

Review

Integrating RNA-Seq Biomarkers and Diagnostic Algorithms to Improve Liver Transplant Outcomes: A Comprehensive Review

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Abstract

Graft rejection, early allograft dysfunction (EAD), and poor diagnostic accuracy are some of the challenges that still need to be addressed, even though liver transplantation (LT) has the potential to help patients with end-stage liver disease (ESLD) significantly. Traditional procedures, such as liver biopsies and liver function tests (LFTs), often fail to identify abnormalities early and with accuracy. Genomic RNA sequencing (RNA-Seq) has recently emerged as a powerful approach for identifying molecular markers of immune activity and graft healing. This study provides a comprehensive review of the current achievements in RNA-Seq applications for liver transplantation by comparing biomarker profiles of liver biopsies and peripheral blood mononuclear cells (PBMCs). We highlight the success and challenges of integrating RNA-seq into clinical processes by critically examining its consistency, diagnostic importance, and translational potential. Furthermore, we evaluated the possibility



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of novel diagnostic algorithms and multi-omics techniques for improving early diagnosis, risk profiling, and personalized immunosuppression. This study highlights the gaps in cross-cohort repeatability, clinical validation, and standardization to improve long-term transplant outcomes. This highlights the need for integrated multicenter approaches driven by biomarkers and provides recommendations for further studies.

Keywords

Liver transplantation; RNA sequencing; biomarkers; acute rejection; diagnostic algorithms

1. Introduction

Liver transplantation is the only option available for patients with end-stage liver disease (ESLD) or certain liver cancers. Chronic viral hepatitis B and C, alcoholism, metabolic dysfunction-associated fatty liver disease (MAFLD), and, in some cases, liver-limited hepatocellular carcinoma (HCC) are pre-existing conditions that may be treated with liver transplant [1-6]. Liver grafts are in high demand owing to the rising incidence of chronic diseases, metabolic syndromes, and obesity. Consequently, the use of marginal donors, including older donors and those with steatotic livers [2-5, 7], is also considered.

Liver grafts are in high demand owing to the rising incidence of chronic diseases, metabolic syndromes, and obesity. Consequently, the use of marginal donors, including older donors and those with impaired liver function, has increased [1, 4, 8-10]. Patient outcomes and transplant survival are adversely affected by early allograft dysfunction (EAD), a significant complication [7, 8, 10]. Improving prognosis and optimizing post-transplant care depend on prompt and precise detection of graft damage [4, 9-11].

Problems with invasiveness, sample variability, and interobserver discrepancies are some of the drawbacks of liver biopsy, the present diagnostic gold standard for transplant damage [9-11]. Therefore, molecular diagnostic methods and non-invasive biomarkers are gaining popularity as a means of supplementing histology and providing a more accurate evaluation of graft health [1, 5, 8, 9, 11]. New molecular markers linked to graft damage and rejection have been uncovered by advances in omics technologies, including transcriptomics using RNA sequencing (RNA-seq) [5, 7, 11]. More customized transplant treatments are possible with the use of integrative methods that combine proteomics, metabolomics, and transcriptomics to evaluate graft quality and anticipate early allograft diseases [3, 5, 7].

The viability evaluation and use of marginal grafts have been greatly enhanced by improvements in organ preservation procedures, such as normothermic machine perfusion [2, 3, 6]. These advancements have contributed to the expansion of the donor pool and improvement of transplant outcomes over the past decade. By improving the dynamic evaluation of graft fibrosis and rejection risk, new customized diagnostic algorithms and machine learning frameworks enable more tailored patient care [5-7, 10]. Improving patient and graft survival after liver transplantation depends on the development of reliable biomarkers and integrated diagnostic algorithms to better identify and treat graft problems [4, 5, 7, 9-11].

The rapid decline in liver function that characterizes acute liver failure (ALF) is a complex clinical illness that may have serious consequences, including coagulopathy and hepatic encephalopathy. Worldwide, the incidence of ALF may vary according to its viral, non-infectious, and metabolic causes. Approximately half of all acute liver failure (ALF) cases in the United States and Great Britain are caused by drug-induced liver damage, with acetaminophen being the most common cause [12]. However, numerous areas, particularly developing nations, continue to rank high for viral hepatitis, including hepatitis A, B, and E [13, 14]. Emphysematous hepatitis is an uncommon infectious disease that has recently been recognized, and it progresses to ALF when gas-producing bacteria invade the liver [15].

Demographic and environmental variables also play a role in the cause of ALF. For example, drug-induced hepatotoxicity is more common in highly industrialized countries, whereas hepatitis virus infections are more common in certain areas owing to geographical variations [13, 14]. Recovery may be complicated when acute liver failure is a subsequent complication of systemic diseases, such as sepsis or severe hypoglycemia [16, 17]. Evidence from studies shows that people with ALF are more likely to have organ system malfunction and an increased risk of death due to infections and other complications [18].

In addition, because the injured liver changes the production of coagulation factors, a hypercoagulable condition may be precipitated by acute liver failure. Significant intra- and extrahepatic thrombotic events may occur in this situation [19, 20], as well as hypoprothrombinemia and impaired fibrinolytic ability. Hepatic damage is inversely related to the degree of coagulopathy, which is clinically indicated by an international normalized ratio (INR) >1.5 [17, 21]. It is critical to manage these consequences while monitoring for infection and organ system breakdown. As hypoglycemia and excessive glucose supplementation may harm liver healing and patient outcomes, maintaining glycemic balance is critical for patient care [16].

The discovery and verification of new biomarkers are crucial for improving the evaluation and management of liver transplantation outcomes. Recent research has shed light on post-transplant patient care by concentrating on biomarkers that indicate graft acceptance and associated problems.

Several studies have shown that specific indicators are useful for graft rejection. One example is the noninvasive detection of acute rejection using donor-derived cell-free DNA, which has gained increasing popularity. This study provides an advance over less specific standard liver function tests by demonstrating how total cell-free DNA concentration and fragment size may be used as indicators to evaluate graft integrity [8]. Furthermore, there have been encouraging developments in the detection of rejection episodes using blood genomic tests that measure mRNA and microRNA [22]. Merola et al. emphasized the need for prospective validation in light of the changing environment, stating that while many genetic and proteomic markers have been reported, their clinical relevance has not been thoroughly investigated because of the lack of comprehensive validation studies [23].

Additionally, the function of preoperative indicators, such as platelet count, in predicting postoperative problems has been evaluated previously. Li et al. highlighted the potential of platelet counts below $60 \times 10^9/L$ on the fifth postoperative day as a biomarker for early risk stratification in liver transplant recipients, as they independently predict serious sequelae [24]. These results support the idea that clinical procedures can benefit from regular laboratory tests to control complications better early.

Tests have been conducted to determine the diagnostic accuracy of biomarkers, such as procalcitonin and presepsin, in detecting infections after donation. Dong et al. provided a comprehensive analysis showing that procalcitonin levels may successfully distinguish between infection problems in liver transplant patients, which helps initiate therapies before they worsen [25]. The use of presepsin in the identification of sepsis, even in the presence of renal function instability, is helpful in critical care settings, according to a study by Yokose et al., which is prevalent in patients undergoing liver transplantation [26].

Transplant research is still tackling the problem of biliary problems head-on, with an emphasis on developing new methods. The use of living donors for liver transplantation increases the risk of biliary issues, such as biliary anastomotic strictures. There is evidence that early administration of endoscopic therapies may enhance their results [27, 28]. According to these results, the quality of life of patients after transplantation may be improved if biliary problems are detected early enough using cutting-edge imaging techniques and treated accordingly.

Finally, alcohol-related indicators were significant in the context of liver transplant eligibility. An effective monitoring technique for relapse prevention, which is crucial for transplant success, might use direct alcohol biomarkers such as phosphatidylethanol (PEth), which has shown better sensitivity in identifying recent alcohol intake than standard methods [29]. Therefore, individuals undergoing liver transplantation due to alcoholism should be closely monitored for changes in behavior or complications that may arise after the procedure.

To improve patient outcomes after liver transplantation, it is necessary to identify and validate new biomarkers. This will allow for better monitoring, faster intervention in the event of difficulties, and more accurate risk assessments. Transplantation treatment techniques seem to benefit significantly from the incorporation of these biomarkers into clinical practice.

We postulate that these gaps may be filled by combining RNA-seq biomarkers with AI-powered algorithms. This will enable the non-invasive monitoring of graft health in real-time. A new paradigm in post-transplant monitoring is proposed by combining the sensitivity of RNA-Seq to identify molecular rejection signs before clinical manifestation with the ability of AI to evaluate complicated multi-omics datasets.

2. Overview of Diagnostic Requirements in Liver Transplantation

2.1 Pre-Transplant Diagnostics

The MELD score is a crucial tool for evaluating the severity of liver disease in patients awaiting transplantation. Objective laboratory test results are used to estimate the likelihood of death in individuals with chronic liver disease. The MELD score considers serum bilirubin, which indicates how well the liver is functioning and how much bile it can excrete; serum creatinine, which indicates how the kidneys are affected by liver illness; and the INR, which evaluates how well the blood clots, which may be hindered when the liver is diseased. The following formula (1) was used to obtain a person's MELD score:

$$\text{MELD} = 3.78 \times \ln(\text{serum bilirubin}) + 11.2 \times \ln(\text{INR}) + 9.57 \times \ln(\text{serum creatinine}) + 6.43(1)$$

Scores between 6 and 40 indicate the severity of liver disease and the risk of death [30].

With its two primary uses, the MELD score is a crucial tool in liver transplantation. First, it ranks patients on the transplant waiting list for allocation purposes. Due to their greater need for transplantation, individuals with higher scores are given priority when organs become available [30]. Research has shown that the MELD score is a reliable predictor of graft and patient survival after transplantation, making it a useful prediction tool [30].

Identifying individuals at high risk of liver transplant rejection requires thorough evaluation using biomarkers. Patients' dietary health, psychological variables, comorbidities, and liver function were assessed using a multidisciplinary approach [30]. To forecast transplant results and identify high-risk individuals, researchers have investigated emerging biomarkers, such as genomic markers and donor-derived cell-free DNA (ddcfDNA) [30]. Essential pre-transplant procedures include identifying high-risk candidates and evaluating the severity of liver disease using the MELD score. This ensures that appropriate candidates receive transplants, which improves outcomes and decreases the risk of complications. As research progresses, the integration of cutting-edge biomarkers with machine learning algorithms can significantly enhance the accuracy of assessments [12].

2.2 Post-Transplant Monitoring

Improving the outcomes of liver transplantation requires early diagnosis of graft failure. The most serious consequences of graft malfunction, which manifests as EAD or primary nonfunction (PNF), include the need for retransplantation or even patient death [31]. Standard traditional liver function tests to assess liver function after transplantation include bilirubin levels, international normalized ratio (INR), serum transaminases (AST and ALT), and other tests. However, these tests may be sensitive but not specific, which can lead to diagnostic delays [1].

Recent research has shown that Factor V (FV) has a predictive value for early graft malfunction. With the addition of ALT levels, FV has demonstrated high specificity and predictive power for graft loss within the first 90 days after transplantation, offering a simple risk stratification tool that may inform clinical decision-making [31]. Furthermore, ddcfDNA is becoming increasingly popular as a biomarker for the early diagnosis of graft malfunctions. Transplant recipients may experience cellular turnover and graft damage before the onset of clinical symptoms if their blood ddcfDNA levels are elevated [1, 9]. According to studies, timely management may be achieved because ddcfDNA levels may increase substantially before severe rejection is detected [1, 8].

