Review

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Nucleic acid biomarkers to assess graft injury after liver transplantation

Elissa Bardhi,[1] Jennifer McDaniels,[1] Thomas Rousselle,[1] Daniel G. Maluf,[1,2] Valeria R. Mas[1,*]

Summary

Many risk factors and complications impact the success of liver transplantation, such as ischaemia-reperfusion injury, acute rejection, and primary graft dysfunction. Molecular biomarkers have the potential to accurately diagnose, predict, and monitor injury progression or organ failure. There is a critical opportunity for reliable and non-invasive biomarkers to reduce the organ shortage by enabling i) the assessment of donor organ quality, ii) the monitoring of short- and long-term graft function, and iii) the prediction of acute and chronic disease development. To date, no established molecular biomarkers have been used to guide clinical decision-making in transplantation. In this review, we outline the recent advances in cell-free nucleic acid biomarkers for monitoring graft injury in liver transplant recipients. Prior work in this area can be divided into two categories: biomarker discovery and validation studies. Circulating nucleic acids (CNAs) can be found in the extracellular environment pertaining to different biological fluids such as bile, blood, urine, and perfusate. CNAs that are packaged into extracellular vesicles may facilitate intercellular and inter-organ communication. Thus, decoding their biological function, cellular origins and molecular composition is imperative for diagnosing causes of graft injury, guiding immunosuppression and improving overall patient survival. Herein, we discuss the most promising molecular biomarkers, their state of development, and the critical aspects of study design in biomarker research for early detection of post-transplant liver injury. Future advances in biomarker studies are expected to personalise post-transplant therapy, leading to improved patient care and outcomes.

Keywords: liver transplantation; biomarkers; molecular diagnostics; transcriptomics; microRNAs; extracellular vesicles; donor-derived DNA; personalized therapy

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Introduction

The field of liver transplantation (LT) has experienced great advances over the last few decades, largely due to the development of novel immunosuppressive drugs, technical refinements in surgery, and effective postoperative management of transplant recipients. Overall, adult and paediatric liver transplant rates continue to increase at a significant rate, reaching nearly 9,000 liver transplants performed in the US alone each year. This represents a 40.8% increase over the past decade as reported by the 2019 Annual OPTN/SRTR Data.[1] Such progress is accompanied by increased rigor in translational and clinical research efforts, resulting in significant improvements in patient outcomes. For instance, with the recent advent of new antiviral treatments, recurrence of hepatitis C after transplantation is no longer the leading cause of graft loss.[1,2] Nonetheless, long-term allograft failure continues to occur, owing to both immunologic and non-immunologic factors, with immunosuppressive toxicity contributing to patient morbidity and mortality.

Finding the right balance between under- and over-immunosuppression remains a holy grail in transplantation. Currently, core biopsies remain the "gold standard" for the diagnosis of post-transplant abnormalities despite increased concerns regarding interobserver variability between histological evaluations.[3,4] Considering the large physical size and high complexity of the liver, core biopsies only capture a small fraction of a single lobe, making up less than 0.002% of the total mass and often leading to inaccurate diagnoses.[5,6] In the absence of reliable tools to measure immunosuppressive burden, clinicians also depend on non-invasive measurements of traditional, non-specific laboratory biomarkers (liver function tests [LFTs]) to guide immunosuppression dosage.[5,7] Thus, there remains a significant need for specific, non-invasive biomarkers that can accurately determine the degree and cause of liver injury post-LT. Recently, cell-free molecular biomarkers have emerged as potential early and sensitive markers of tissue injury and function.

Multi-omics approach to identifying biomarker signatures

The prognostic and diagnostic applications of molecular biomarkers have been widely studied throughout the transplant community. High-

[1]*Surgical Sciences Division, Department of Surgery, School of Medicine, University of Maryland, Baltimore, MD 21201, USA;* [2]*Program of Transplantation, School of Medicine, University of Maryland, 29 S Greene St, Baltimore, MD 21201, USA*

* Corresponding author. Address: Surgical Sciences Division, University of Maryland Baltimore School of Medicine, HSF3 Building, 670 W Baltimore Street, Room 7179, Baltimore, MD 21201, USA.
E-mail address: vmas@som.umaryland.edu (V.R. Mas).

throughput technologies allow for the generation of large amounts of data and information at different molecular levels.[8] Recent studies using multi-omics techniques have identified unique molecular signatures that confer significantly more information than the microscopy and staining measures currently used for medical diagnostics.[9–15] Such advanced technology paired with extensive patient databases has allowed for the discovery of a diverse set of biomarkers, shifting the focus away from a single marker to a combination of various biomarkers. Because of the complexity of the aetiology of post-transplant liver injury, prognostic and diagnostic tests will likely need to integrate multiple layers of biological information (*e.g.*, transcriptomics, epigenetics, proteomics) in order to be both sensitive and specific (especially in the presence of coexisting conditions). By definition, biomarkers are measurable and reproducible objective indicators of medical state, observed externally from the patient, which can give information on normal biological mechanisms, pathogenic processes, or responses to an exposure or intervention.[16]

Common molecular biomarkers include nucleic acids (*e.g.*, microRNAs [miRNAs] and cell-free DNA), epigenetic patterns (*e.g.*, DNA methylation and histone modifications), and the more traditional protein-based markers (*e.g.*, glycoproteins, antigens, and antibodies) that correspond to disease progression and severity. Less common markers may include proteomic and metabolomic biomarkers, which are explored using multi-analyte technologies that assess the complete set of proteins or metabolites of a biological sample.[9] However, findings from proteomic and metabolomic biomarker discovery studies have not been readily transferred to clinical trials due to their high complexity, relative novelty, and the intricate analytical techniques required to interpret such data.[17,18] Therefore, this review will focus on the biomarkers that have been described using an important number of clinical samples and that are thus more likely to be transferred to the clinical setting promptly. Principal applications of these biomarkers in transplantation include: i) predicting the progression or development of disease, ii) diagnosing specific post-transplant conditions, iii) assessing the severity or extent of graft injury, and iv) monitoring the response to therapeutic interventions such as immunosuppressants and steroids.[19] Overall, this article describes the recent scientific advances in the discovery and implementation of circulating molecular biomarkers in LT.

