Assessing Donor Liver Quality and Restoring Graft Function in the Era of Extended Criteria Donors

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Abstract

Liver transplantation (LT) is the final treatment option for patients with end-stage liver disease. The increasing donor shortage results in the wide usage of grafts from extended criteria donors across the world. Using such grafts is associated with the elevated incidences of post-transplant complications including initial nonfunction and ischemic biliary tract diseases, which significantly reduce recipient survival. Although several clinical factors have been demonstrated to impact donor liver quality, accurate, comprehensive, and effective assessment systems to guide decision-making for organ usage, restoration or discard are lacking. In addition, the development of biochemical technologies and bioinformatic analysis in recent years helps us better understand graft injury during the perioperative period and find potential ways to restore graft function. Moreover, such advances reveal the molecular profiles of grafts or perfusate that are susceptible to poor graft function and provide insight into finding novel biomarkers for graft quality assessment. Focusing on donors and grafts, we updated potential biomarkers in donor blood, liver tissue, or perfusates that predict graft quality following LT, and summarized strategies for restoring graft function in the era of extended criteria donors. In this review, we also discuss the advantages and drawbacks of these potential biomarkers and offer suggestions for future research.

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Introduction

Liver transplantation (LT) is a life-saving treatment option for patients with end-stage liver disease. In recent decades, good short- and long-term outcomes after LT have been achieved because of improvements in surgical technologies and organ preservation.1 Graft quality is believed to play a dominant role in early graft function and thereby dramatically influences graft survival and mortality after LT.2–4 Over the last decade, the disparity between the need for LT and the organ shortage is widening, which leads to the expanded usage of grafts from the extended criteria donors (ECDs).1 Traditionally, ECDs are donors with underlying medical diseases such as diabetes, or hypertension, advanced age, high-degree liver steatosis, prolonged ischemia time, pathogenic infection, prolonged intensive care unit stay, hypernatremia, and donation after circulatory death (DCD).5–7 ECD graft quality is routinely considered inferior because of their increased rate of post-transplant complications, such as primary graft nonfunction (PNF),2 early allograft dysfunction (EAD),4 and ischemic-type biliary lesions (ITBLs).8,9

PNF is early graft loss after LT and requires emergency regrafting, which occurs following 2–10% of LTs.10–12 ECDs include DCD donors13 and those with severe steatosis,14 prolonged ischemia time,15–17 and high donor bilirubin level18 sharply increase the risk of PNF, thereby reducing patient and graft survival. Unlike PNF, EAD represents marginal, usually reversible, graft function during the first postoperative week, and results in a higher morbidity and mortality.1 Compared with 1–10% seen in donation after brain death (DBD) LT, the incidence of biliary complications after DCD LT is approximately 10–30%,19–22 in which the time from asystole to cross-clamp is considered as a ma-
Moreover, advanced donor age, prolonged ischemia time, microvascular thrombosis, bile salt toxicity and immune injury may be the underlying mechanisms of the development of biliary complications. Therefore, ECDs should be well defined and precisely allocated to appropriate recipients. More importantly, in the era of ECD, effective systems need to be established to assess donor liver quality and guide the decision for organ usage or discard. Based on clinical risk parameters (Fig. 1), models like donor risk index, Eurotransplant donor risk index, and discard risk index were constructed to evaluate the risk of graft failure or discard, serving as useful tools to make decisions for organ allocation. However, those scoring models mainly focus on donor characteristics and cannot assess the degree of liver injury. Furthermore, combining clinical parameters with advanced molecular profiles, imaging, or histopathology may contribute to the development of better systems. In recent years, with the rapid development of multi-omics, single cell technology, and bioinformatic analysis, significant achievements have been made in revealing the molecular profiles that are closely related to poor graft outcomes, and which can provide novel biomarkers for evaluation of graft viability.

Herein, we provide a review of potentially useful biomarkers in donor blood, liver tissue, and graft perfusate, which have been associated with impaired graft quality or predictive for the occurrence of EAD, PNF, and biliary complications after LT. In this review, we mainly focus on studies using human liver grafts. Given that the available biomarkers were insufficient in the field of LT, we also include experimental studies that have been performed in animal models. Furthermore, we summarize potential therapies for graft repairment during LT. Finally, we describe the pros and cons of the potential biomarkers, accompanied with suggestions for future graft assessment and restoration.