Noninvasive imaging techniques, including magnetic resonance elastography (MRE) and ultrasound, can indicate liver graft health by measuring liver morphology and stiffness. These methods provide helpful data without the risks associated with invasive operations, such as liver biopsy [32].

To identify those at high risk of liver transplant rejection, a thorough evaluation, including the use of biomarkers, is necessary. Patients' dietary health, psychological variables, comorbidities, and liver function were assessed using a multidisciplinary approach [30]. The use of genetic markers and emerging biomarkers, such as ddcfDNA, to predict transplant success and identify high-risk patients is currently being investigated [30]. Essential pre-transplant procedures include identifying high-risk candidates and evaluating the severity of liver disease using the MELD score. This ensures that appropriate candidates receive transplants, which improves outcomes and decreases the risk of complications. As research progresses, the integration of cutting-edge biomarkers with machine learning algorithms can significantly improve the accuracy of assessments [30].

Preventing and treating rejection episodes, including both cellular and humoral components, requires close monitoring of the immune response in patients undergoing liver transplantation (LT). Liver transplant recipients are at an increased risk of acute and chronic rejection when donor-specific antibodies (DSAs) are present. Patients at increased risk of rejection may be identified by monitoring DSA levels both before and after transplantation [33]. Highly sensitive detection of DSAs is now possible with modern methods such as Luminex assays [33]. To fully understand the potential for acute rejection, it is essential to evaluate T cell-mediated reactions. Cytokine profiles and other biomarkers may provide insight into the recipient's immunological state and the likelihood of rejection [33].

A non-invasive method for monitoring graft viability and immune response is emerging: liquid biopsies, mainly through cell-free DNA (cfDNA) analysis. Elevated cfDNA levels, which are associated with rejection episodes, may indicate graft injury [1, 9]. This method allows real-time tracking of graft integrity and immunological activity. Gene expression profiling is a cutting-edge diagnostic tool that can predict the likelihood of rejection. A possible technique for customized immunosuppressive medication is the use of gene sets associated with acute rejection [33]. Emerging research suggests that acute cellular rejection (ACR) may be indicated by microRNAs and protein-based biomarkers in serum and bile [34]. Because these biomarkers provide accurate diagnostic information, invasive biopsies may become unnecessary in the future.

Essential aspects of pre-transplant monitoring for liver transplantation include immune response monitoring and early identification of graft malfunction. The capacity to forecast and oversee graft health may be improved by combining biochemical indicators with noninvasive imaging methods and cutting-edge molecular diagnostics such as cfDNA analysis and DSA monitoring. Through ongoing research, these methods are expected to improve the results in patients undergoing liver transplantation.

2.3 Role of Imaging and Biopsy

Conventional diagnostic technologies, including imaging (ultrasound, computed tomography (CT), magnetic resonance imaging (MRI)) and liver biopsy, are vital for liver transplantation to evaluate liver function, diagnose diseases, and track post-transplant results. However, certain constraints and obstacles may affect the success of these strategies.

Essential imaging techniques for liver evaluation include ultrasonography, computed tomography (CT), and magnetic resonance imaging (MRI), each with its inherent benefits and drawbacks. Liver structure and blood flow can be observed in real time using ultrasound, which is noninvasive, accessible, and extensively used. It relies on the operator and may not provide sufficient information regarding small lesions. Results may be affected by patient characteristics, such as obesity or intestinal gas, and ultrasonography cannot always distinguish between benign and malignant tumors [32, 35]. It is used to view intricate anatomical features, providing high-resolution images. Patients who require numerous follow-ups should be concerned about the fact that CT involves exposure to ionizing radiation, even though it has several benefits. It is also less helpful in measuring liver function and has the potential to overlook minor lesions [32, 35].

Unlike ionizing radiation, MRI produces high-contrast soft-tissue images. It can evaluate liver function using methods such as magnetic resonance elastography and provide high-resolution images of the liver lesions. However, MRI may be expensive, time-consuming, and not always

accessible. Patients with claustrophobia or certain types of implants may also find it less effective [32, 35].

Each imaging technique has its advantages and disadvantages. Ultrasound is non-intrusive; anyone can have one, although it may not be detailed. Despite the high-resolution images produced, CT scans expose patients to radiation. Although expensive and time-consuming, MRI provides excellent soft-tissue contrast without the use of radiation. The unique clinical setting and patient characteristics dictate the choice of an imaging modality.

Histological data on the liver's architecture, inflammation, and fibrosis provided by liver biopsy are the diagnostic gold standard for liver illnesses. However, several problems remain. Patients with coagulopathy or other comorbidities may find the risks of infection, discomfort, bleeding, and the intrusive nature of the surgery especially worrisome [32]. Another restriction is the possibility of sampling error, which may lead to misdiagnosis if the biopsy sample does not accurately reflect the liver. Not including focal lesions in the biopsy increases the risk of missing them [32]. Inconsistencies in diagnosis and inter-observer variability may also arise from pathologists' different approaches to histological evaluation [32]. Finally, post-transplant outcomes, such as EAD, may be difficult, if not impossible, to predict using conventional histological rating methods [32]. While liver biopsies are essential for understanding liver histology, they are not without drawbacks, including invasiveness, potential for sampling errors, inter-observer variability, and limited reliability in predicting post-transplant outcomes. Since these limitations are already present, it underscores the need for supplementary non-invasive diagnostic methods to improve the evaluation and tracking of liver disease.

The ability to integrate data is crucial in liver diagnostics because conventional methods often operate in silos, making it challenging to combine imaging, biopsy, and laboratory results into a cohesive picture of a patient's illness [32]. In addition, because invasive methods, such as biopsy, have limitations, there is a growing need for alternatives that do not involve invasive procedures. The need for noninvasive diagnostic tools has prompted the development of imaging and biomarkers that may provide the same or better results without the dangers of invasive procedures [32]. Furthermore, imaging technology, genetics, and molecular biology are experiencing rapid technical breakthroughs. These advancements may necessitate changes in clinical practice, allowing more accurate and thorough evaluations of liver health [32].

2.4 Emergence of Algorithmic Approaches

Artificial intelligence (AI) and machine learning (ML) have become game-changing technologies in liver transplantation. These technologies enhance clinical decision-making by delivering predictive analytics, boosting diagnostic accuracy, and enhancing patient care.

Clinical practitioners may miss trends in large datasets, including EHRs, imaging studies, and lab findings; however, AI and ML systems can sift through these data and identify them. Donor and recipient details, past results, and current clinical data can all be input into prediction models to determine the chance of graft survival [30]. To forecast EAD, machine learning models have been trained to include clinical variables such as recipient demographics, donor risk index, and MELD score [30].

Conditions such as acute rejection episodes and hepatocellular carcinoma (HCC) recurrence may affect transplant results, and AI systems can help in diagnosing these issues [36-38]. Radiological

images can be analyzed using AI and image recognition algorithms to identify malignancies and evaluate liver conditions before transplantation [30]. Deep learning algorithms may better understand liver biopsy images, which may detect rejection or fibrosis histological characteristics with greater precision than conventional approaches [30].

By customizing immunosuppressive treatment according to specific patient risk profiles, AI may aid in the development of customized medical techniques. For instance, by analyzing patient data, algorithms might propose appropriate medication regimens that lessen the likelihood of rejection and its side effects [30]. A new measure called the medication level variability index (MLVI) evaluates medication adherence and drug level fluctuations and may be used to forecast unfavorable outcomes for liver transplantation [30].

Liver transplant outcomes may be better understood using AI that integrates multi-omics (genomics, proteomics, and metabolomics) data with clinical information. Biomarkers indicating graft rejection or malfunction can be identified using this comprehensive method [30]. Artificial intelligence programs can decipher these complicated statistics, and studies have shown that ddcfDNA levels can be tracked after transplantation to predict severe rejection [30].

Although artificial intelligence and machine learning show considerable promise in liver transplantation, some obstacles remain. The efficacy of artificial intelligence models depends on the quality and consistency of the data used for training; hence, the quality and standardization of the data play vital roles in their performance, as inconsistent data and biased or erroneous predictions [30]. Clinical integration is another obstacle; when integrating AI technologies into clinical processes, user interfaces, educating clinicians, and the need to process data in real time must be carefully considered [30]. Concerns regarding algorithmic bias, informed consent, data privacy, and other ethical issues have also emerged [30]. Notwithstanding these obstacles, AI and ML have the potential to optimize patient care, improve diagnostic accuracy, and enhance predictive analytics, all of which will have a profound impact on clinical decision-making in liver transplantation. As these technologies continue to evolve, their integration into clinical practice will require ongoing research, collaboration among stakeholders, and a focus on ethical considerations to ensure that they benefit patients and health care providers [30].

3. RNA-Seq Biomarkers in Liver Transplantation

3.1 Basics of RNA-Seq Technology

The transcriptome, or the whole collection of RNA transcripts in a cell or organism at a given moment, can be studied using RNA-Seq, a potent technique for transcriptome analysis. By directly sequencing RNA molecules, RNA-Seq provides a more comprehensive and accurate picture of gene expression than previous approaches, such as microarrays. Novel transcripts, splice variants, and fusion genes can be detected in this way, even if microarrays often fail to do so [39].

This process usually includes multiple stages. It must first be extracted from a sample (such as blood or tissue) and then treated to eliminate impurities to purify RNA. The sample type dictates the technique of analysis; for example, tissue samples are subjected to Trizol [39], or blood samples are analyzed using PAXgene Blood RNA Kits [40]. Testing for RNA integrity (RIN) using a bioanalyzer is a critical quality control technique [39, 41]. Reverse transcriptase was used to transform the extracted RNA into cDNA to prepare a cDNA library for sequencing. A sequencing-appropriate library is generated by fragmenting the cDNA, adding adapters, and amplifying the fragments. Our

selection of library preparation kits includes the NEBNext® Multiplex Small RNA Library Prep Set for Illumina®, among others [42], TruSeq Stranded Total RNA with Ribo-Zero Globin [43], and NEBNext Ultra II Directional RNA Library Prep kit [41]. To sequence DNA, scientists use high-throughput sequencing systems, such as the Illumina HiSeq 2500/2000 [42], NovaSeq 6000 [44], Nextseq 500 [43], and DNBSEQ T1 [45], to sequence the cDNA library. This has led to the creation of a large dataset consisting of short-sequence reads.

In the final step of data processing, transcript abundance was determined by aligning sequence reads to a reference genome. To identify splice variants, differentially expressed genes, and other interesting aspects, this data was evaluated. The STAR software suite [41, 43, 44], RSEM [43], DESeq2 [41, 43], limma [44], and Seurat [45] are commonly used for such analyses.

Compared with traditional methods, such as microarrays, RNA-Seq has several benefits. It offers improved sensitivity and dynamic range for identifying transcripts with varying abundances, particularly those with low abundance that microarrays would miss. This is required to identify uncommon transcripts or transcripts that are expressed at low levels in specific cell types. RNA-Seq also has better precision and resolution than microarray probes, allowing for more accurate quantification of transcript abundance and the discovery of novel transcripts and splice variants. Contrary to microarrays, which depend on pre-designed probes targeting known genes, RNA-Seq does not require any previous knowledge of the transcriptome; hence, it permits the identification of novel transcripts and isoforms.