Key points

- Molecular evaluation of biofluids (*i.e.*, blood, urine, perfusate, and bile) are providing new opportunities to understand how specific perturbations in nucleic acids can signal the early stages of disease.
- Circulating nucleic acid (CNA) biomarkers (*i.e.*, cell-free RNA/DNA) confer significantly more information than current prognostic and diagnostic methods, emerging as early and sensitive measures of liver injury and function.
- Extracellular vesicles (EVs) carry a heterogeneous composition of RNAs, DNAs, lipids, and proteins, and have been found to protect molecular biomarkers from degradation in the extracellular environment.
- Advances in EV analytic approaches have enabled the investigation of donor-specific EVs in recipient biofluids, demonstrating the capacity for these structures to facilitate intercellular and interorgan communication following liver transplantation.
- Circulating miRNAs provide information on causal transcriptional processes occurring at a molecular level, leading to the identification of dysfunctional canonical pathways and novel therapeutic targets.
- Donor-derived cell-free DNA (dd-cfDNA) is released from necrotic or apoptotic cells in the transplanted organ and may consequently be useful as an early predictor of post-transplant injury or rejection.
- Large multicentre clinical trials have generated vast amounts of data and information at various molecular levels, demonstrating a promising opportunity for cell-free biomarkers to be introduced into clinical care.

Extracellular nucleic acid markers

Since the discovery of cell-free nucleic acids by Mandel in 1948, researchers have demonstrated that nucleic acids in body fluids have the potential to detect, track, and discriminate between patients with and without disease.[20] Circulating biomarkers offer a viable non-invasive approach to early disease diagnosis. While most nucleic acids are located within cells, a small quantity termed “circulating nucleic acids” (CNAs) can be found in the extracellular environment. In the more studied cancer field, researchers have found traces of tumoural-shed nucleic acids in the blood, urine, and stool.[21,22] The information stored within CNAs has various benefits related to cancer detection and treatment. These circulating biomarkers offer additional information regarding methylation or transcriptional and translational status via non-coding RNA, which may be suitable for the early detection of cancer.[23]

Despite the robust presence of CNA in biological fluids, the origin and function of these biomarkers in the extracellular environment remain poorly understood. Given the high levels of nucleases within circulating biofluids, it has been demonstrated that nucleic acids packaged into membranous structures are protected from immediate degradation upon release.[24] These structures often contain a heterogenous composition of RNAs, DNAs, lipids, and proteins, which vary depending on cellular origin and physiological state.[25] In the last decade, a novel method of cell-to-cell communication mediated by extracellular vesicles (EVs) has emerged. These 0.05–1 μm membrane-bound vesicles are involved in normal physiological processes, as well as being implicated in pathological conditions.[26] They are released by cells into the bloodstream and other bodily fluids, shuttling bioactive molecules from their cell of origin to recipient cells via phagocytic or non-phagocytic pathways and delivering their contents directly into the cytoplasm of target cells.[27] This emerging field of interest has spurred a plethora of EV studies that employ high-throughput technologies including proteomics, transcriptomics, and lipidomics.[28]

Recent studies have demonstrated that EVs may facilitate intercellular and interorgan communication.[29–32] It has also been suggested that through the internalisation of EVs, bioactive contents such as miRNAs can alter gene expression and mediate a physiological response in target cells. Thus, decoding the composition, biogenesis, and biological functions of EV cargo can further elucidate cellular crosstalk within transplant organs. Such information can be combined with physiological data to elucidate the cellular pathways responsible for differential liver graft outcomes. Fig. 1 depicts EVs and their pertinent molecular contents, which can function as liver-specific biomarkers.

The pursuit of non-invasive biomarkers of allograft rejection has led to the evaluation of EVs in a range of biofluids. Specifically, using bulk analyses to evaluate EV cargo, researchers have been able to identify markers of varying specificity, sensitivity, and usefulness. Recently, EVs bearing donor-human lymphocyte