Potential biomarkers in donor blood

Donor serum alanine transferase (ALT), aspartate transferase (AST), total bilirubin, gamma glutamyl transpeptidase, and sodium concentration may reveal the underlying liver dysfunction and ischemic injury prior to graft procurement. Over the past decades, numerous studies have demonstrated that such laboratory disorders in donor blood are independent risk factors for early graft dysfunction following LT. In recent years, novel biomarkers in donor blood have been found to useful for predicting graft outcomes. By analyzing data from over 10,000 nondiabetic donors, Ezekian et al. showed that elevated donor serum hemoglobin A1c (HbA1c) >6.5% was associated with increased rate of PNF and decreased graft and patient survival. HbA1c is known to be a useful biomarker representing the average plasma glucose concentration within the last 3 months, serving as an early warning of diabetes. The
liver undergoes glycogen deposition and hepatic steatosis resulting from diabetes. Therefore, it is worth noting that HBa1c may be a valuable marker for further stratifying marginal graft quality. In a large prospective study of 815 participants, Piemonti et al. identified increased serum donor interleukin 6 (IL6) and C-X-C motif chemokine ligand 10 (CXCL10) concentration as good DNA (GcfDNA), which is function, graft failure and inferior graft survival after DBD LT. IL6 is responsible for transforming naive B cells into mature plasma cells, as well as activating the production of IL17 to inhibit regulatory T lymphocyte (Treg) function. Altering LT histopathology is a useful strategy for macrophages, natural killer (NK) cells and dendritic cells (DCs), thereby shaping immune immunity. More interestingly, Polara et al. found that elevated circulating mitochondria-derived damage-associated molecular patterns (mtDAMPs) in donor plasma were associated with severe inflammation response and the development of EAD following DBD LT in a group of 55 recipients. The major source of mtDAMPs may be the mitochondria released from graft tissue or cell death during organ procurement, suggesting that mtDAMPs might quantitatively assess graft injury.

Potential biomarkers in donor grafts

The liver, a multifunctional organ in the body, is mainly engaged in metabolism, synthesis, storage, detoxification, and complex immune activities. After implantation, the donor graft becomes the new center of the recipient to perform those functions. Therefore, the graft features could significantly regulate hepatic homeostasis and influence outcomes after LT (Table 1). Donor grafts could be gained for histological assessment and quantification of liver injury during LT. Histopathology is the gold standard for the diagnosis of steatosis, fibrosis, necrosis, inflammation, and cellular infiltration in liver grafts. In our center, pretransplant, and post-reperfusion liver biopsies are routinely performed, offering valuable clues for graft quality assessment (Supplementary Table 2), including biliary epithelium, mura lumen, peribiliary vascular plexus, thrombosis, intramural bleeding, peribiliary gland, and inflammation, to quantify biliary duct injury.

Genetic variants

With the advent of genome-wide association studies and pretransplant genetic analysis, a series of genes and variants have been found to be susceptible to graft injury. Heme oxygenase-1 (HO-1), a regulator of immune response, is considered to be cytoprotective gene of ischemia-reperfusion injury (IRI) during LT and is modulated by a single-nucleotide polymorphism A (~1413) T. Buix et al. reported that, compared with recipients of a liver with an A-allele genotype (n=245), recipients of livers with an HO-1 TT-genotype (n=61) had dramatically elevated serum hepatic transaminases after LT and a higher incidence of PN. HLA-C, which is the major inhibitory ligand for immunoglobulin-like receptors, inhibits the cytotoxic activity of NK cells, and therefore reduced liver inflammatory damage. In a large LT cohort of 459 patients, Hanvesaku et al. found that donor grafts with at least one HLA-C2 allele were associated with less incidence of graft dysfunction and rejection. After LT, graft-derived cell-free DNA (GcfDNA), which is continuously released into recipient circulation because of cellular turnover, is a promising noninvasive biomarker to assess graft quality. Previous studies have showed that the elevated GcfDNA was a signal of early graft injury after LT, particularly acute cellular rejection. For example, a prospective study conducted by Schutz et al. demonstrated that GcfDNA increased by more than 50% 1 day following LT, probably because of the IRI. However, GcfDNA rapidly decreased to a median of <10% within 7–10 days without the recipient experiencing early graft injury over a 1 year observation period. This suggested that GcfDNA may be a precise and superior biomarker to predict early graft dysfunction compared with conventional liver function tests.