In contrast to microarrays, which usually only work with a selection of known genes, RNA-Seq allows for total transcriptome analysis, including coding and non-coding RNAs. Compared to relative measurements from microarrays, it provides a more precise and quantitative assessment of gene expression by digitally counting transcripts, thereby dramatically improving the quantification of gene expression. In conclusion, RNA-Seq delivers a more comprehensive picture of gene expression than microarrays because it is superior at detecting new transcripts and splice variants.

In the linked papers, RNA-Seq has been used in liver transplantation research, showing that it can detect rejection-related gene expression profiles [40, 43] and classify immune cell types that play a role in transplant results [45]. These findings highlight the value of RNA-Seq in elucidating the intricate molecular processes involved in LT.

These studies show how RNA sequencing has replaced more conventional approaches, such as microarrays, in liver transplantation studies and how it has improved the sensitivity, resolution, and capacity to identify new transcripts. This data thoroughly analyzes the contribution of RNA-Seq to the development of liver transplantation.

Although microarrays were formerly used in transplant genomics, RNA-Seq presents clear benefits for monitoring liver transplantation (Table 1). Multicenter validation studies have demonstrated that it has greater sensitivity and may identify transcripts associated with early rejection that microarrays have missed [40, 43]. To further improve biomarker findings for graft evaluation, it is possible to profile non-coding RNAs (e.g., miR-223 [42]) and splicing variants.

Table 1 Comparison of RNA-Seq vs. Microarray in Liver Transplant Biomarker Studies.

Feature	RNA-Seq	Microarray	References
Sensitivity	High — detects low-abundance transcripts	Moderate — limited by probe design	[40, 43, 44]
Specificity	High—base—level resolution for unique transcripts	Moderate — cross-hybridization may reduce accuracy	[40, 44]
Detection of Novel Transcripts	Yes — can identify novel genes, isoforms, and SNPs	No — limited to known probe sequences	[44, 45]
Dynamic Range	Wide — up to 5 logs of expression	Narrower — 2–3 logs	[40, 44]
Quantification Accuracy	Digital read counts provide accurate quantification	Analog signal intensity may vary	[41, 43, 44]
Application in Liver Transplant Studies	Identifies novel rejection/tolerance signatures (e.g., FOXP3/IFNG, 59-probe classifiers)	Limited to known markers (e.g., IFNG, IL2 expression)	[40, 41, 43]
Data Complexity	High — requires bioinformatics expertise	Moderate — established pipelines	[44, 45]
Cost	Higher	Lower (but narrowing)	[46]
Scalability for Multi-Omics	Excellent — integrates with proteomics/metabolomics	Limited integration capabilities	[7, 11]

3.2 Biomarker Discovery through RNA-Seq

The linked papers include several studies that used RNA-Seq to examine the gene expression patterns in individuals who underwent liver transplantation. Differentially expressed genes and pathways in patients with sustained graft function or operational tolerance compared to those with rejection are the targets of these investigations.

Several stages are often included in the use of RNA-Seq for research on liver transplantation. The initial step involved collecting samples from patients who underwent liver transplantation. It can be performed several times after transplantation or using PBMCs. Patients with stable graft function or operational tolerance and those with rejection were included in the sample pool [41, 42, 47]. Subsequently, the samples are prepared for RNA extraction and sequencing, which creates a massive database of gene expression levels for each sample [41, 42, 47]. The next step is bioinformatics analysis, which involves using tools to examine the RNA-Seq data. As part of this process, the authors used differential gene expression analysis to identify genes that are either highly upregulated or downregulated in rejection samples compared to control samples [44], examination of biological pathways to determine which ones are more abundant among genes with differential expression [44], and methods for constructing networks of genes that regulate gene expression, as well as for identifying regulatory genes and the relationships between them [43]. Finally, using the known genes and pathways, the next step is to identify biomarkers that may indicate tolerance or rejection. Research often seeks gene expression ratios (e.g., FGL2/IFNG) as potential biomarkers because of their use in early diagnosis, prognosis, and individualized treatment [41].

An efficient diagnostic approach is essential, such as the discovery of biomarkers for liver illnesses. This is especially true when deciding between invasive procedures, such as liver biopsy, and noninvasive options, such as PBMCs. Although they provide different insights into disease states, both methods have shown distinct usefulness in therapeutic practice.

MAFLD and HCC are two examples of liver illnesses for which liver biopsies are the most reliable diagnostic tools [48, 49]. Important histological features, such as fibrosis and steatosis, may be more easily identified using this invasive technique, which histologically visualizes and evaluates liver tissue [50]. Liver biopsies have their uses, but they also come with hazards such as discomfort, bleeding, and complications, especially in individuals with advanced liver disease [51]. In addition, there is a chance of sample mistakes; since a biopsy only takes a small slice of the liver, it is possible to miss localized lesions, leading to different opinions on the severity of the disease [52].

In contrast, PBMC profiling offers a non-invasive approach that is gaining popularity for detecting indicators of liver disease [53-55]. Research has shown that PBMCs may mimic the tumor microenvironment, enabling the detection of gene expression changes comparable to those observed in liver tissue [53, 55]. One example is the potential use of PBMC variations in lncRNAs as biomarkers for HCC diagnosis and prognosis. Transcriptomic profiles of tumor tissue samples and PBMCs show encouraging agreement [55]. This not only indicates that PBMC profiling is versatile but also has the potential for less intrusive diagnostics, such as the capacity to track reaction times to treatments.

There seems to be some agreement between the two methods concerning the transcriptome profiles obtained from PBMCs and liver tissue, according to the study. The consistent detection of specific inflammatory markers and gene changes in both types of colorectal cancer samples is

supported by comparable gene expression patterns [53]. While PBMCs may shed light on systemic reactions, direct tissue samples may be more specific for diagnosing liver disease in particular regions [56].

In patient scenarios where repeated biopsies are not feasible, the comparative benefits of using PBMCs become even more apparent. Noninvasive approaches are crucial for continuous evaluation without the hazards of biopsy, especially in patients with chronic illnesses [52, 57]. Integrating PBMC profiling into larger diagnostic paradigms for liver illnesses is relevant because the development of non-invasive biomarker testing, such as serum markers and imaging, has shown efficacy equivalent to that of established approaches [49, 58].

Liver biopsies are the gold standard for confirming liver disease, but there is a non-invasive option that may supplement tissue biopsy results: biomarkers extracted from PBMCs. The consistency between the two methods paves the way for more comprehensive diagnostic techniques that prioritize patients without sacrificing the precision required for liver disease diagnosis.

Further validation in larger cohorts is required, as studies often have disadvantages, including small sample sizes. Identifying relevant biomarkers is challenging due to factors such as the diversity of liver transplant recipients and the intricacy of their immune responses. Nevertheless, the data presented facilitate a broad understanding of the RNA-Seq approach to identifying genes and pathways involved in graft rejection and tolerance, with several investigations elucidating specific gene expression ratios as potential indicators. Access to data and analyses from different studies is necessary to obtain more precise information on particular genes and pathways.

Liver transplant patients with graft dysfunction (GD) can be effectively identified using RNA-Seq, which is very effective in detecting differential gene expression in GD. Liver biopsies were performed in transplant patients with GD (rejection, fibrosis) and control patients (those whose graft function was steady or who had operational tolerance). Gene expression patterns can shift over time, making the timing of biopsy collection important. Liver biopsies were processed by RNA-Seq sequencing after RNA extraction. The result was a massive database detailing the gene expression levels for each sample. Bioinformatics methods were used to compare gene expression levels between the control and GD groups. To identify genes that were substantially upregulated or downregulated in the GD group, statistical techniques such as DESeq2, edgeR, and limma were used. Standard methods for assessing the significance of differential expression include the p-value and fold-change. After identifying differentially expressed genes, the next step is to analyze pathways using tools such as GOseq, DAVID, or Reactome to determine which biological pathways are more abundant among these genes. This study sheds light on the molecular and biological pathways that contribute to graft failures. Possible biomarkers for diagnosis, prognosis, or therapy response monitoring may include genes and pathways strongly linked to GD. These must be validated in separate cohorts before being employed in therapeutic settings (Figure 1).

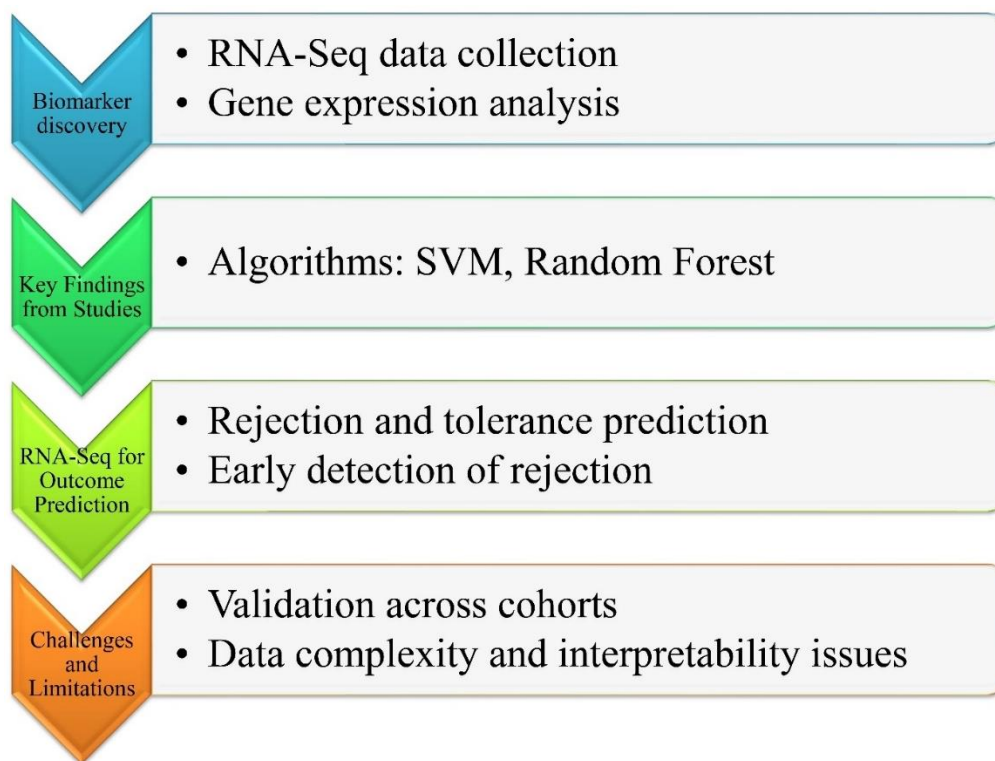


Figure 1 Overview of RNA-Seq applications for biomarker discovery, outcome prediction, and associated challenges.

3.3 Types of Biomarkers

There is encouraging evidence that circulating RNA molecules, such as microRNAs (miRNAs) and other non-coding RNAs, may be biomarkers for various disorders, including post-liver transplantation problems. They are appealing for non-invasive diagnostic and prognostic uses because they are present in readily available body fluids such as serum and plasma. Table 2 categorizes the different types of biomarkers used in the context of LT.

Table 2 Types of Biomarkers in Liver Transplantation.