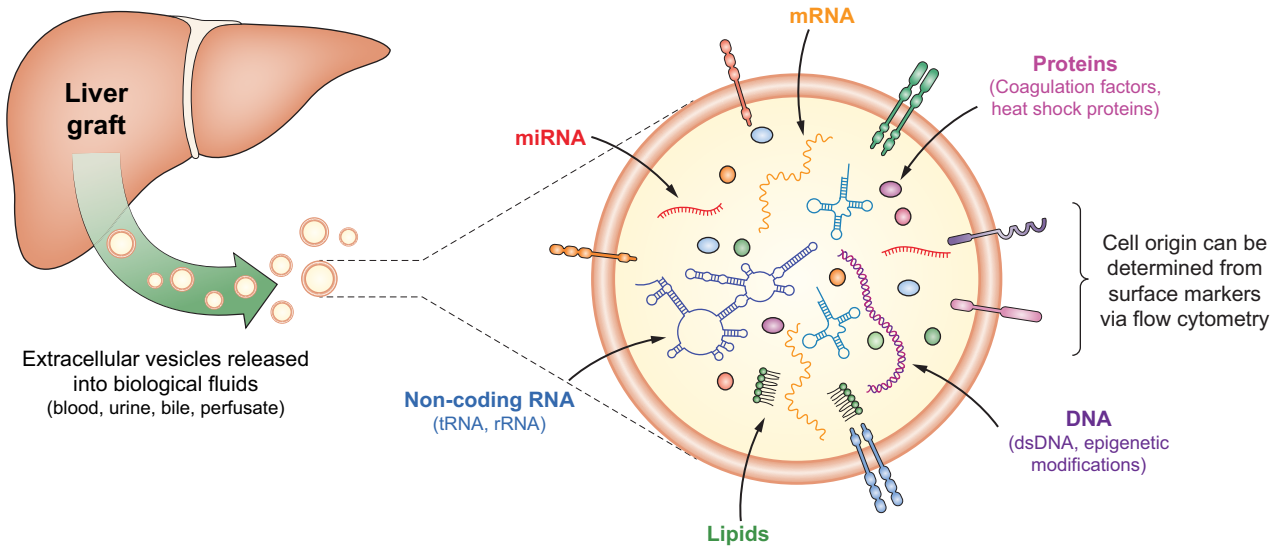

Fig. 1. EVs released from graft tissue function as well-protected messenger complexes that allow for the transfer of lipids, proteins, and nucleic acids to neighbouring or distant targets. By carrying biomolecules from tissues to biofluids, EVs may serve as non-invasive sources of clinical biomarkers. Characterising the cellular sources using surface markers can provide biological information about the functional state of liver-specific parent cells. dsDNA, donor-specific DNA; EV, extracellular vesicle; miRNA, microRNA.

antigen (HLA) have garnered interest as potential biomarkers of allograft function. Mastoridis *et al.* used ImageStream flow cytometry to detect and characterise circulating organ-specific EVs.[33] The EVs from 3 liver transplant recipients were labelled with a pan-EV marker (CFSE [carboxyfluorescein diacetate succinimidyl ester]), a *bona fide* marker of exosomes (CD63), and probes for donor and recipient HLA (such as HLA-B27 and HLA-B8). The investigators confirmed that donor-specific EVs were detectable in the circulation after LT. Further multiparametric analyses were employed to interrogate donor EV cargo for co-stimulatory/inhibitory molecules, thereby providing additional support for the application's potential to characterise disease and impart functional insights. Recently, the same group employed advanced imaging flow cytometry to explore the kinetics of EV release and the extent to which donor EVs might induce cross-dressing (a phenomenon where recipient cells display membrane proteins from the transplant donor) following LT.[34] Typically, the cross-dressing process occurs after organ transplantation when donor leukocytes and/or parenchymal cells release vast amounts of small EVs, including exosomes (30–150 nm in diameter).[34] These EVs are taken up and displayed as patches on the surface of host dendritic cells, causing

Table 1. miRNAs associated with various post-transplant liver conditions.[42–79]

Condition	Differentially expressed miRNAs when compared to normal livers		
Acute rejection/T cell-mediated rejection	• miR-34a[42] ↑ Ⓢ • miR-22[42] ↑ Ⓢ • miR-148a[42] ↑ Ⓢ • miR-192[42] ↑ Ⓢ • miR-193b[42] ↑ Ⓢ • miR-194[42] ↑ Ⓢ	• miR-210[42] ↑ Ⓢ • miR-885-5p[42] ↑ Ⓢ • miR-122-5p[42–46] ↑ ⓈⓊ • miR-155-5p[43,44] ↑ Ⓢ • miR-181a-5p[43,44] ↑ Ⓢ	• miR-483-3p[54] ↑ Ⓢ • miR-301a[55] ↑ Ⓣ • miR-18b[56] ↑ Ⓜ • miR-340[56] ↑ Ⓜ • miR-106b[56] ↑ Ⓜ
Hepatitis C virus	• miR-155[46] ↑ Ⓜ • miR-29[46] ↓ Ⓜ • miR-223[46] ↓ Ⓜ	• miR-199[46] ↑ Ⓜ • miR-122-5p[46,47] ↑ Ⓜ • miR-449a[57] ↑ Ⓣ	• miR-885-5p[58] ↑ Ⓢ • miR-122[59] ↑ Ⓢ
Alcohol-related liver disease	• miR-122[48] ↑ Ⓢ • miR-17[60] ↑ Ⓑ • miR-107[60] ↑ Ⓣ • miR-192[61] ↑ Ⓢ • miR-30a[61] ↑ Ⓢ	• miR-155[61,62] ↑ Ⓢ • miR-103[63] ↑ Ⓣ • miR-34a[64] ↑ Ⓣ • miR-182[65] ↑ Ⓢ	• miR-27a[66] ↑ Ⓢ • miR-212[67] ↑ Ⓣ • miR-291b[68] ↑ Ⓣ • miR-223[69] ↑ Ⓢ
Non-alcoholic fatty liver disease	• miR-21[47] ↑ Ⓢ • miR-34a[47] ↑ Ⓢ • miR-451[47] ↑ Ⓢ • miR-122[47,49,50] ↑ Ⓢ • miR-24[50] ↑ Ⓢ	• miR-30c[51] ↑ Ⓢ • miR-31[69,70] ↑ Ⓣ • miR-9 [71] ↑ Ⓢ • miR-10b[72] ↓ Ⓢ • miR-15b[72,73] ↑ Ⓢ	• miR-16[74] ↑ Ⓢ • miR-17[70] ↓ Ⓣ • miR-19[75] ↑ Ⓢ • miR-27b[76] ↑ Ⓢ • miR-30b[77] ↓ Ⓢ
Hepatocellular carcinoma	• miR-21[45,47] ↑ Ⓢ • miR-223[47] ↓ Ⓣ • miR-122a[47] ↓ Ⓣ • miR-145[47] ↓ Ⓣ	• miR-193a-5p[54] ↑ Ⓢ • miR-214-3p[54] ↑ Ⓢ • miR-365a-3p[54] ↑ Ⓢ • miR-885-5p[58,78] ↑ Ⓢ	• miR-155[78] ↑ Ⓢ • miR-532-3p[78] Ⓤ • miR-765[78] Ⓤ • miR-122[79] ↑ Ⓢ
Drug-mediated hepatotoxicity	• miR-193a-5p[52] ↑ Ⓢ • miR-1290[52] ↑ Ⓢ • miR-21-5p[52] ↑ Ⓢ • miR-194-5p[52,53] ↑ Ⓢ	• miR-122-5p[52,53] ↑ Ⓢ • miR-27b-3p[52,53] ↑ Ⓢ • miR-125b-5p[52,53] ↑ Ⓢ • miR-483-5p[53] ↑ Ⓢ	• miR-99a-5p[53] ↑ Ⓢ • miR-320b[53] ↑ Ⓢ • miR-192-5p[53] ↑ Ⓢ • miR-23a-3p[53] ↑ Ⓢ

MiRNA (miR), microRNA.

