO4 duplication and CES1 and ACOT1 deletions, have been associated with fibrogenesis and dysregulated lipid metabolism (Lim et al., 2026). In a recent study, researchers used machine learning to examine the genome-wide cfDNA

fragmentation patterns in 1,576 patients with liver disease (Annapragada et al., 2026). The researchers demonstrated that these fragmentomic signatures, in addition to supporting liver disease diagnosis, can also capture molecular changes linked to chronic disease burden, expanding the clinical utility of cfDNA-based diagnostics.

Beyond its diagnostic utility, cfDNA methylation profiling is now being explored for monitoring therapeutic response in MASLD, adding a new dimension that, when combined with other existing multi-omics techniques, may improve clinical outcomes and disease management (Figure 3). Because cfDNA has a short half-life (15–140 min) in the circulation, it provides a near-real-time snapshot of liver status rather than a record of past damage. Serial assessment of cfDNA methylation levels, fragmentation size distribution, cfDNA abundance, and fibrosis-associated epigenetic markers may provide early indications of therapeutic response. A recent study demonstrated the potential of a multianalyte cfDNA blood test, showing it to be more effective than an established imaging protocol for detecting liver disease, as has already been demonstrated for hepatocellular carcinoma (Taggart et al., 2026). Similarly, integration of cfDNA profiling with transcriptomic and proteomic markers may further facilitate monitoring responses to pharmacotherapy in patients with MASLD/MASH.

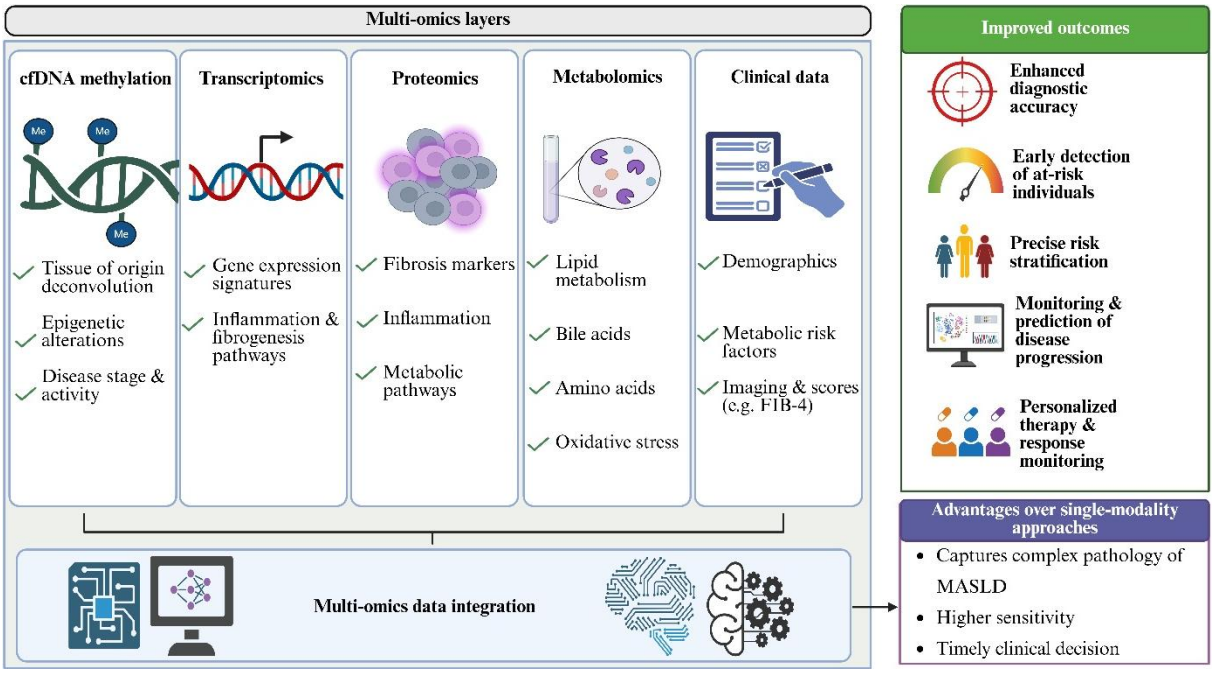

Figure 3: Integrated multi-omics approach with cfDNA methylation for MASLD. cfDNA methylation represents an epigenetic information layer that complements transcriptomic, proteomic, metabolomic, and clinical datasets. Integration of these multi-omics modalities using bioinformatics and machine-learning approaches improves the identification of disease-specific molecular signatures associated with MASLD initiation and progression. Combined analysis enhances diagnostic sensitivity and specificity, facilitates early detection of at-risk individuals, enables accurate fibrosis risk stratification, supports monitoring of disease progression and therapeutic response, and provides a framework for personalized medicine. This integrated systems-biology strategy may overcome limitations of single-modality biomarkers and improve clinical decision-making in MASLD. Created using *BioRender.com*.

Taken together, cfDNA methylation signatures may improve early-stage detection and therapeutic monitoring, leading to better treatment outcomes. Future research should seek to develop more cost-effective, validated, and highly reproducible assays for incorporation into clinical practice.

5. CONCLUSION

This review offers a comprehensive assessment of the clinical utility of circulating cfDNA methylation signatures for diagnosis, risk stratification, and therapeutic monitoring of MASLD. This tool can be applied in conjunction with the current diagnostic approaches, which are usually a combination of serum biochemistry (to examine liver health and function), imaging techniques (to assess steatosis), and fibrosis assessment indices (to estimate the extent of liver scarring). At present, successful incorporation of cfDNA analysis into routine clinical practice requires overcoming several biological, technical, and clinical challenges. Before clinical implementation, cfDNA methylation assays require standardized blood collection, processing, storage and analysis, assessment of cfDNA contributions from non-hepatic cells, and independent prospective validation against clearly defined disease-stage outcomes. Nevertheless, this approach may encourage further investigation into cfDNA-associated epigenetic signatures and support protocol standardization to aid clinical application. Large, multicenter prospective cohorts with standardized diagnostic protocols that evaluate dynamic changes in the cfDNA methylation profile will be critical for identifying highly tissue-specific methylation signatures capable of distinguishing simple steatosis, MASH, and progressive stages of fibrosis with high accuracy. Future studies may also consider integrating multi-omics data with cfDNA methylation profiles to further enhance the specificity of this technique.

Conflict of interest

The authors declare that they have no conflicts of interest.

Data availability

Not applicable.

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Author contribution

Nisha Sharma: Data analysis, writing the draft, original draft preparation; Sumeet Kumar Singh: Writing the draft; Umashanker Navik: Writing – reviewing and editing, supervision; Ralf Weiskirchen: Writing – reviewing and editing, supervision.

Artificial Intelligence (AI) – assisted technology

The authors used Grammarly software to improve the readability and language of the manuscript. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article. The figures for this review were created using *BioRender.com*.

Ethics approval and consent to participate

Not applicable.

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