Biomarker Type	Examples	Detection Methods	Clinical Applications	Advantages	Clinical Validation Status	References
Circulating miRNAs	miR-223, let-7e-5p	qPCR, RNA-Seq	Acute rejection detection	Non-invasive, stable in serum	Retrospective cohort	[42]
Gene Expression Ratios	FOXP3/IFNG	RNA-Seq, Nanostring	Operational tolerance prediction	High specificity to graft	Phase II trial (LITMUS)	[41]
Exosomal miRNAs	miR-21-5p, miR-4532	RNA-Seq (urine)	Rejection monitoring	Organ-specific cargo	Preclinical	[59]
Donor-derived cfRNA	Graft-specific mRNAs	ddPCR, RNA-Seq	Early graft injury	Real-time monitoring	Multicenter prospective	[9]
T-cell Transcripts	CD8A, IFNG	scRNA-Seq, bulk RNA-Seq	Rejection subtyping	Mechanistic insights	Retrospective	[45]

Some biological functions, such as those of the immune system and liver, are mediated by non-coding RNAs (ncRNAs) called miRNAs, which also regulate gene expression. Changes in their blood levels may indicate liver problems, such as rejection or fibrosis. Several studies have examined miRNAs as potential indicators of acute rejection (AR) in patients who have undergone liver transplantation. One study found that patients with AR had downregulated miR-199a-3p and increased miR-223 and let-7e-5p [42]. Different studies have investigated serum extracellular vesicles for a panel of miRNAs that may indicate AR [42]. In addition to miRNAs, many other non-coding RNAs, including circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs), are being studied as potential biomarkers.

RNA-Seq provides an alternative to invasive liver biopsies by allowing the collection of circulating RNAs from the blood. This method makes it easier to identify problems early on, as changes in circulating RNA levels may occur before the appearance of the clinical symptoms. Tracking circulating RNA levels over time may help in assessing the efficacy and potential effects of therapy. However, several obstacles and restrictions limit the utility of circulating RNA biomarkers in clinical practice. First, many studies are still in their nascent phases and require additional validation in large, independent cohorts to confirm their therapeutic value. Second, to ensure that results can be repeated and compared across studies, it is essential to standardize the sample collection, RNA extraction, and analyses. Third, there is a chance that specific circulating RNAs are not condition-specific, which might cause false-positive or negative results. Therefore, it is crucial to make these tests more sensitive and specific. Finally, correct interpretation of the data requires a thorough understanding of the molecular processes underlying the variations in circulating RNA levels.

The most reliable way to determine whether a transplanted liver is healthy and to diagnose rejection is by performing a biopsy. By sequencing the RNA in these biopsies, tissue-specific RNA biomarkers may be identified, allowing for a more complex understanding of graft health.

Gene Expression Profiling involves comparing the gene expression patterns of biopsies taken from patients suffering graft malfunction (such as rejection or fibrosis) with those taken from patients who have stable graft function. Once accomplished, differentially expressed genes can be identified as potential biomarkers. This method has been used in several studies, leading to the discovery of several genes and pathways linked to fibrosis and rejection. One study, for instance, used RNA-Seq to build a gene expression signature that predicted liver transplant rejection. This signature identifies a 59-probe classifier that differentiates acute rejection from other causes of graft malfunction [40]. Another study focused on the ratio of the FOXP3 gene to the IFNG gene, using liver biopsies as a possible biomarker of operational tolerance [41]. Activation of T cell receptors and interferon signaling are two examples of immune-related pathways highlighted by a pan-organ transcriptome atlas linked to allograft fibrosis [44].

After genes with differential expression have been found, the next step is to perform pathway analysis to identify the enriched biological pathways. This finding sheds light on the molecular mechanisms underlying transplant failure. Therefore, KEGG and Reactome databases are often used in studies [44]. This cutting-edge method allows for a more in-depth examination of graft cellular heterogeneity by analyzing gene expression at the single-cell level. The function of CD8⁺ tissue-resident memory T lymphocytes in liver transplant rejection has been investigated using single-cell RNA sequencing [45].

Graft biopsies provide precise tissue-specific RNA biomarkers that reduce the likelihood of false-positive results in the transplanted liver. In contrast to circulating biomarkers, which may represent

systemic alterations rather than graft-specific events, this method directly evaluates graft health. RNA-Seq stands out for its complete perspective of gene expression and the molecular information it provides, which might uncover minor alterations that other approaches might miss. Liver biopsies are associated with several risks, including infection, discomfort, and bleeding. Because the biopsy only takes a small slice out of the liver, it cannot be a good indicator of the graft's health, which adds another layer of difficulty regarding the sampling bias. Furthermore, RNA-Seq requires specific equipment and knowledge, which are both complicated and costly. Standardizing biopsy techniques, RNA extraction, and analysis methodologies is essential to guarantee the consistency and reliability of investigations.

Exosomes, which are vesicles released by nano-sized cells, carry various chemicals, including RNA (mRNA, miRNA, and lncRNA). It is becoming increasingly apparent that exosomal RNAs may serve as biomarkers for multiple disorders, including post-transplant problems. They are appealing for non-invasive diagnostic and prognostic uses because they are present in readily available body fluids (plasma, serum, and urine).

Immune responses and liver function are only two physiological processes affected by miRNAs, which are small non-coding RNAs that influence gene expression. Changes in exosome levels may indicate the existence of problems such as fibrosis, rejection, or changes in liver health. One research looks at miRNAs expressed variably in serum extracellular vesicles as possible indicators of acute rejection after liver transplantation (miR-223, let-7e-5p, miR-486-3p, miR-199a-3p, miR-148a-3p, miR-152-3p) [42]. A three-miRNA signature (hsa-miR-21-5p, hsa-miR-31-5p, and hsa-miR-4532) was highlighted in another study that investigated the use of urine exosomal miRNAs for the diagnosis of acute rejection in kidney transplantation patients [59]. This study focused on kidney transplantation; however, the same concepts may be applied to liver transplantation as well.

Since exosomes may be extracted from readily available body fluids, exosomal RNA biomarkers have several benefits, one of which is that they do not require invasive liver biopsy. Exosomes are more stable than free RNA in bodily fluids because their lipid bilayer prevents the destruction of RNA payloads. It is possible to detect problems early on when changes in exosomal RNA levels occur before the appearance of the clinical symptoms. It is also possible to evaluate the efficacy of therapy and anticipate the results by tracking the changes in exosomal RNA levels over time. Nevertheless, one must consider the obstacles and constraints of such a system. Further testing in large, separate cohorts is required to verify the practicality of exosomal RNA biomarkers for clinical use. Methodologies covering the standardization of sample collection, exosome isolation, RNA extraction, and analysis are essential to guarantee reproducibility and comparability of investigations. Some exosomal RNAs may have insufficient specificity and sensitivity, resulting in incorrect positive or negative results. Therefore, trustworthy interpretation requires a thorough understanding of the molecular processes driving variations in exosomal RNA levels and the use of exosomal RNA, especially miRNAs, as biomarkers, as shown in several studies. As prospective indicators of acute rejection after liver transplantation, Wang et al. focused on miRNAs found in serum extracellular vesicles [42]. Seo et al. discovered a way to detect acute rejection in kidney transplant patients by analyzing urine exosomal miRNAs, and the same concept may be used in liver transplantation [59]. As potential indicators of liver transplant success, Vidal-Correoso et al. investigated cell-specific extracellular vesicles and the miRNAs they contained, which were released into the organ preservation solution after cold ischemia storage [60]. The feasibility of using exosomes extracted from preservation solutions as biomarkers was emphasized in this study.

3.4 Immune Cell Profiling and Single-Cell Technologies in Biomarker Discovery

The importance of immune cell profiling and single-cell technologies in the search for transplant rejection biomarkers lies in the need to investigate the immunological status, which includes different types of immune cells and molecular markers in great detail. The use of coding and non-coding RNAs as potential biomarkers has expanded due to recent developments in RNA-seq. However, when discussing transplant outcomes, especially in the case of liver transplantation, the focus is frequently on defining immunological components rather than ncRNAs. To map the immunological landscape after liver transplantation, recent studies have highlighted the efficacy of scRNA-seq and multicolor flow cytometry (FACS). These tools enable the study of complex immunological responses to allograft implantation. For example, Peereboom et al. drew attention to the fact that single-cell technologies may pinpoint specific alloreactive T- or B-cell clones, which reveals important information about the pathways that play a role in transplant tolerance and rejection [61]. Barbetta et al. demonstrated that these technologies offer a fresh perspective on complex immunological interactions, which is crucial for determining the operational tolerance of transplant recipients and tailoring their immunosuppressive treatments accordingly [62].

Researchers can measure various immune populations that may directly impact transplant outcomes by characterizing immune cell subsets using methods such as multi-color FACS. In particular, Kildey et al. demonstrated complex immunological interactions inside the allograft by investigating the functions of different subsets of T cells and natural killer (NK) cells in the rejection of kidney transplants [63]. Acute rejection episodes may be correlated with changes in regulatory T cells and other immune cell types, highlighting the prognostic usefulness of these profiles in transplantation [64]. Nguyen et al., who previously highlighted the growing importance of RNA-seq in identifying circular RNAs, now support these methods and call for a more comprehensive application that integrates transcriptome data with immunological profiles [65]. Crucially, phenotyping of immune cells extends beyond detecting rejection to understanding the mechanisms of operational tolerance in transplant recipients. It is necessary to employ a more comprehensive approach that encompasses various types of immunological mediators, beyond T and B lymphocytes, as Hjortdal et al. suggested that circulating myeloid cells may play a crucial role in transplant inflammation [66]. Based on the results of recent liver transplant studies, we can gain a deeper understanding of immune activity patterns and their relationship with transplantation outcomes by combining cell type analyses with modern single-cell techniques [67]. Improving biomarker identification techniques and enabling prompt treatments to avoid transplant rejection are the next steps that may be taken by capitalizing on these advanced immunological insights. Zhang et al. used targeted investigations to identify serum CXCL8 as a non-invasive indicator of subclinical rejection, showcasing the promise of combining immune cell profiling with clinical biomarkers [68]. Various investigations of immune population gene expression patterns have shown encouraging findings for potential predictive biomarkers in liver and kidney transplantation [69].

3.5 Advantages of RNA-Seq in Early Rejection Detection

Compared to more conventional omics methods, such as classical proteomics, RNA-Seq has been shown to be an effective technique for the early diagnosis of liver rejection. A key benefit of RNA-Seq is that it can detect differentially expressed genes (DEGs) that can be used as sensitive biomarkers for acute rejection events, by providing a complete picture of the transcriptome. In

contrast to microarray methods, RNA-Seq can identify low-abundance gene expression, opening the door to the discovery of new biomarkers [70, 71]. In the context of liver transplantation, where small changes in gene expression may signal impending rejection, the ability to identify weakly expressed genes is particularly relevant [72]. Another benefit of RNA-Seq over proteomic analysis is its higher resolution. There is a clear correlation between mRNA levels and protein production; however, proteomics is limited by problems with post-translational modifications [73]. The transcriptional alterations that occur in response to graft rejection may be better understood using RNA-Seq, which directly quantifies mRNA levels. Research has shown that RNA-Seq is a reliable tool for detecting significant increases in genes associated with immunological responses, which have been linked to acute rejection in patients undergoing liver transplantation [74]. One of the most critical aspects of RNA-Seq is the sequencing depth that can be achieved. By detecting more genes at higher sequencing depths, we can provide a complete picture of the transcriptome landscape during the early stages of liver rejection [75]. Compared to conventional proteomic methods, this quality allows for the discovery of complex gene networks and pathways active during acute rejection, which in turn helps doctors evaluate graft viability and make more accurate predictions of outcomes [76].