↑: upregulated; ↓: downregulated; Ⓑ: bile; Ⓢ: serum/plasma; Ⓤ: urine; Ⓣ: tissue; Ⓜ: peripheral blood mononuclear cells.

alloreactive T cell stimulation or tolerization. The authors conclude that cross-dressing can be transiently detected in the circulation shortly after LT in association with the release of donor-HLA EVs. In LT recipients, most circulating cells exhibiting donor-HLAs are indeed cross-dressed cells and not passenger leukocytes.[34]

Such advances in EV analytic approaches have enabled the investigation of donor-specific allograft-derived EV release following LT and demonstrated the capacity for these to cross-dress recipient cells through the transfer of donor major histocompatibility complexes. Given the presence of cross-dressed cells in experimental and clinical transplantation and the recognised impact of these cells on the overall alloresponse, these pathways could be critical for the design of tolerance-promoting protocols.[35]

In addition to functioning as "biomarker carriers," EVs may hold therapeutic potential based on their drug delivery capabilities and regenerative properties.[36] A recent report showed human liver stem cell-derived EVs can reduce liver injury and promote regeneration during normothermic machine perfusion in a donation after circulatory death donor model with prolonged warm ischaemia.[37] Further identification of the cellular origins of EVs (*i.e.*, stellate cells, hepatocytes, endothelial cells) and their intended recipient cells will be critical for targeted interventions.

MiRNAs as markers of injury

Ongoing investigations into the transcriptomic profiles associated with biological fluids have revealed the potential for non-coding RNAs to serve as biomarkers of graft injury. Non-coding RNAs (*e.g.*, miRNAs, long non-coding RNAs, transfer RNAs, small-interfering RNAs) represent specific, sensitive, and stable markers of liver injury. MiRNAs have garnered heightened interest because of their upstream roles in modulating transcriptional programmes and orchestrating both physiological and pathological processes. Given the regulatory role of miRNAs in mediating gene expression on the post-transcriptional level, it is not surprising that physiological and pathological changes can induce alterations in the circulating miRNA expression profiles. MiRNA sequences are highly conserved across species, allowing for tissue-specificity and easy detection through reverse-transcription quantitative PCR (RT-qPCR).[38] Additionally, circulating miRNAs are rapidly released from cells in response to hypoxic stress and injury, and can enable real-time assessments of the dynamic conditions related to transplant injury.[39,40] It has also been demonstrated that critical facets of adult tissue repair mechanisms are subject to control by miRNAs.[41] The release of shedding vesicles containing miRNA is known to be highly regulated and depends on the activation state of the source cells.[26] These features establish miRNAs as ideal biomarkers for a variety of clinical applications. Circulating miRNAs' potential as sensitive markers of liver injury has been reported in many studies. Table 1 summarises the differentially expressed miRNAs found to be associated with acute rejection, HCV, alcohol-related liver disease, non-alcoholic fatty liver disease, hepatocellular carcinoma (HCC), or drug-mediated hepatoxicity.[42–79] Of note, miR-122, miR-34a, miR-223, miR-192, miR-885-5p, miR-155, and miR-21 were related to 3 or more of these conditions, demonstrating their broad potential to assess multiple forms of liver injury. Mir-122 is the only miRNA that was found to be associated with each of these conditions.[42–53]

As one of the earliest examples of tissue-specific miRNAs, miR-122 is of heightened interest as a marker of liver injury.[80,81] Upon its discovery, investigators found that miR-122 accounted for 72% of all miRNAs cloned from the livers of mice.[82] Other miRNAs such as miR-21, miR-29, miR-192, and miR-194 are also highly enriched in the liver and may function as specific markers of post-transplant injury.[83–87]

Investigators have used clinical models to explore the quantitative relationship between miRNA expression and degree of liver injury. Changes in liver-specific miRNAs in the plasma of mice are both dose- and exposure duration-dependent.[88,89] Wang *et al.* found that expression of miR-122 and miR-192 increased more rapidly than LFTs after drug overdose (≤1 hour), and progressively increased as the dose of acetaminophen increased.[83] Similarly, Chang *et al.* reported that patients with non-alcoholic fatty liver disease and advanced fibrosis (grade 3-4) had significantly lower levels of serum miR-122 than those with lower grade fibrosis (grade 1-2), demonstrating an inverse relationship between the severity of fibrosis and miR-122.[90] In contrast, most studies have demonstrated that elevated miR-122 serum levels correlate with hepatic injury and inflammatory activity in patients with HCV, acute rejection, alcohol-related liver disease, and non-alcoholic fatty liver disease.[42–53] These discrepancies might be due to differences in the aetiology of liver disease, thus, the relationship between miR-122 and fibrotic injury needs to be further explored.

MiRNAs can also provide information on causal transcriptional processes occurring at a molecular level, leading to the identification of dysfunctional canonical pathways and novel therapeutic targets. Of note, Shaked *et al.* investigated the miRNA profiles of longitudinal serum biopsies to identify early predictors of acute rejection.[91] Using a prospective study design, the investigators demonstrate that a two-miRNA (miR-483-3p and miR-885-5p) combination model could predict acute cellular rejection (ACR) up to 40 days before biopsy-proven rejection. The findings of this study suggest that serum miRNAs could identify ACR without the need for a liver biopsy. Consequently, these markers could replace or, more likely, contribute to the diagnostic value of liver biopsies. Critically, immunosuppression could also be personalised for individual patients based on miRNA measurements during protocolised immunosuppression minimisation. Validated, non-invasive markers of rejection could be used to predict clinical rejection episodes, thereby providing a unique tool for physicians to optimise immunosuppression levels while avoiding ACR. In summary, these findings may give rise to more precise postoperative immunosuppression regimens, addressing the critical need to mitigate the toxic effects of excess immunosuppression, which is associated with system-wide comorbidities.