RNAs

Protein-coding associated RNAs, for example messenger RNA (mRNA) and noncoding RNAs including microRNAs (miRNAs), circular RNAs (circRNAs) and long noncoding RNAs (lncRNAs) are believed to be reliable markers to evaluate graft injury because of their organ specificity. Nrf2 transcription factor, which is activated by reactive oxygen species, is known to protect against liver IRI via activating phase II antioxidants. Zaman et al. demonstrated that grafts (n=6) with increased Nrf2 mRNA expression before IRI were associated with lower liver injury. Interestingly, donors with low Nrf2 mRNA levels (n=8) were significantly older than those with high levels, suggesting that older grafts experienced severe IRI and inferior graft quality. Additionally, Resch et al. reported that high gene expression of the major histocompatibility complex class I related chain A (MICA) mRNA in zero hour biopsies (n=88) was associated with mild graft injury and prolonged graft survival. During LT, MICA had an important role in linking the innate and adaptive immune responses via interacting with NK cells, mucosal-associated invariant T, CD8+T cells, miR-22, a regulator of a series of pathways such as cell cycle, metabolism and kinase signaling, is relevant to cell survival, glucose metabolism, and protein translation. Khorsandi et al. reported that low expression of graft miR-22 was associated with the incidence of PN after DCD LT (n=21). Another study of 42 human LTs showed that high expression of donor graft miR-146b-5p was associated with the development of EAD. Downregulation of miR-146b increased the production of tumor necrosis factor receptor-associated factor 6, which activated the nuclear factor-kappa B (NF-κB) pathway, and in turn enhanced T-cell function. In our previous study, we found that elevated donor graft miR-103 and miR-181 were significantly associated with the development of new-onset diabetes mellitus (NODM) in recipients following LT (n=30). NODM not only increased the risk of biliary stricture and cholangitis but also resulted in poor graft survival, serving as an indicator of poor graft quality as well. The two miRNAs targeted several genes related to glucose homeostasis and insulin signal transduction, which may have been the underlying mechanism.

In a cohort of 115 human LTs, Wang et al. reported that low levels of donor graft circFOXN2 and circNEXTIN3 that regulated miR-135b-5p and miR-149-5p and had roles in hepatic IRI were associated with the incidence of EAD. In a mice model of IRI (Qu et al. identified 13 differentially expressed circRNAs (e.g., Chr1:83031528|83031748, Chr10:89473752|89483524) in postperfusion livers that were involved in more severe IRI in steatotic livers. In a rat LT model, Chen et al. demonstrated that IncRNA LOC103692832 in rat grafts was related to early graft injury following LT that was mediated by the expression of apoptosis-related genes like HMox1 and ATF3. Nevertheless, the mechanisms of these potentially involved circRNAs and IncRNAs are still unclear, and further prospective or multi-
## Table 1. Biomarkers from donor livers potentially useful for the prediction of graft outcomes following transplantation