In addition, scRNA-seq has improved our ability to study the cellular heterogeneity of transplanted liver tissues. To identify certain cell types linked with rejection, scRNA-seq may shed light on the distinct roles of different immune cell populations in the transplant environment [77, 78]. Studies on macrophage populations in liver transplant recipients have revealed unique transcriptional patterns, providing insights into immune function after transplantation and paving the way for more precise treatment approaches [79].

Its promise in diagnosing liver rejection is further highlighted by the combination of RNA-Seq and machine-learning methods. The use of machine learning algorithms to sift through RNA-Seq's massive datasets has allowed the creation of prediction models that improve graft rejection prognosis [74]. These developments point to the future of RNA-Seq as a foundational tool in precision transplant medicine, especially with the ever-increasing amount of biological data available. When monitoring and treating liver transplantation patients, RNA-Seq is more capable than proteomics in identifying disease markers and achieving superior clinical results. It is a favored approach over traditional proteome analysis because of its high sensitivity, thorough transcriptome profiling, and ability to identify cellular dynamics during early liver rejection.

3.6 Clinical Applications

Early rejection diagnosis is essential for improving liver transplantation outcomes. Liver biopsies, the gold standard in traditional procedures, are invasive and do not always accurately reflect the actual state of the grafts. Therefore, the development of noninvasive methods for early rejection identification is of great interest.

Circulating miRNAs and other ncRNAs are being studied as possible noninvasive indicators of AR. Researchers often compare miRNA expression patterns in patients with consistent graft function to those in patients with AR in these investigations. Potential biomarkers can be identified by identifying differentially expressed miRNAs.

A plasmatic score for reliable prediction and diagnosis of liver allograft rejection was identified in one investigation utilizing an miRNA signature (miR-155-5p, miR-181a-5p, and miR-122-5p) and

CXCL-10 [47]. Several studies have investigated miRNAs in serum extracellular vesicles as potential indicators of acute rejection in individuals who have undergone liver transplantation [42], revealing the differential expression of several miRNAs in patients with acute rejection. The third study aimed to identify potential indicators associated with rejection in liver transplant patients with hepatocellular carcinoma by analyzing changes in miRNA gene expression patterns [39].

Gene expression profiles linked to AR can be determined by sequencing the RNA of PBMCs. These signatures can be utilized to develop diagnostic algorithms for the early identification of rejection in kidney transplant recipients.

One study developed a blood-based biologically relevant biomarker (a 59-probe classifier) that can predict AR before AR-associated graft injury. [40]. A high negative predictive value indicated that the classifier effectively excluded AR.

To uncover gene expression signatures or miRNA profiles linked to AR, RNA-seq data from blood or liver biopsies have been used in many studies. According to biomarker profiles, patients may be divided into groups with varying levels of risk. Therefore, immunosuppressive methods may be fine-tuned for individuals at high risk of adverse reactions, which may lower the incidence of AR and the risk of adverse effects in patients at low risk.

Patients may be categorized into high- and low-risk categories based on the results of a study that created a gene expression profile using blood to predict AR [40]. Using an miRNA signature and CXCL-10, another study identified a plasma score that accurately predicted and diagnosed liver transplant rejection, enabling risk stratification [47]. Research on miRNAs in serum extracellular vesicles has found that some miRNAs are expressed differently in patients with AR, which might lead to stratification [59].

RNA-Seq biomarkers have been the subject of some research to determine whether patients have reached operational tolerance (OT), a point at which they may safely stop using immunosuppressants. Improving patients' quality of life and decreasing long-term adverse effects is possible by identifying those with biomarker profiles that suggest OT and considering discontinuation of immunosuppressive therapy.

According to research, the FGL2/IFNG ratio in PBMCs is used as a gene expression biomarker to identify operationally tolerant patients after LT [41]. We may use this biomarker to divide patients into two groups: those who will likely attain OT and those who are not.

RNA-seq biomarkers may also be used to predict the likelihood of other types of graft failure, such as fibrosis. Patients may be stratified according to their risk of fibrosis, which could lead to early intervention measures that stop or slow the progression of the disease.

Immunosuppressive regimes may be tailored to each patient's requirements, mitigating rejection and minimizing side effects through stratification. Healthcare professionals can maximize the use of their resources by prioritizing the treatment of patients who are most in need and excluding those who pose little danger. Preventive measures made possible by the early detection of high-risk patients can enhance patient outcomes and graft survival. Medical expenditures may be significantly reduced in the long run if biomarker-based stratification successfully lowers the rate of rejection and other complications. Nevertheless, it is vital to consider the constraints and difficulties. Further validation in large, separate cohorts is required to verify the clinical value of these stratification techniques. Standardizing procedures for sample collection, RNA extraction, and result analysis is essential to guarantee the consistency and reliability of the results. Additionally, RNA-Seq and other cutting-edge methods can be expensive and require specialized tools and expertise.

Potent algorithms and user-friendly tools are also necessary to integrate biomarker-based stratification into clinical practice (Figure 2).

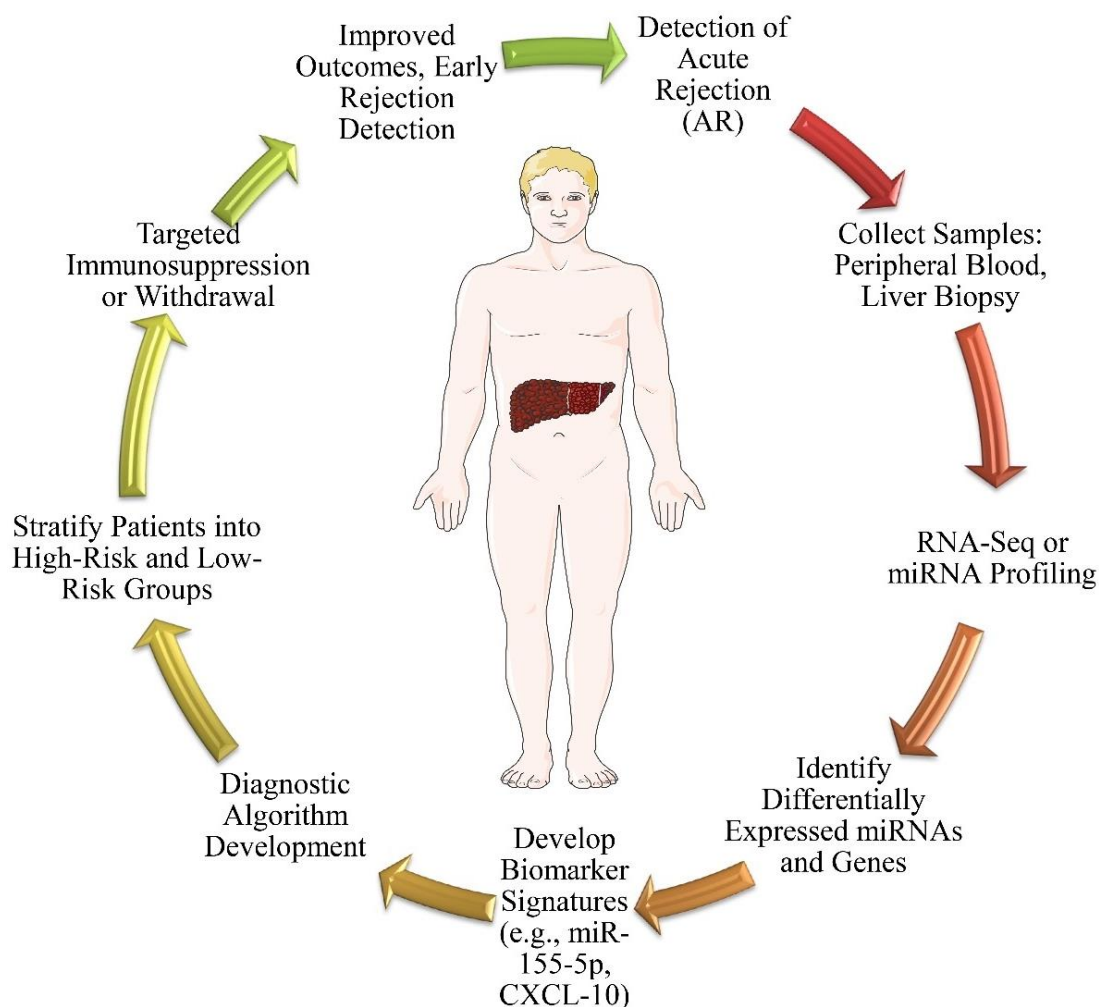


Figure 2 Overview of the RNA-Seq-based biomarker pipeline for improving outcomes in liver transplantation.

4. Integration of Diagnostic Algorithms and RNA-Seq Biomarkers

4.1 The Need for Integration

While histology and clinical data are already part of liver transplant diagnostic procedures, they are not always precise or predictive enough to guarantee the best possible results. However, there are some significant drawbacks to algorithms that rely solely on clinical data and conventional histopathology.

LFTs, such as ALT and AST, are associated with rejection; however, they cannot distinguish rejection from other diseases, including steatohepatitis [80]. The existing gold standard for healthcare, histological evaluation, is vulnerable to inter-observer heterogeneity because it relies on expert pattern identification and the identification of comorbidities [80]. The low-to-moderate reported kappa values for TCMR-related diseases underscore the need for enhanced diagnostic accuracy. Another contentious issue is the incidence and method of diagnosing antibody-mediated rejection (ABMR) after LT [80].

Liver biopsies, the conventional gold standard for fibrosis assessment, are intrusive and unpopular among patients [81]. This may cause essential treatments to be postponed and reduce the frequency of assessments. Although there are variations in transcription between liver allografts that reject and those that do not, our knowledge of the specificity and accuracy of molecular biomarkers in various biological specimens remains limited [82]. Algorithms require molecular data to understand the intricate relationship between damage and rejection. For instance, employing HLA crossmatches or similar markers to screen for ACR in juvenile liver transplantation is impossible, emphasizing the need for alternative diagnostic methods [43].

Study limitations are common because many different disorders can cause liver failure and require LT [43]. Constructing cohorts of a single liver illness large enough to establish strong predictions is challenging because these diseases may have different impacts on the peripheral blood transcriptome [43].

Using models such as Classification and Regression Trees or binary mixed model trees, algorithms may make accurate predictions about the onset and progression of acute liver failure [83]. However, they are not always good at tailoring treatments to individual patients' needs or anticipating long-term effects. A vital area of study that must be addressed is the identification of repeatable, non-invasive biomarkers of graft rejection [82].

Providing tailored forecasts and treatment plans is challenging for algorithms that lack the molecular data. Identifying patients prone to rejection and customizing immunosuppressive regimes according to individual molecular profiles is essential for improving outcomes [43].

A vital area of study that must be addressed is the identification of repeatable, non-invasive biomarkers of graft rejection [82]. Researchers have found that various cohorts and platforms identify biomarkers differently. Some studies have linked specific gene expression profiles to liver transplantation rejection [82]. However, it remains unclear whether these results can be reliably reproduced in other studies. Problems with validation, inadequate sample sizes, or a lack of standardization in methods and procedures may contribute to contradictory findings [84]. The predictive power of biomarkers may not withstand independent prospective trials, even when they show promise in retrospective studies [84]. An example is the re-analysis of 20 biomarkers for liver transplantation tolerance that had already been published; a few of these demonstrated robust predictive ability in a separate study [84].