In a recent report, Ruiz *et al.* reported that 3 upregulated plasma miRNAs (miR-155-5p, miR-122-5p, and miR-181a-5p) can accurately diagnose T cell-mediated rejection (TCMR) with AUROCs of 0.87, 0.91, and 0.89, respectively.[43] Furthermore, they report that miR-155-5p was able to discriminate all patients who presented TCMR. This study demonstrates that miRNA expression levels in plasma can differentiate TCMR from other causes of graft dysfunction and may serve as a useful tool in clinical practice. Because this is a single-centre study with a limited

sample size, results should be interpreted cautiously and must be validated in a larger patient population. In addition, only a few miRNAs were examined, and the methodologies used for their analysis vary from other works.

Similarly, Muthukumar *et al.* reported a miRNA signature in serum that can diagnose and differentiate acute rejection from other forms of liver injury.[42] The group first identified a panel of 9 miRNAs that is diagnostic of acute rejection of human liver when compared to a cohort without signs of rejection. To reduce confounding variables this analysis was limited to patients who received transplants for non-immunological and non-viral liver diseases. Remarkably, the authors found that a diagnostic model using a combination of miR-210 and miR-34a grouped all the patients correctly. These results suggest that serum miRNAs could identify acute rejection without the need for a liver biopsy. The investigators then tested the specificity of these serum markers by comparing the acute rejection cohort to recurrent HCV samples. While HCV rarely poses an ongoing threat to post-transplant outcomes, the investigators still demonstrate the ability of circulating miRNAs to successfully differentiate liver injury caused by different aetiologies. These miRNA signatures can be detected significantly earlier than the current established diagnostic methods, allowing for more timely intervention and guided treatment. Ultimately, these studies support the utility of cell-free miRNA as diagnostic non-invasive biomarkers and also demonstrate the applicability of new bioinformatics tools (*i.e.*, logistic regression models and AUROCs) in the discovery of biomarkers.

Furthermore, as the study of machine perfusion accelerates, there is an even greater demand to identify and validate markers of liver viability and functionality during perfusion. Perfusate is an especially advantageous choice as a source of biomarkers for 3 critical reasons: i) there is a large volume readily available, ii) collection is non-invasive, and iii) it can be rapidly analysed while the liver is awaiting transplantation and/or being perfused. Therefore, as scientists promptly work to extend the limits of cold ischaemia time (CIT), there is a growing window of opportunity in which graft quality can be non-invasively examined in real-time. During CIT the liver is under significant hypoxic stress and releases uniquely packed EVs in response to extreme conditions. Few groups have explored the prognostic potential of circulating miRNAs released during CIT. Using advanced molecular techniques, it takes about 1 hour to isolate RNA, 1 hour for cDNA conversion, and 1 hour for qPCR set up and analysis. Given the CIT is 6-9 hours for most of the liver donors (range: 5-13 hours), there is ample time to collect perfusate and run prognostic tests while the patient awaits implantation.[92] Selten *et al.* set out to determine whether miRNA expression in perfusate correlated with early allograft dysfunction after LT.[93] The investigators found that hepatocyte-specific miR-122 and the miR-122/miR-222 ratios in perfusate served as potential biomarkers of early allograft dysfunction. The two-miRNA ratio model may aid in the development of therapies during graft preservation, thereby reducing post-transplant complications. This work demonstrates the maladaptive physiological and pathological processes that may begin manifesting in the donor organ under ischaemic conditions prior to transplantation. Such findings can be used to assemble a panel of miRNAs that can be tested at pre-implantation and may predict future graft function and outcomes. Decoding these early signals that are constitutively released into the perfusate may also allow for a better understanding of the role of cell-to-cell communication in the propagation of graft injury.

While the use of miRNA biomarkers holds great promise, the identification of robust controls for data normalisation remains a challenge in miRNA biomarker research, and the use of an exogenous "spike-in" control during RNA isolation remains essential. During RNA extraction, exogenous oligonucleotides (*e.g.*, cel-miR-39, cel-miR-54, cel-miR-238) are added at known concentrations in order to assess RNA yield, signal the presence of nucleases, and monitor reverse-transcription efficiency.[94] After conducting qPCR, spike-in expression values between patient samples can be compared, and outlier samples may be identified and considered for exclusion from further data analysis. In a recent publication, Roest *et al.* systematically investigated the challenges associated with the isolation of RNA from a range of human biofluids related to LT, including serum, urine, bile, and perfusate.[95] Using 4 different isolation techniques and complete sample sets from matched patients, the investigators demonstrate i) the large variability in RNA yield between biological samples, ii) the significant contamination of anticoagulant RT-qPCR inhibitors detected in perfusate samples, and iii) the benefits of using a spike-in control to determine RNA loss during workup. However, the investigators also warn against using spike-in controls to normalise qPCR data, instead confirming that it serves as a reliable indicator of RNA yield.[95] Still, investigators are left without a set of standards for data normalisation in miRNA discovery research. There are limited consistent "housekeeping" miRNAs in human biofluids, and the heterogeneity of these samples makes the identification of one increasingly challenging.[96] Recently, Faraldi *et al.* investigated the efficiency of 4 different normalisation strategies for analysing circulating miRNAs, including i) the global mean of miRNA expression, ii) the mean of endogenous controls (33 stable miRNAs ranked by NormFinder[97] algorithm), iii) exogenous oligonucleotides (3 "spike-in" miRNAs), and iv) the single most stable endogenous miRNAs.[94] The investigators found that global, endogenous, and single-miRNA methods performed similarly in reducing the technical variability among their experimental replicates. Interestingly, they report that exogenous spike-in normalisation methods (using both individual and mean Ct values) generated marked differences in the fold-change expression levels (opposite trend), suggesting that this strategy may lead to misinterpretation of results.[94] Other groups have also demonstrated that the relative logarithmic differences between two endogenous miRNAs can be used to create "self-normalising miRNA pairs," which circumvent the need for a reference miRNA in circulating biofluids.[98–100] Currently, it is clear that different normalisation methods significantly influence experimental outcomes and thus, the lack of an accepted standardised control strongly limits the clinical applicability of miRNA biomarkers.