| Biomarker | Study | Sample | Model(s) | Group | Key point |
|-----------|-------|--------|----------|-------|-----------|
| Genetic variant | Heme oxygenase-1 A/T-allele genotype | Buis et al. (2008)\(^{35}\) | Pretransplant biopsies | Human LT A-allele genotype (n=245) vs. TT-allele genotype (n=61) | Graft with TT-allele genotype had elevated serum transaminases after LT and a higher incidence of PNF |
| HLA-C2 allele | Hanvesakul et al. (2008)\(^{36}\) | Pretransplant biopsies | Human LT | 459 livers biopsies | Donor grafts with HLA-C2 allele were associated with less incidence of graft dysfunction |
| GcfDNA | Levitsky et al. (2021)\(^{37}\) | Recipient blood | Human LT | Normal function (n=94) vs. Acute dysfunction (n=68) | Elevated GcfDNA represented early graft injury after LT |
| GcfDNA | Schutz et al. (2017)\(^{38}\) | Recipient blood | Human LT | / | Elevated GcfDNA could predict early graft injury |
| RNA Nrf2 mRNA | Zaman et al. (2007)\(^{39}\) | Pretransplant biopsies | Human LT | 14 donor liver biopsies | Higher Nrf2 mRNA expression before IRI were associated with lower liver injury |
| MICA mRNA | Resch et al. (2021)\(^{40}\) | Pretransplant biopsies | Human LT | 88 liver biopsies | High expression of MICA mRNA could reduce graft injury |
| MiR-22 | Khorsandi et al. (2015)\(^{41}\) | Post-reperfusion biopsies | Human DCD LT | PNF (n=7) vs. non-PNF (n=7) | Graft miR-22 was associated with PNF |
| MiR-146b-5p | Li et al. (2017)\(^{42}\) | 1.5 hours after LT | Human LT | EAD (n=22) vs. non-EAD (n=20) | Graft miR-146b-5p was associated with EAD |
| MiR-103 and miR-181 | Ling et al. (2017)\(^{43}\) | Pretransplant biopsies | Human LT | NODM (n=15) vs. non-NODM (n=15) | Graft miR-103 and miR-181 were significantly associated with the development of NODM |
| CircFOXN2 and circNEXTIN3 | Wang et al. (2021)\(^{44}\) | Pretransplant biopsies | Human LT | EAD (n=29) vs. non-EAD (n=86) | Two circRNAs were associated with EAD |
| LncRNA LOC103692832 | Chen et al. (2019)\(^{45}\) | 12 hours after LT | Rat DBD LT model | / | Graft IncRNA LOC103692832 was related to early graft injury |
| Protein | Sirtuin 1 | Nakamura et al. (2017)\(^{46}\) | 2 hours after LT | Human LT | 51 liver biopsies | High graft Sirtuin 1 was associated with superior liver function |
| Heme oxygenase-1 | Nakamura et al. (2018)\(^{47}\) | 2 hours after LT | Human LT | 51 liver biopsies | Enhanced Sirtuin 1 expression and protected against IRI |
| YAP | Liu et al. (2019)\(^{48}\) | 3 hours after LT | Human LT | 60 liver biopsies | Improved early liver function |
| FGF15 | Gulfo et al. (2020)\(^{49}\) | Post-reperfusion biopsies | Rat DBD LT model | / | Low graft FGF15 was associated with more severe hepatic damage and inhibited regeneration |
| CEACAM1 | Nakamura et al. (2020)\(^{50}\) | Pretransplant biopsies | Human LT | 60 liver biopsies | Hepatic CEACAM1 could prevent early graft injury |
| Hepatic occult collagen deposition | Hirao et al. (2021)\(^{51}\) | Pretransplant biopsies | Human LT | Low level (n=140) vs. High level (n=54) | Increased risks of severe IRI and EAD |

(continued)
Liver IRI. A previous study showed that high Sirtuin1 expression has an important role in autophagy induction involved in inflammatory responses, cellular aging, and stress resistance. Sirtuin1, a histone/protein deacetylase that regulates oxidative stress, 

Proteins

Sirtuin1, a histone/protein deacetylase that regulates inflammatory responses, cellular aging, and stress resistance, has an important role in autophagy induction involved in liver IRI. A previous study showed that high Sirtuin1 expression in grafts post-reperfusion sharply inhibited proinflammatory cytokine levels accompanied by superior liver function and improved patient survival. HO-1 is a rate-limiting enzyme that converts heme to biliverdin, free iron, carbon monoxide, and has anti-inflammatory and anti-oxidative activity. In addition, Nakamura et al. showed that high HO-1 levels in post-reperfusion liver biopsies were associated with good liver function, dramatically enhanced Sirtuin1/LC3B expression, and protected against hepatic IRI by inducing autophagy. Notch1, a highly conserved transmembrane receptor, has been shown to reduce cellular apoptosis or necrosis and inflammatory response. Kageyama et al. demonstrated high Notch1 expression in grafts was correlated with low serum ALT levels, consistent with alleviated liver damage. In addition, Liu et al. found that high graft YAP expression after LT was linked with well-preserved histopathology and improved liver function at 1–7 days following LT. YAP is an effector of Hippo pathway dependent turnover of proteins. 