In retrospective studies, the identification of tolerance biomarkers may be complicated by the presence or absence of immunosuppression [84]. The ability of blood gene expression to identify rejection may be influenced by other variables, such as persistent HCV infection [82]. An in-depth analysis of how immunosuppressive medications affect gene expression is required [82].

Combining gene expression data with other clinical measures, such as sequential liver function tests, is necessary to demonstrate the clinical usefulness of transcriptional biomarkers in more extensive clinical studies [82]. An ongoing multinational clinical study is investigating the therapeutic value of biomarker-guided immunosuppression in LT [84].

Individual indicators lack specificity because many genes linked to rejection are also implicated in other liver diseases. For example, while LFTs, such as ALT and AST, are related to rejection, they cannot differentiate rejection from other disorders, such as steatohepatitis [82]. It is challenging to identify exact indicators because of the similarity in gene expression between livers that have been approved and those that have been rejected for donation [11].

In particular, in individuals with confounding variables such as persistent HCV infection, specific biomarkers may not be sensitive enough to identify rejection [82]. Because existing indicators are inadequate for screening ACR in pediatric liver transplantation, there is a clear need for additional sensitive and specific biomarkers [82].

An accurate diagnosis may require more than the use of an individual biomarker. The predictive power of rejection may be enhanced by multivariate logistic regression analysis incorporating numerous gene expression datasets [82]. However, identifying the best mix of biomarkers and proving their effectiveness remains challenging, especially when using multiple biomarkers. Biomarker validation and identification may be inconsistent because of a lack of uniformity in the methodologies used for RNA extraction, sequencing, and data analysis. The use of alternative platforms and normalization techniques, such as microarrays *versus* RNA-Seq, may also impact the findings. Expertise in bioinformatics is required to decipher complex RNA-Seq data. Extracting appropriate routes and functional networks from vast datasets is challenging.

Finally, specificity, sensitivity, and validation are significant obstacles to the independent use of RNA-seq biomarkers, despite their potential. Reliable and accurate prediction of liver transplant outcomes requires the integration of several biomarkers into complex diagnostic algorithms that incorporate clinical data and histological information. The following sections discuss how these difficulties can be addressed through such integration.

4.2 Examples of Integrated Approaches: Algorithmic Models Incorporating RNA-Seq Data

Improving liver transplant outcomes may be possible by combining RNA-seq data with sophisticated diagnostic algorithms. This integration has been the subject of several studies, all of which have shown promise in improving diagnostic accuracy and predictive capacity in ways that conventional approaches cannot.

Rejection and tolerance are two liver transplant outcomes linked to gene expression profiles identified using RNA-seq data. Next, classification algorithms are fed these signatures as features to determine the probabilities of the various outcomes.

Studies such as Bonaccorsi-Riani et al. [82] and Madill-Thomsen et al. [80, 85] have discovered gene expression patterns in liver transplant biopsies that are associated with ACR. Although RNA-Seq is a sister technique to microarray data, the underlying idea of finding differentially expressed genes and using them to construct a classifier was the same in both studies. The need for larger, more thoroughly vetted datasets to enhance the sensitivity and specificity of these models is emphasized as a limitation of these investigations. Validating biomarkers across separate cohorts is challenging, as shown by Pérez-Sanz et al., which highlights the need for strong validation methods [84].

Identifying gene expression signatures linked to transplant tolerance has also been a subject of research in this field. After reviewing the literature on liver transplant tolerance biomarkers, Pérez-Sanz et al. discovered that only a small subset of these indicators had robust predictive power in a separate study [84]. This highlights the need for thorough validation procedures before clinical use and the challenges in biomarker validation.

Gene regulatory networks constructed from RNA-seq data can be included in algorithmic models for outcome prediction. This method provides a comprehensive understanding of biological processes by considering the interplay between pathways and genes.

To determine which expression modules contribute to rejection after pediatric liver transplantation, Ningappa et al. used a network-based strategy [43]. To develop gene regulatory networks and identify modules associated with rejection, they utilized RNA-seq data and combined gene-centric and network-centric approaches. Support vector machines (SVMs) were used for classification. LASSO was utilized for feature selection in the research. Specifically, the findings provide insights into the potential of network-based techniques to enhance the precision of rejection predictions.

The most effective methods combine RNA-seq data with other data, including imaging, histological, and clinical information. By combining multiple types of data, this multimodal approach can enhance the predictive power of models.

Analytical and predictive algorithmic models utilizing RNA-seq data have been employed in several investigations, including those referenced in the linked papers. Here are some concrete instances and the constraints they impose:

Several research projects have used random forests, an ensemble learning technique. The study used a random forest method to select the features that were most predictive of tolerance after liver donation [84]. The authors praised random forests for their ability to handle high dimensionality, withstand noise, avoid overfitting, and ease of application [84]. Another study employed logistic regression analysis, which included a decision tree component similar to that found in random forests [81]. Compared to regression models, random forests outperformed them in predicting the likelihood of HCC [83].

Along with Extreme Gradient Boosting (XGBoost) and random forest, SVM was included as one of the three techniques utilized in feature gene extraction in a study [86]. To identify the genes involved in the intersection feature, which are necessary for risk modeling, the outcomes of the three algorithms were integrated.

Another approach used for feature gene selection is XGBoost [86]. In addition, the study concluded that XGBoost was the best model to construct the SurvMLSHAP model following feature selection because of its performance metrics in the test set, including accuracy, precision, recall, and F1 score [86]. The model predictions and feature relevance were interpreted using SHAP (SHapley Additive exPlanations) values [86].

Various machine learning models have been used in previous studies. To forecast allograft rejection, another study used a transfer learning-optimized for prediction (TOP) framework to construct models for all organs simultaneously and for individual organs [44]. Vidal-Correso et al. used a logistic method to generate a prediction model [60]. The other study used DESeq2 for differential gene expression analysis and the umap package for dimension reduction before k-means clustering [11].

Overfitting becomes an even more critical restriction when working with RNA-seq data. A model is said to be overfitted when it memorizes all features and noise in its training data to the point that it fails to generalize successfully to novel, unseen data. Due to the high expense and technical difficulty of producing RNA-Seq data, many biological research studies use small datasets, which makes this issue even more troublesome. Although not all research's sample sizes are specified in the linked papers, overfitting is a possible issue, particularly in studies with small samples. To account for this, Srivastava used a power analysis to determine the statistical confidence of the study based on the sample size [11]. Overfitting is another issue with medical AI that Su highlighted in his assessment; he emphasized the need for strong validation using separate datasets [83].

Techniques such as cross-validation, discussed in this review, are crucial for mitigating overfitting. Additional measures to avoid overfitting include incorporating regularization methods into models. Several computational models were used to analyze RNA-Seq data in relation to liver illness and transplantation. Overfitting, which may occur with small datasets, is a shortcoming of these models that must be thoroughly examined and corrected using validation methodologies and suitable statistical approaches.

To determine which rejected donor livers could be good candidates for transplantation, Srivastava et al. used transcriptomics, histopathology (including AI-based image analysis), and clinical data [11]. The study shows how combined data modalities might enhance liver transplant decision-making, but does not reveal the precise algorithm for this.

Biomarker identification and classification using integrated techniques have great potential; however, many obstacles remain. Massive, well-annotated datasets are required to train and verify these complex models, making data availability and standardization crucial. The key to guaranteeing repeatability is standardization of RNA-Seq procedures and data analysis pipelines. Notably, many of the sophisticated machine learning algorithms used in this study are opaque or "black boxes," concealing the process by which they make their predictions. More interpretable models must be developed to facilitate their use in clinical settings. Before widespread implementation, these models must undergo rigorous clinical validation to prove their clinical value and safety in patients. Evaluated studies should provide detailed information on the algorithms utilized, as well as tables and figures summarizing the various models' performance measures (such as AUC, accuracy, sensitivity, and specificity) to address these obstacles. Furthermore, it is essential to critically evaluate the limitations of this study and the difficulties in using its results in clinical practice.

Although RNA-Seq is an excellent tool for studying gene expression, combining transcriptomic data with data from other omics layers, such as proteomics and metabolomics, is necessary to obtain a complete picture of the mechanisms underlying liver transplantation.

The combination of transcriptomics, proteomics, and metabolomics can improve the predictive capabilities of diagnostic systems. Gene expression (transcriptomics) shows how much protein can be produced, whereas proteomics counts the amount of protein directly and shows how well the cells function. Metabolomics provides insights into the biochemical effects of gene expression and protein function within cells. By combining these different types of data, we can obtain a more comprehensive understanding of the biological processes that lead to liver donor rejection, tolerance, and other complications. For example, a combination study could reveal that a specific pattern of gene expression is associated with a particular biochemical profile that can predict refusal.

Novel biomarkers undetectable by transcriptomics alone may be more easily discovered using multi-omics approaches. Clinical outcomes may correlate better with proteins and metabolites than with mRNA transcripts. Researchers have identified biomarkers that increase the sensitivity and specificity of transplant outcome prediction by combining data from several omics platforms.

By combining data from multiple omics studies, we can gain a deeper understanding of the molecular factors that influence the response to LT. Ultimately, this may pave the way for the development of more precise treatment approaches for this disease. A good example is the development of medications that target specific metabolic pathways dysregulated in rejecting livers, aiming to prevent or cure rejection.

The limitations of single-omics methods include transcriptomics. Protein and mRNA levels are not always proportional, and post-translational modifications significantly affect protein activity. In

addition to revealing changes in gene expression and protein levels, metabolomics may uncover alterations that are missed by other metrics. A more comprehensive and accurate view of the biological system can be achieved by integrating multi-omics data, which can help overcome these limitations.

Despite these potential advantages, there are significant barriers to integrating multi-omics data. Due to the high dimensionality and complex data of multi-omics datasets, advanced computational approaches are necessary for their analysis. Data normalization, standardization, and alignment must be carefully considered when integrating data from several omics platforms. A carefully designed experiment is essential to ensure that the data are relevant and comparable across studies. However, multi-omics research requires specialized equipment and significant time and resource investments. A thorough understanding of the appropriate biological systems is also necessary to understand the findings of multi-omics investigations.

Future studies should aim to create reliable computational tools for analyzing and integrating data from several omics studies. Large datasets with numerous annotations are required to train and test these algorithms. To guarantee repeatability, it is essential to establish standardized data-gathering and processing processes. Researchers may improve therapeutic outcomes and deepen our knowledge of complicated biological systems by overcoming these obstacles and realizing the full potential of multi-omics data.

The sensitivity and specificity of traditional diagnostic procedures, such as LFTs and histology, are limited. LFTs may indicate liver damage, but cannot differentiate between rejection and other diseases, such as steatohepatitis [80]. Despite its status as the gold standard, histological evaluation has limitations, including the potential for subjectivity and the risk of overlooking subtle rejection indicators (especially in the presence of comorbidities) [44]. RNA-Seq can detect rejection, indicating slight variations that are ignored by conventional approaches, as it provides a holistic picture of gene expression. Machine learning techniques applied to RNA-Seq data may further improve sensitivity and specificity by revealing intricate gene patterns and interactions that would otherwise go unnoticed when using traditional methods, such as histological slide examination or fundamental LFT analysis. One example is the improvement in rejection prediction accuracy that may be achieved by integrating many gene expression indicators rather than relying on individual biomarkers [84].