Beyond identifying expression patterns that are significantly associated with graft survival and recurrent disease, miRNAs can be used to explore the molecular mechanisms and specific cell types involved in mediating post-transplant injury. Technologies based on immunoaffinity-capture surfaces and beads, such as flow cytometry and ELISA, have enabled the characterisation of EV populations expressing cell-specific antigens.[101,102] Thus,

Table 2. Current challenges associated with biomarker discovery research.

Challenge	
Working with human samples	• Recruitment: enrolling patients with diverse backgrounds from multiple transplant centres remains a challenge • Small sample size: acquiring a large number of study participants is necessary for significant findings • Clinical data organisation: collecting, storing, and sorting a significant volume of longitudinal patient data can be difficult to manage • Study timeline: retention of patients during follow-up is costly, time-consuming, and difficult • Variable post-transplant drug regimens: immunosuppressive treatment, dosage, and management may change over time, presenting a confounding variable that is difficult to adjust for
Study Design	• Lack of standardised study endpoints: patient/graft survival, acute rejection episodes, biochemical data (*i.e.*, liver enzymes), disease recurrence and development, and non-immunological injury (nephrotoxicity) must all be assessed in relation to post-transplant outcomes • Lack of control group: post-transplant control groups are difficult to define • Identification of best markers: there is no standardised method for narrowing down the top candidate markers when using high-throughput approaches
Technical challenges	• Isolating nucleic acids: circulating nucleic acids are characterised by low concentration and high degradation rates • Experimental methods: utilising high throughput technologies can be costly and time-consuming • Normalisation methods: multiple approaches can be used to normalise cell-free nucleic acids
Building predictive models	• Team science: biostatisticians are needed to aid in the development and implementation of statistical and mathematical methods • Overfitting: over-testing the training data can result in a model that appears very accurate but has memorised the key points in the data set rather than learned how to generalise, requiring an independent dataset to validate findings.

liver-specific EV populations can be studied for their protein (*e.g.*, western blotting), lipid (*e.g.*, mass spectrometry), and nucleic acid (*e.g.*, RT-qPCR, DNA, and RNA sequencing) content, allowing for the continued study of novel circulating molecular markers.[102]

However, it is important to note that miRNAs have a variate distribution in biofluids, which may subsequently affect findings. Some are known to be associated with proteins, others with vesicles, and others with both. For example, in alcohol-related liver disease and inflammatory liver injury, miR-122 and miR-155 are mainly associated with EVs, whereas in drug-induced liver injury, these miRNAs predominate in protein-rich fractions.[18] Such variations are important to consider when investigating the origin, function, and target of pertinent miRNA markers from differing biological fluids.[62]

Lastly, it may be useful to discern the mechanisms and pathways by which master miRNA regulators drive liver injury by quantifying the expression of novel downstream targets. Studying the molecular pathways and biological functions associated with significant miRNAs could lead to translational and therapeutic advances in LT. In future work, gain- and loss-of-function approaches could be used to target top miRNA candidates and thereby to identify significant molecular interactions that orchestrate liver injury in clinical models.[103,104]

Cell-free DNA as a marker of graft injury

In the current era of immunosuppression, acute rejection rates in LT remain acceptably low, while immunosuppressant toxicity continues to have severe consequences on long-term outcomes. Thus, a non-invasive marker to personalise immunosuppression remains a critical need. The transplant field is in need of biomarkers that are practical, cost-effective, and reproducible, with a rapid (same-day) turnaround time. Analytic performance shows high specificity and sensitivity for graft complications, and such biomarkers should have diagnostic or prognostic utility at the earliest disease stages. Donor-derived cell-free DNA (dd-cfDNA) is a promising new biomarker for the detection of graft injury.[92] One of the earliest studies concluded that plasma dd-cfDNA levels can function as markers of cell death, released from necrotic or apoptotic cells in the transplanted organ, and may consequently be useful in predicting rejection.[105]

There are various approaches for detecting dd-cfDNA; for instance, the use of preselected single nucleotide polymorphisms and high-throughput sequencing for readouts have been reported.[94–96] Currently, the most common methods include shotgun sequencing or droplet digital PCR (ddPCR). This data is expressed either as GcfDNA percentage (graft cfDNA/total cfDNA) or absolute quantification in copies/millilitre.[104,106,107] In a recent multicentre study, the detection of dd-cfDNA in plasma by ddPCR allowed for earlier and more sensitive discrimination of acute rejection compared with conventional LFTs.[108] Specifically, LFTs and plasma dd-cfDNA were both monitored in 115 adult liver transplant recipients at 3 different transplant centres as part of a prospective, observational, multicentre cohort trial. Multivariable logistic regression modelling demonstrated that GcfDNA provided additional information on graft integrity beyond LFT measurements. Diagnostic sensitivity and specificity were 90.3% (95% CI 74.2%-98.0%) and 92.9% (95% CI 89.3%-95.6%), respectively, for dd-cfDNA at a threshold value of 10%.