Metabolites

Cortes et al. used metabolomic profiling of 124 graft biopsies to identify significantly increased lysophospholipids, bile acids, phospholipids, sphingomyelins, and histidine metabolism products that were predictors for EAD. Based on the metabolic features, an EAD predictive model was established and further determined in a validation set (n=24) to center studies with larger samples are needed to verify the results.

| Biomarker | Study | Sample | Model(s) | Group | Key point |
|-----------|-------|--------|----------|-------|-----------|
| Metabolite | Lysophospholipids, bile acids, phospholipids, sphingomyelins, and histidine metabolism products | Cortes et al. (2014) | Pretransplant biopsies | Human LT | EAD (n=48) vs. non-EAD (n=48) | Predictors for EAD |
| Lactate and phosphocholine | Faitot et al. (2018) | Pretransplant biopsies | Human LT | EAD (n=7) vs. non-EAD (n=35) | Predictors for EAD |
| single cell RNA sequencing | Yang et al. (2021) | 24 hours after LT | Rat steatotic LT model | Fatty graft (n=3) vs. Control graft (n=3) | A pro-inflammatory phenotype of KCs that highly expressed colony-stimulating factor 3 and a subset of DCs with high expression of XCR1 were enriched in the steatotic grafts |
| Metabolite | Wang et al. (2021) | Grafts gained from procurement, at the end of organ preservation and 2 h after reperfusion | Human DBD LT | n=1 | Showed a dynamic transcription profile of intrahepatic cells during LT |

DBD, donation after brain death; DCD, donation after circulatory death; DCs, dendritic cells; EAD, early allograft dysfunction; GcfDNA, graft-derived cell-free DNA; IRI, ischemia-reperfusion injury; KCs, Kupffer cells; LT, liver transplantation; MICA, major histocompatibility complex class 1 related chain A; NODM, new-onset diabetes mellitus; PNF, primary nonfunction.
have 91% sensitivity and 82% specificity. Likewise, Faitot et al.54 described a dynamic transcription profile of the heterogeneity and relevance between cells. In a review by Verhoeven et al.71 in 2014, ALT, AST, lactate dehydrogenase, lactate, adenine nucleotide level, hyaluronic acid, thrombomodulin and inflammatory markers (e.g., hypoxia-inducible factor-1α, and tumor necrosis factor-α) in metabolites in perfusate may be associated with graft outcomes. In a study published on BioRxiv, we established a graft-tolerant mouse LT model and identified two stages of graft recovery, which included an acute and stable phases.70 We also found that the interaction between CD206+MerTK+ macrophages and CD49a+CD49b− NK cells regulated metabolic and immune remodeling of the graft.70

**Potential biomarkers in perfusate**

The donor graft and perfusate keep interacting during preservation. Molecules including nucleic acids, proteins, and metabolites in perfusate may be associated with graft outcomes. In a review by Verhoeven et al.71 in 2014, ALT, AST, lactate dehydrogenase, lactate, adenine nucleotide level, hyaluronic acid, thrombomodulin and inflammatory markers (e.g., hypoxia-inducible factor-1α, and tumor necrosis factor-α) in perfusate and perfusate pH were useful biomarkers to assess graft quality. Machine perfusion (MP) such as hypothermic machine perfusion (HMP), hypothermic oxygenated perfusion (HOPE), and normothermic machine perfusion (NMP) continuously inject the perfusion fluid into the graft blood vessels to form a circuit, mitigating IRI and maintaining cellular metabolism in graft.72 So far, a series of current and ongoing clinical trials have shown that they were superior in reducing ischemic complications compared with static cold storage (SCS).73–76 In addition, the development of detection technology and MP have facilitated the discovery of a series of novel perfusate biomarkers for graft viability evaluation and are summarized as below and in Table 2.57–79