Preventing irreparable transplant damage requires an early diagnosis of rejection. Current approaches often depend on finding rejection after it has occurred, when significant harm has already been done. In contrast, RNA-Seq may identify pre-clinical or pre-histopathological molecular alterations that suggest rejection. Severe rejection may be averted with prompt care upon early identification, and long-term results can be improved. In pediatric liver transplantation, when the present indicators are inadequate for screening severe cellular rejection, the capacity to identify rejection sooner is of utmost importance [43].

Distinguishing rejection from other diseases that can produce comparable clinical and histological symptoms is a significant obstacle in liver transplantation. By revealing distinct gene expression profiles linked to each disorder, RNA-seq may aid in their differentiation. In situations where rejection is not the leading cause of liver failure, this enhanced distinction allows for a more precise diagnosis and focused therapy, sparing patients the need for immunosuppression. For example, Madill-Thomsen et al. emphasized how RNA-Seq may differentiate liver transplant biopsies from other illnesses, including damage, steatohepatitis, and rejection [80, 85].

One way to improve diagnostic precision is by using RNA-seq data. Determining a patient's unique gene expression profile allows doctors to personalize the diagnosis and therapy for the patient. The efficacy of treatment and the incidence of side effects may both be enhanced by this individualized strategy.

The most reliable way to determine rejection is liver biopsy; however, this invasive process is not without hazards and limitations. RNA-Seq, which uses easily accessible samples such as peripheral blood, is a less intrusive option for monitoring rejection. The need for frequent biopsies may be reduced by integrating RNA-Seq data with algorithms. This will minimize patient pain and danger. Table 3 provides an overview of the various algorithmic models that incorporate RNA-Seq data for the analysis and prediction of liver transplant outcomes.

Table 3 Algorithmic Models Incorporating RNA-Seq Data.

Category	Key Findings	Examples & Studies	Algorithm/Method Used	Performance Metrics	Challenges	Potential Solutions	Clinical Application
Algorithmic Models Incorporating RNA-Seq Data	RNA-Seq enhances diagnostic accuracy for liver transplant outcomes.	[80, 82, 85]	SVM, Random Forest	AUC = 0.85, Accuracy = 82%	Need for large, well-validated datasets.	Standardized RNA-Seq protocols and validation strategies.	Early rejection detection and risk stratification.
Gene Regulatory Networks	Network-based approaches improve rejection prediction.	[43]	LASSO, SVM	Sensitivity = 78%, Specificity = 80%	Complexity in modeling gene interactions.	Advanced computational tools for network analysis.	Identifying critical pathways for targeted therapy.
Multi-Omics Integration	Combining transcriptomics, proteomics, and metabolomics enhances predictive power. RNA-Seq detects subtle rejection-related changes earlier than LFTs or histology.	[11]	Deep Learning, Multi-Modal AI	AUC = 0.90	High costs and data complexity.	Improved data normalization and integration methods.	Predicting transplant tolerance and complications.
RNA-Seq vs. Traditional Methods	RNA-Seq detects subtle rejection-related changes earlier than LFTs or histology.	[80, 85]	Logistic Regression, Decision Trees	Accuracy = 85%	Interpreting RNA-Seq data is complex.	Machine learning models for better pattern recognition.	Personalized treatment adjustment.
Non-Invasive Monitoring	RNA-Seq from blood samples	Studies highlight potential for	Ensemble Learning	Sensitivity = 90%	There is a need for further	Prospective clinical trials for validation.	Routine monitoring without

reduces biopsy
dependence.

non-invasive
rejection
monitoring
[41, 42]

validation
before
clinical use.

invasive
biopsies.

4.2.1 Clinical Validation Challenges and Pathways to Translation

The development of RNA-seq and AI models in the last few years has opened up exciting new possibilities for the discovery of biomarkers that can improve clinical outcomes in liver transplantation. Nevertheless, despite their potential, there is a severe lack of prospective studies validating RNA-seq and AI models for improving liver transplant outcomes. There are substantial obstacles to implementing these promising biomarker findings in practical clinical procedures because of this gap.

Although a growing body of research is using RNA-seq to define liver diseases, most of it is either retrospective or exploratory, according to the evidence [68, 72, 87]. The difficulties of effectively implementing direct-acting antivirals, as pointed out by Huang et al., require meticulous scheduling considerations in relation to transplant operations; however, the authors did not provide confirmation of prospective results [88]. Furthermore, findings from retrospective analyses, rather than rigorous prospective trials, primarily support our knowledge of biomarkers in addressing problems such as ACR [76, 82]. To improve transplant success prediction and complication management, existing prospective studies have often failed to thoroughly investigate RNA-seq and AI integration [82, 89].

A strong foundation is also necessary to enable future assessments of RNA-seq and AI models, as our understanding of immunological tolerance in liver transplantation has undergone significant changes. Vionnet and Sánchez-Fueyo emphasized the significance of identifying biomarkers that might lead to tailored immunosuppressive treatments. However, well-designed studies will help to bring these biomarkers into clinical practice [90]. Existing studies highlight the gap between the capabilities of current technologies and their practical use, emphasizing the critical need to create a framework that integrates RNA-seq techniques into clinical trials.

Multiple critical components should be incorporated into a framework for the clinical translation of RNA-seq biomarkers and AI algorithms in liver transplantation to address these challenges. First, the definition of clinically relevant RNA-seq biomarkers must be standardized to ensure their reliability and consistency across all research locations. Finding specific genetic markers in different cohorts may improve assessment consistency, as shown in the studies by Keyvani et al. and Macparland et al. [78, 91]. It is essential to actively register prospective clinical studies with efforts such as those of ClinicalTrials. The government will support and register them. For example, the current research cited by Bohne et al. is an excellent example of how to study immunosuppressive methods and biomarkers [92]. It would be possible to test the RNA-seq results using a comparable approach in future studies.

The empirical foundation for treatment options may be enhanced by using AI to identify the links between reported RNA-seq biomarkers and clinical results. By identifying essential gene markers related to T-cell-mediated rejection, Shao et al. demonstrated the potential of machine learning. [74]. The success of RNA-seq studies depends on careful planning and execution, which may be achieved via multidisciplinary teamwork, including genomicists, clinical researchers, and transplant surgeons [88, 90]. For RNA-seq validation to be widely used in clinical settings, a system must be established to fund novel research and provide instructional frameworks for physicians on how to understand and use these large datasets. In summary, RNA-seq has excellent potential for improving the usefulness of biomarkers in liver transplantation, but there are not enough high-quality prospective studies to support these findings. Improved clinical outcomes for liver transplant

patients may be achieved by addressing this constraint using a systematic and comprehensive framework. This will enable the translation of results from RNA-seq analytics and AI applications.

4.3 Benefits of Integration: Personalized Patient Management and Targeted Therapies

RNA-seq data can reveal gene expression patterns associated with rejection, tolerance, and other related issues. With more precise risk stratification, doctors may be able to identify high-risk patients and personalize their treatment. Individuals with high-risk gene expression profiles may require additional immunosuppression or closer monitoring. Bonaccorsi-Riani et al. found that gene expression analysis may predict immunosuppression withdrawal rejection, enabling tailored treatment [82]. Similarly, Ningappa et al. demonstrated that network-based methods can enhance rejection prediction accuracy in pediatric liver transplantation, resulting in more individualized risk assessment and treatment [43].

Over- or under-immunosuppression may occur in some individuals due to the standardization of current immunosuppression protocols. RNA-seq data analysis may reveal unique responses to various drugs, allowing the selection of the best immunosuppressant regimen for each patient. With this individualized strategy, immunosuppressive side effects and the likelihood of rejection may be reduced.

Successful liver transplantation results are highly dependent on the delicate balance between immunosuppression and immunological rejection of the transplanted organ. To optimize treatment regimens and improve patient prognosis, understanding the immunological processes underlying the two main types of transplant rejection, ACR and chronic rejection, is crucial. To reduce the likelihood of acute rejection and the side effects of long-term immunosuppression, precise regulation of immunosuppressive treatment is a crucial component of this management.

Advanced immunosuppressive regimens have reduced the incidence of ACR in liver transplantation, although ACR still complicates approximately 25% of procedures [93]. Mycophenolate mofetil (MMF) and calcineurin inhibitors (CNIs), such as tacrolimus and cyclosporine, are the main immunosuppressants used in these patients. Although these substances successfully lower the rate of rejection, they also increase the risk of opportunistic infections and cancer [94, 95]. Improving patient tolerance and reducing toxicity are two areas where the possible advantages of moving from CNIs to mTOR inhibitors have been investigated [96]. The goal of this change in immunosuppressive treatment is to improve long-term results by creating an environment that is more conducive to graft acceptance, in keeping with the ideas of "lighter immunosuppression" [97].

Immunosuppression withdrawal (ISW) and other methods for reducing immunosuppression are currently the subject of much research, particularly in children [98]. To avoid acute rejection episodes when immunosuppression is removed, this technique requires careful monitoring for de novo DSA [98]. The findings of these studies highlight the need for individualized changes based on immunological profiles, as some patients may tolerate lower levels of immunosuppression [99]. There is hope for new immunotherapeutic approaches that target inducing operational tolerance, which is defined as the maintenance of transplant function without immunosuppression, as some patients may be able to reach this condition [100].

Patients are more likely to develop infections, including tuberculosis and invasive fungal infections, in the early post-transplant period due to severe immunosuppression [101]. Essential aspects of transplant care include infection prevention measures such as prophylactic antimicrobials

and close monitoring. Comprehensive studies have shown that infection risk assessment is a predictive tool for improving immunosuppressive treatment strategies [95].

A constant issue is striking a compromise between avoiding acute rejection and reducing long-term adverse effects. Contrary to individuals undergoing thoracic organ transplantation, studies suggest that lowering overall immunosuppressive doses is associated with a decreased frequency of problems, such as cancer, in liver transplant recipients [102, 103]. One issue that is still being closely studied is how to create individualized treatment plans that consider each person's response and any possible hazards associated with the treatment.

In the early stages, RNA-Seq may identify minor molecular alterations that indicate rejection or other problems before they become clinically apparent. This paves the way for prompt action, which may halt the disease course and mitigate its effects. Given that the present indicators are inadequate for screening acute cellular rejection, the capacity to identify rejection early is of paramount importance in pediatric liver transplantation [43].

By analyzing RNA-Seq data, we can identify the exact molecular targets and pathways contributing to rejection and other complications. This knowledge may be used to create tailored medicines that target fundamental illness processes to improve therapeutic success and decrease adverse effects. Medications that target specific metabolic pathways could be developed if the dysregulated pathways in rejecting livers could be pinpointed.

Patients' reactions to therapy can be tracked using RNA-Seq. Changes in gene expression patterns may determine whether a treatment is effective or whether changes are required to improve efficacy. This paves the way for maximizing results via dynamic modification of treatment procedures in response to individual reactions.

5. Current Challenges and Limitations

5.1 Technical Challenges

Although high-throughput RNA-seq technologies are excellent for identifying biomarkers, many technical problems are associated with their use in clinical settings. Here are some of the difficulties encountered.

The high expense of RNA-seq, which includes all aspects of the process from sample preparation to data processing, prevents its widespread application in clinical practice. This is even more important in situations where resources are scarce. Massively parallel sequencing has been used to identify SNPs that are specific to donors and recipients [46]. Some ddPCR methods require expensive fluorescent probes, further increasing the cost [46].

Regarding population-based research or regular clinical monitoring, a considerable number of samples are too large for current RNA-seq procedures to manage. As mentioned in the scRNA-seq workflow description, the processing time and complexity of the approaches [45] emphasize this restriction. Many studies have limited sample sizes, making it difficult to generalize their results [104].