Additional studies using different biomarker detection methods have explored the potential of dd-cfDNA biomarkers. Using probe-based ddPCR, Beck *et al.* have demonstrated that the plasma dd-cfDNA fraction was between 5%-10% at day 10 post-transplant in stable liver transplant recipients, whereas in cases of rejection it reached approximately 20% and gradually increased to 55–60%.[103] Macher *et al.*, who instead quantified cfDNA by RT-qPCR, also showed higher total cfDNA and dd-cfDNA serum levels in patients with liver transplant injury (acute rejection, hepatic arterial and venous thrombosis, and profound cholestasis ending in multiple organ failure) compared

Table 3. Biomarkers to assess graft injury after liver transplantation.

Clinical trial title and identifier	Institution	Enrolment	Start date	End date	Description
Serum Markers of Ischemia-Reperfusion Injury in Liver Transplant Patients [NCT00698399]	Vanderbilt University Medical Center	20	Mar 2008	Feb 2010	Observational trial of markers (TMAO, NGAL, cystatin-C, and allantoin) in the serum of patients who are undergoing LT surgery.
Gene Expression in Liver Allograft Rejection and Recurrent Hepatitis C [NCT01428700]	University of Pennsylvania	275	Aug 2011	Jan 2013	Observational study evaluating whether certain patterns of biomarkers in blood post-LT can be used to determine if the transplanted liver is being rejected or sustaining injury.
Pilot Study of Immunosuppression Drug Weaning in Liver Recipients Exhibiting Biomarkers of High Likelihood of Tolerance	Hospital Clinic of Barcelona	25	Sep 2011	Jan 2013	Non-randomised prospective study in which gradual weaning of immunosuppressive drugs will be offered to LT recipients exhibiting a favourable peripheral blood gene expression profile.
Discovery and Validation of Proteogenomic Biomarker Panels in Liver Transplant Recipients [NCT01672164]	Northwestern University Feinberg School of Medicine	202	Aug 2012	Dec 2015	The main focus of this study is to develop blood and/or urine tests that will help to detect early signs of rejection in LT patients.
The Relationship of Hepatobiliary microRNA Expression Profile and Clinical Outcome in Liver Transplantation [NCT02307890]	University of Edinburgh	100	Aug 2014	Aug 2020	Observational study correlating miRNA levels in bile duct biopsies taken during LT with the incidence of IC following LT.
Liver Immunosuppression Free Trial (LIFT) [NCT02498977]	King's College London	148	Oct 2015	Oct 2021	Prospective interventional study analysing the risk/benefit ratio of employing transcriptional biomarkers to guide immunosuppression withdrawal post-LT.
Plasmatic Factor V as a Predictor of Graft Dysfunction After Liver Transplantation [NCT03396016]	University Health Network, Toronto	140	Apr 2018	Apr 2021	Observational study validating the use of coagulation cofactor Factor V as a predictive biomarker of graft function after LT.
Enteric Microbiome and Liver Transplantation [NCT03666312	IRCCS Ospedale San Raffaele	275	Sep 2018	Aug 2021	Observational study of the faecal microbiome of LT patients in combination with a large panel of clinical, lab and functional parameters correlated to different clinical outcomes.

(*continued on next page*)

Table 3 (*continued*)

Clinical trial title and identifier	Institution	Enrolment	Start date	End date	Description
Liquid Biopsy-based Monitoring System for Relapse of HCC After Liver Transplantation: A Multi-center and Prospective Study [NCT03708705]	Zhejiang University	500	Nov 2018	Jul 2020	Prospective study aiming to develop a liquid biopsy-based biomarker system for relapse of HCC tumour post-LT using genomic and proteomic information.
Monocytic Expression of HLA-DR After Liver Transplantation (EdMonHG) [NCT03995537]	Hospices Civils de Lyon	100	Feb 2020	Sep 2023	Observational study examining the expression of monocytic surface markers (HLA-DR) in blood and their association with post-LT immune dysfunction (acute cell rejection and sepsis).
Non-invasive Rapid Assessment of Patients with Liver Transplants Using Magnetic Resonance Imaging With LiverMultiScan.	Leiden University Medical Centre; King's College Hospital NHS Trust	131	Aug 2020	Oct 2020	Prospective biomarker trial comparing the accuracy of a new test (LiverMultiScan) against an existing test (liver biopsy) in the assessment of LT recipients.
Role of Fecal Microbiota in Predicting Graft Rejection and Sepsis Among Recipients of Living Donor Liver Transplant in First Year. [NCT04621812]	The Institute of Liver and Biliary Sciences	100	Nov 2020	Oct 2022	Observational study analysing the role of gut microflora an early biomarker for graft dysfunction and its influence on immune remodelling for the prediction of post-LT infection or rejection.
Molecular Assessment and Profiling of Liver Transplant Recipients [NCT04793360]	Icahn School of Medicine at Mount Sinai	1500	May 2021	Dec 2025	Prospective observational study to assess the correlation between clinical events (*e.g.*, rejection, recurrent disease, biliary obstruction), graft histology, dd-cfDNA levels, and gene expression profiling

dd-cfDNA, deceased donor cell-free DNA; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; IC, ischaemic cholangiopathy; LT, liver transplantation; NGAL, neutrophil gelatinase-associated lipocalin; TMAO, trimethylamine N-oxide.

to recipients with stable graft function.[109] In contrast, increases in total cfDNA levels but not dd-cfDNA were observed in patients with complications that did not compromise the donated organ (biliary peritonitis and surgical wound infection).

A recent study aimed to evaluate the feasibility of measuring dd-cfDNA through short tandem repeats analysis by quantitative fluorescent-PCR and, to assess the role of the concentration and fragment size of total cfDNA as biomarkers of acute rejection.[110] Short tandem repeat amplification by quantitative fluorescent-PCR may be an alternative, easily implementable, strategy for rapid dd-cfDNA quantification in clinical laboratories. The results of this pilot study indicate that dd-cfDNA increases very early, even 1-2 weeks before the diagnosis of acute rejection, so it could be useful as a prognostic biomarker to improve patient risk stratification.