| Biomarker                      | Study                  | Model          | Group                                          | Key point                                                                 |
|-------------------------------|------------------------|----------------|-----------------------------------------------|---------------------------------------------------------------------------|
| Bile production               | Pavel et al. (2019)77  | NMP            | 5 discarded human DCD livers                  | Earlier production of bile and higher bile flows during NMP were linked to better bile duct histology |
| Biliary bicarbonate, pH, and glucose | Matton et al. (2019)78 | NMP            | 23 human donor livers                        | High biliary bicarbonate and pH and low glucose were associated with bile duct injury |
| Bile/perfuse ratio and bile/ perfusate Na+ ratio | Linares-Cervantes et al. (2019)56 | A porcine DCD LT model; NMP | /                                            | Bile/perfuse glucose ratio ≤0.7 and bile/ perfusate Na+ ratio ≥1.1 were correlated with successful LT |
| CDmiRs                        | Verhoeven et al. (2013)79 | Human LT; SCS  | Drafts developed ITBL (n=20) vs. Grafts without biliary strictures (n=37) | CDmiRs could be predictive of bile duct injury and ITBL                   |
| miR-122                       | Selten et al. (2017)80 | Human DCD/ DBD LT; SCS | EAD (n=35) vs. non-EAD (n=48)                | High miR-122 level could predict EAD                                        |
| FMN                           | Muller et al. (2019)81 | Human DCD/ DBD LT; HOPE | 53 donor livers                          | High FMN level could predict severe graft dysfunction following LT          |
| D-dimer                       | Karangwa et al. (2017)62 | NMP            | 12 discarded human livers                      | High D-dimer level was associated with graft damage                        |
| NGA2F                         | Verhelst et al. (2018)83 | Human DCD/ DBD LT; SCS | PNF (n=3) vs. non-PNF (n=63)                  | Increased NGA2F level could predict PNF                                      |

**CDmiR, cholangiocyte-derived miRNA; DBD, donation after brain death; DCD, donation after circulatory death; EAD, early allograft dysfunction; FMN, flavin mononucleotide; HOPE, hypothermic oxygenated perfusion; LT, liver transplantation; NGA2F, agalacto-core-alpha-1,6-fucosylated biantennary glycan; NMP, normothermic machine perfusion; PNF, primary graft nonfunction; SCS, static cold storage.
of bile and higher bile flows during NMP contributed to better bile duct histology. In addition, Matton et al.\textsuperscript{78} showed that high biliary bicarbonate and pH, and low biliary chloride in human liver grafts (n=23) during NMP were significantly associated with high risk of bile duct injury. In a porcine LT model, Linares-Cervantes et al.\textsuperscript{5} demonstrated that a bile/perfusate glucose ratio < 0.7 and a bile/perfusate Na\textsuperscript{+} ratio ≥ 1.1 within 4 h of NMP predicted graft survival after LT. Given that the role of donor graft miRNAs in predicting post-transplant outcomes, perfusate miRNAs may serve similarly. Furthermore, miRNAs have been shown to be stable in perfusate for at least 1 day.\textsuperscript{79} Verhoeven et al.\textsuperscript{79} showed that cholangiocyte-derived miRNAs (CDmiRs) in perfusate were predictive of bile duct injury and the development of ITBL. They also found that a significantly elevated hepatocyte-derived miRNA to CDmiRs ratio was associated with the incidence of ITBL. Moreover, Selten et al.\textsuperscript{80} reported that both high miR-122 levels and a high miR-122/miR-222 ratio in SCS perfusate predicted the development of EAD and poor graft survival after LT in 83 recipients.

Flavin mononucleotide (FMN), a critical molecule of generating electrons for ubiquinone reduction in mitochondrial complex 1, was shown to be associated with mitochondrial injury.\textsuperscript{81} Muller et al.\textsuperscript{81} preserved 53 grafts with HOPE and demonstrated that a high perfusate FMN level after 30 min of HOPE was strongly linked to severe graft dysfunction. Wang et al.\textsuperscript{82} infused 23 DCD livers with normothermic regional perfusion and found that the levels of perfusate FMN in transplantable grafts (n=15) were dramatically lower than those in nontransplantable grafts (n=8). D-dimer, a product of fibrin degradation, is a small protein fragment released during fibrinolysis. Karangwa et al.\textsuperscript{82} preserved 12 discard donor livers with NMP and showed that D-dimer levels > 3,500 ng/mL were significantly associated with graft liver injury, suggesting that it was predictive of poor graft function.