One of the biggest obstacles is determining how to incorporate RNA-seq data processing into pre-existing EHRs and LISs. Integrating and interpreting RNA-seq findings in a clinical environment is difficult because of the absence of defined data formats and processing workflows in clinical laboratories. A further hurdle to this integration is the need for specific bioinformatics skills.

Important data required for prompt clinical decision-making may not be available for extended periods because of the time required for sample processing, sequencing, and data analysis. Sequencing-based methods are characterized by “an unacceptable turnaround time” [46].

5.2 Data Limitations

Research on biomarkers of liver transplant rejection is often plagued by studies with insufficient sample sizes, which severely restricts the reliability and applicability of the results. According to one study, the small sample size hints at a small proportion of event incidence that may be underpowered to resolve the primary study outcome with clarity [104]. The results obtained concerning biomarker connections with rejection have direct implications for reliability. The limitations in generalizability and the possibility of bias stem from small samples that do not adequately reflect the range of individuals who received liver transplants. In addition, the authors of another study were honest about the drawbacks of their unicenter investigation, including its small sample size and brief follow-up time [104]. It is difficult to conduct thorough subgroup studies with small samples to identify possible variations in biomarker expression across different patient subgroups (e.g., depending on age, sex, and underlying liver disease). When studies have small sample sizes, the reported relationships between biomarkers and transplant outcomes are more susceptible to bias and confounding factors.

5.3 Regulatory and Clinical Translation Hurdles

Obtaining FDA clearance for novel biomarkers is a lengthy and complex procedure that requires thorough safety and effectiveness demonstration studies and clinical validation trials. One study addressing the absence of regulatory clearance for specific medicines in liver transplant patients with HCC [36] depicts this obstacle. To verify the practical use of RNA-seq biomarkers for predicting and controlling liver transplant rejection, large-scale and well-planned clinical studies are required. Thorough preparation and considerable financial investment are needed for these studies. Some ethical considerations when using AI-driven diagnostic tools that rely on RNA-seq data include data privacy, algorithmic bias, and the possibility of misinterpretation of the findings. It is essential to guarantee patient consent and ensure data security. Patients' right to privacy in RNA-seq biomarker development and validation is of paramount importance. Adherence to applicable data privacy standards (such as HIPAA) is crucial. Algorithms trained on RNA-seq data may be biased in a manner typical of these datasets. Equal access to and benefits from AI-driven diagnostics can only be achieved by addressing these biases.

Concerns regarding algorithmic bias in RNA-seq data have been raised in relation to the diagnostic algorithms used to improve liver transplant success rates. Misleading predictions and inaccurate diagnostic power may result from training datasets that do not adequately reflect the characteristics of ethnic minorities. These issues may be exacerbated by several computational and experimental biases that occur during RNA-Seq processing.

The GC content of genes is a major contributor to RNA-seq data biases. Sequencing machines have a bias towards reading genes that are abundant in guanine (G) and cytosine (C) bases, resulting in unequal read distributions across all genes [105]. Furthermore, the precision of differential expression analysis is affected by the inherent biases introduced by sequencing depth and gene length. For example, larger genes tend to collect more reads than shorter genes [106, 107]. Data

processing controls are essential for differential expression analysis because of their sensitivity to these biases [108]. Discordant measurements of gene expression levels among studies may also result from experimental design biases, such as adaptor ligation and PCR amplification [108, 109].

Another potential source of bias in RNA-seq data processing is the algorithm used for analysis. Various bioinformatics methods may provide diverse findings when analyzing circular RNA, highlighting the need for algorithmic uniformity to reduce variability [110]. By offering diagnostics on library bias, effective normalization approaches, such as those in the LiBiNorm package, aim to compensate for biases across datasets [111]. Achieving the universal applicability of biomarkers discovered for liver transplant outcomes requires these methodological advances.

These biases affect the diagnostic algorithm's prediction accuracy and gene expression levels. Models may struggle to generalize well to underrepresented groups if the training datasets consist of data from only one ethnicity or demographic group. Undermining the overall purpose of individualized care, this may result in ethnic minorities receiving inadequate treatment or incorrect diagnoses [112].

Additionally, the need for more inclusive databases that accurately reflect population genetic and demographic variations was emphasized. Identifying biases throughout the experimental and analytical stages of RNA-Seq may improve biomarker robustness and reliability in clinical applications. Finally, enhancing liver transplantation biomarker diagnostics and prognoses requires addressing algorithmic bias in RNA-Seq data. Diversity in training datasets and enhanced computational methods are needed to verify algorithms across various populations to improve health outcomes.

5.4 Unrealized Potential of Multi-Omics Integration

In the context of enhancing diagnostic algorithms and outcomes for illnesses such as liver disorders, there is enormous, yet largely untapped, potential for synergy between RNA-Seq and other omics technologies, including proteomics and metabolomics. Integrating data from many omics platforms may help us better understand biological systems and disease development by illuminating intricate relationships that would otherwise go unnoticed.

A significant step forward in multi-omics analysis is the creation of frameworks that can integrate different types of omics data. To illustrate the underlying biological variances, researchers may use tools such as the Multi-Omics Factor Analysis (MOFA+) framework to examine many omics profiles simultaneously, which helps them uncover hidden variables [113]. By enhancing the readability of the combined information, this approach facilitates the identification of relevant insights into the genetic basis of disorders by correlating gene expression levels with changes in protein and metabolite levels.

In addition, Gao et al. showed that cellular diversity and spatial organization can be learned by combining single-cell RNA-seq with spatial transcriptomic data [114]. Because of the key role that spatial organization plays in the development of liver diseases, this lends credence to the idea that combining RNA-Seq with proteomics and metabolomics may shed light on the complicated relationships between tissues.

It has also been demonstrated that integrative techniques significantly enhance predictive capabilities. For example, colon cancer prognosis prediction was shown to be more accurate when studies included gene expression, microRNA expression, and DNA methylation data, rather than

relying on individual assessments [115]. These results imply that clinical decision-making for treatment plans is informed by integrated analytics, which also improves our understanding of cancer biology.

Pharmacogenomics, the study of how genetic diversity impacts drug reactions, may be better understood from a therapeutic standpoint using integrated multi-omics data. Individual differences in medication metabolism and effectiveness may be better understood by combining pharmacogenomic and pharmacometabolomic data in clinical trials, as shown in a case study [116]. According to these findings, combining RNA-seq biomarkers with proteomic and metabolic metrics may help develop personalized treatment plans for patients undergoing liver transplantation.

Emerging technologies, such as graph convolutional networks, can seamlessly combine different forms of omics data, which may lead to improved biomarker detection and classification. This highlights the potential for integrating multiple omics [117]. The regulatory mechanisms that control these interactions may be better understood using this method, which also helps in understanding the complex nature of these illnesses.

However, due to the challenges of data integration and interpretation, there has been limited translation of these integrative methodologies into clinical practice, despite notable breakthroughs. Data normalization, missing values, and high dimensionality problems are common in multi-omics studies. Methods such as graph-linked embedding, which integrate data from several omics studies, show how these problems can be efficiently addressed [118]. To fully utilize these thorough studies, collaboration across disciplines is essential, as highlighted by the creation of integrative frameworks.

Finally, better computational techniques, more precise clinical applications, and improved analytical frameworks may help achieve the promise of multi-omics integration, especially between RNA-seq and proteomics/metabolomics analyses. We anticipate that new diagnostic algorithms and treatment approaches will emerge as a result of the synergistic insights gained from integrated omics data, which will significantly improve patient outcomes in liver transplantation and other areas of study.

6. Future Directions

6.1 Advances in RNA-Seq Technologies

The cellular heterogeneity of the liver is obscured by the fact that current RNA-seq technologies examine large tissue samples. To gain a deeper insight into the molecular pathways contributing to liver transplant failure, single-cell RNA-seq allows for gene expression profiling at the single-cell level. Novel biomarkers for specific cell types involved in rejection or other consequences may be identified using this approach. One possible avenue for improved immunosuppression is the identification of particular subsets of immune cells (such as T cells) that play a role in rejection [119, 120].

When using short-read RNA-Seq, crucial details, such as splice variants and other isoform detections, may go unnoticed. Long-read sequencing tools can circumvent this limitation by providing a more comprehensive view of the transcriptome and identifying new biomarkers for allograft failure. As a bonus, long-read sequencing might enhance mutation discovery, which could be pivotal in elucidating the etiology of specific problems such as recurrent primary liver disease [121].

6.2 Development of Hybrid Tools

Biomarker identification and analysis can be made significantly more efficient and accurate by applying AI and ML algorithms to RNA-seq data. Artificial intelligence can identify intricate linkages and patterns in high-dimensional RNA-sequencing data that conventional statistical approaches may overlook. This can potentially improve models that anticipate allograft rejection, other problems, and the likelihood of long-term graft survival in the long run. Numerous studies have demonstrated that AI can accurately predict liver transplant outcomes using various datasets, including imaging and electronic health records (EHRs) [83].

The potential for continuous evaluation of allograft health may be realized by integrating RNA-Seq biomarkers with real-time monitoring techniques, including the use of wearable biosensors. If possible, patients may benefit from earlier intervention in cases of rejection or other problems. Although this is a more far-fetched strategy, post-transplant treatment might be transformed by combining continuous monitoring with RNA-Seq data processing.

6.3 Focus on Predictive Models

Existing methods for assessing liver transplant success consider only the immediate results. Nevertheless, the success of transplantation depends on the graft's ability to survive long term. Improving long-term outcome prediction may be as simple as building a model that uses RNA-seq biomarkers and other clinical data. In the long run, optimal graft survival and patient well-being may be achieved through individualized treatment plans. Studies have shown that AI and ML can predict long-term results in various transplant contexts [120-122].

6.4 Collaborative Efforts

RNA-Seq analysis is more effective than WES in studies with larger sample sizes. The development of reliable prediction models and the identification of strong biomarkers will depend on the completion of massive RNA-Seq datasets collected from several centers. Several transplant facilities must collaborate to establish uniform procedures for collecting and analyzing data.

A diverse team is required to integrate diagnostic algorithms with RNA-sequencing biomarkers successfully. To ensure that the created tools are relevant to clinical practice and based on solid science, researchers, physicians, and bioinformatics specialists must collaborate. This collaborative approach is essential for the application of research results in clinical practice. Close cooperation between these groups is necessary to use AI in healthcare [119] effectively.

7. Conclusions

By enhancing the precision of early diagnosis and patient care, the integration of RNA-Seq biomarkers with current diagnostic algorithms may completely transform the field of liver transplantation. Technical obstacles, such as high cost and data complexity, limit the clinical applicability of RNA-Seq, despite it being a more accurate and non-invasive alternative to conventional procedures. Integrating RNA-seq with proteomics, metabolomics, and machine learning models is the way forward for liver transplant diagnosis in the future. To ensure that these cutting-edge technologies are beneficial in the clinic, they must be thoroughly validated, and researchers from various institutions must collaborate. The use of RNA-Seq in diagnostics has the

potential to significantly improve patient outcomes and decrease liver transplant risks with ongoing technological improvements and collaborative efforts.

Author Contributions

EA gathered and analyzed the studies. BS participated in the design of the study and drafted the manuscript. EA and BS conceived of the study and participated in its design and coordination and data analysis. All authors have read and approved the final manuscript.

Competing Interests

The authors declare that no conflicts of interest exist.

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