In summary, these studies show that dd-cfDNA is a biomarker with promising clinical utility in LT. In cases of acute rejection, dd-cfDNA has repeatedly outperformed LFTs, allowing for the earlier and more sensitive discrimination of this complication. Currently, the main limitation associated with cfDNA is specificity. However, it has been reported that unique methylation patterns on DNA fragments can serve as fundamental determinants of cell identity. Lehmann-Werman *et al.* recently described a method to detect cfDNA carrying hepatocyte-specific methylation patterns.[111] The authors identify 3 genomic loci that are specifically unmethylated in hepatocytes. Probing cfDNA methylation sites allows for the i) identification of the cellular origins of markers, ii) quantification of injury in specific cell types, and iii) identification of novel therapeutic targets in transplant injury.

Advancing precision medicine: the now and the future

The prognostic and diagnostic value of non-invasive biomarkers has been widely explored in LT. However, findings have not yet been translated into routine clinical use, owing to small sample sizes, and the lack of proper control groups or independent validations. The main limitations of CNA analysis include its low quantity and compromised quality (potential degradation) in biological fluids. The current challenges associated with biomarker research are summarised in Table 2. Many recent analyses are also limited to tissue-derived biomarkers, which are useful for integrative analyses but have limited implications for clinical practice. While the use of tissue miRNA expression for diagnosis is not as practical as non-invasive markers, significant miRNA discoveries in liver tissue can be used to guide future investigations in biological fluids. Within the liver, several microRNAs including miR-21, miR-221/222, and miR-181b promote liver fibrosis through the TGF-β or NF-κB pathways, whereas miR-29b, miR-101, miR-122, and miR-214-3p prevent fibrosis by inhibiting collagen synthesis or suppressing activation of the TGF-β pathway.[112] Serum miR-34a was found to be upregulated in patients with fibrosis in a stage-dependent manner, and miR-571 and miR-513-3p were elevated in patients with cirrhosis.[74,86] MiR-29a was downregulated in patients with fibrosis in an inversely stage-dependent manner.[113] Also, while miR-122 is significantly downregulated in HCC tissue samples, many groups have reported the upregulation of circulating miR-122 in the serum of patients with HCC compared to that of controls.[54,114,115] More research is needed to understand the correlation between tissue and peripheral biomarker signatures.

Statistical significance does not readily translate into clinical utility in biomarker research. The pipeline from discovery to clinical practice for biomarkers is complex, and independent validations of biomarker-guided practice using a large population of human patients with longitudinal follow ups are needed. Table 3 outlines the pertinent biomarker clinical trials identified in LT in chronological order (retrieved from the clinicaltrials.gov database). Of note, the Liver Immunosuppression Free Trial (LIFT) [NCT02498977] is currently studying the utility of transcriptional tissue biomarkers to guide immunosuppression withdrawals and is in phase IV of clinical testing. Such biomarkers improve the accuracy and speed by which physicians can detect post-transplant complications. While this work is undoubtedly restricted by the need for tissue biopsies, it represents an important starting point for the use of molecular diagnostics in transplantation. The LiverCare Kit [NCT04793360] utilises Streck and PAX gene tubes to assess dd-cfDNA and gene expression patterns in the blood of transplant patients, specifically monitoring clinical events related to rejection, recurrent disease, and biliary obstruction. Typically, many discovery biomarker studies utilise plasma or serum for analysis; however, the utilisation of whole blood for biomarker surveillance is more transferrable to clinical settings where physicians may not have access to large centrifuges. Thus, the reproducibility and efficacy of biomarkers need to be validated using clinically relevant biological samples.

Limitations in study design can also significantly delay the utilisation of molecular diagnostic markers in clinical settings. Biomarker studies can be retrospective (*i.e.*, using previous data) or prospective (*i.e.*, following a large number of patients). Prospective designs allow for the longitudinal collection of pre-diagnostic samples, making this the optimal study design for identifying early, prognostic markers of injury.[116] Marker levels likely increase as liver injury progresses, thus, samples collected closer to the occurrence of physical symptoms will be more informative than those taken after a clinical diagnosis.

Furthermore, since current diagnostic methods and cohort groupings are often based on histological findings of core biopsies, predictive biomarkers for post-transplant conditions are limited by biopsy findings. Establishing clinically meaningful surrogate endpoints for biomarker discovery research is an ongoing challenge in LT. Compared to related fields, it is less clear which outcomes are most useful in the prediction of long-term liver graft function. This issue emphasises the need for more diverse surrogate markers of injury to improve study design and predictive models.

Despite a decade of research, no LT biomarkers are currently available for use in clinical practice. Large multicentre clinical trials have generated vast amounts of data and information at various molecular levels, demonstrating a promising opportunity for cell-free biomarkers to be introduced into clinical care. This article summarised how recent advances in sequencing and biotechnological methodologies are contributing to the identification of new transcriptomic and genomic biomarkers that could be used in post-transplant management. Data-driven approaches have allowed for the integration and interpretation of the structural and functional features of EVs, miRNA, and cf-ddDNA. Molecular profiles of biofluids such as blood, urine, perfusate, and bile are being integrated with post-LT challenges, providing new opportunities to understand how specific perturbations in biological fluids can signal the early stages of disease.

Abbreviations

ACR, acute cellular rejection; CIT, cold ischaemia time; CNA(s), circulating nucleic acid; dd-cfDNA, donor-derived cell-free DNA; ddPCR, digital droplet PCR; EV(s), extracellular vesicles; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; LFTs, liver function tests; LT, liver transplantation; miRNA, microRNA; RT-qPCR, reverse-transcription quantitative PCR; TCMR, T cell-mediated rejection.

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Conflict of interest

The authors of this manuscript have no conflicts of interest to disclose.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

EB (Literature review, Writing –original draft, review & editing), JM (Writing – review & editing), TR (Writing – review & editing), DM (Supervision; Writing – review & editing), VM (Literature review; Writing – original draft, review & editing).

Supplementary data

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