In a multicenter cohort study, Verhelst et al.\textsuperscript{93} compared the glycome patterns in SCS perfusate in PNF (n=3) and non-PNF (n=63) groups and found that increased NGA2F, a single under galactosylated biantennary glycan, predicted the development of PNF with 100% accuracy. That highlighted the essential role of omics, especially the metabolomics, in discovering potential perfusate markers of poor graft function during LT.

Potential strategies for restoring graft function

In recent years, in vivo and ex vivo potential protective interventions that have been used to restore graft function are listed in Table 3.\textsuperscript{85,102} During the process of ex vivo therapies, the role of MP is apparent because it provides a platform for graft preconditioning.

Gene therapy

Previous in vivo studies were performed to treat liver IRI by using small interfering RNA (siRNA). Jiang et al.\textsuperscript{85} silenced toll-like receptor 4, a critical mediator of inflammation, in a hepatic IRI mouse model, resulting in significant reduction of serum transferases and histological injury. In another study, Zinn et al.\textsuperscript{96} downregulated nuclear high mobility group box 1 by transfecting mice with siRNA and found that it effectively inhibited the expression of serum inflammatory cytokines and protected the liver against IRI. Although the efficacy of hydrodynamic injection has been shown in these animal models, it is difficult to use in the clinic because of off-target effects. Recent studies of graft perfusates showed a potential to solve this problem. For example, Gillooly et al.\textsuperscript{87} found that Fas siRNA directly added to the perfusate was successfully delivered to rat livers during HMP and NMP. This technology ensured that the siRNA only targeted the grafts, opening a new door for graft reconditioning. Anti-sense oligonucleotide, another gene modulation agent, was demonstrated to significantly reduce miR-122 expression and inhibit hepatitis C virus replication or reinfestation after LT in a porcine LT model with NMP, further confirming the possibility of ex vivo gene therapy in grafts.\textsuperscript{98}

Cell therapy

In vivo cell therapies such as tolerogenic DCs, Tregs, and mesenchymal stem cells (MSCs) have a role in immunomodulation. In a rat LT model, we innovatively treated acute rejection with a combination of galectin-1-induced tolerogenic DCs and apoptotic lymphocytes, which resulted in prolonged survival of the treated rats, with 37.5% surviving over 100 days, compared with untreated, all of which died within 14 days.\textsuperscript{90} In a phase I clinical trial, Sanchez-Fueyo et al.\textsuperscript{90} demonstrated that autologous Tregs transfer was safe and effective in reducing antidonor T cell responses after LT by intravenously administering autologous Tregs to the LT candidates. In addition, Shi et al.\textsuperscript{91} found that human MSCs injection in LT recipients suppressed acute rejection and improved graft histology by upregulating the Treg/T helper 17 cell ratio. Compared with in vivo treatment, ex vivo technology provides novel strategies for graft restoration. For instance, Verstegen et al.\textsuperscript{92} showed in a porcine LT model that MSCs directly added to the perfusate during HOPE were effectively distributed to the porcine grafts, which continued to maintain their paracrine activity after distribution.

Extracellular vesicles

It has been reported that the above tolerogenic cells had the potential to undergo spontaneous malignant transformation.\textsuperscript{103} Therefore, some investigators began to use MSC-, DC- and trig-derivated extracellular vesicles (EVs) as alternatives to cell therapy. In in vivo mice and rat IRI models, MSC-derived EVs had a diverse set of functions including mitochondrial autophagy,\textsuperscript{104,105} inhibition of immune response\textsuperscript{106,107} and liver regeneration.\textsuperscript{108,109} Zheng et al.\textsuperscript{83} found in a rat IRI model that DC-derivated EVs could protect liver against IRI through modulating differentiation of Tregs. In a rat LT model, Chen et al.\textsuperscript{84} demonstrated that injection with Tregs-derived EVs after LT suppressed the proliferation of CD8\textsuperscript{+} cytotoxic T cells and prolonged liver graft survival. Compared to the in vivo injection, the ex vivo technology has the potential to directly target donor grafts without concern for off-target effect. Rigo et al.\textsuperscript{95} successfully delivered human liver stem cells-derived EVs into the rat livers during NMP, leading to less histological damage and lower levels of AST and lactate dehydrogenase in the treated group.

Anti-inflammatory agents

Liver IRI is characterized by the activation of pro-inflammatory cytokine response. Therefore, adding anti-inflammatory agents to perfusate may regulate immune response and alleviate graft damage. In a porcine LT model, Golderacaena et al.\textsuperscript{96} put alprostadil, n-acetylcysteine, carbon monoxide, and sevoflurane into the NMP perfusate, showing significantly decreased interleukin-6, tumor necrosis factor-α, and AST during NMP; and lower AST and bilirubin levels in serum after LT in the treated group.\textsuperscript{96} In addition, Yu et al.\textsuperscript{97} used Mcc950, which strongly inhibited the nucleotide-binding
Glucocorticoids

They also found that Mcc950 significantly reduced inflammatory cytokines and histological injury, and prolonged long-term survival after LT.

Vasodilators

During the ischemic phase of LT, rapid adenosine triphosphate depletion and lack of blood flow result in mitochondrial dysfunction and liver sinusoidal endothelial cell (LSEC) injury. After reperfusion, the injured LSECs produce insufficient vasodilators but also express P-selectin to accumulate platelets, which result in microcirculation disorder. Hara et al.98 inhibited the accumulation of platelets by adding prostaglandin E1 (PGE1) to the perfusate under normothermic conditions. PGE1 ameliorated serum liver enzymes and histologic necrosis, and significantly improved bile production and energy status. In addition, Nassar et al.99 added a prostacyclin analog (epoprostenol) to NMP perfusate to preserve porcine livers.

Table 3. Potential therapies to restore donor liver function

| Therapy               | Study                       | Target                  | Model          | Outcome                                                                 |
|-----------------------|-----------------------------|-------------------------|----------------|-------------------------------------------------------------------------|
| Gene therapy          | Jiang et al. (2011)85       | Toll-like receptor 4 siRNA | Mice-IRI in vivo | Reduce liver IRI                                                        |
|                       | Zhao et al. (2017)86        | High-mobility group box 1 siRNA | Mice-IRI in vivo | Reduce liver IRI                                                        |
|                       | Gillooly et al. (2019)87    | siRNA against the Fas receptor | Rats HMP and NMP | Absorbed by rat donor livers during HMP and NMP                       |
|                       | Goldaracena et al. (2017)88 | Antisense oligonucleotide | Porcine LT NMP | Prevent HCV replication or reinfection after LT                        |
| Cell therapy          | Peng et al. (2018)89        | DC+ apoptotic lymphocytes | Rat LT in vivo | Prolong rat survival                                                    |
|                       | Sanchez-Fueyo A et al. (2020)90 | Tregs                  | Human LT in vivo | Reduce antidonor T cell responses and play the potential role of graft rejection |
|                       | Shi et al. (2017)91         | MSCs                    | Human LT in vivo | Suppress acute rejection and improve graft histology                    |
|                       | Verstegen et al. (2020)92   | MSCs                    | Porcine LT HOPE | Absorbed by porcine grafts and continue to maintain paracrine activity after distribution |
| Extracellular vesicles| Zheng et al. (2018)93       | EVs deprived from DCs    | Rat IRI in vivo | Modulate differentiation of Tregs and protect liver against IRI         |
|                       | Chen et al. (2019)94        | EVs deprived from Tregs  | Rat LT in vivo | Prolong liver graft survival                                            |
|                       | Rigo et al. (2018)95        | EVs deprived from human liver stem cells | Rats NMP | Absorbed by hepatocytes and reduce liver injury |
| Anti-inflammatory agents | Goldaracena et al. (2016)96 | Alprostadil, n-acetylcysteine, carbon monoxide, and sevoflurane | Porcine LT NMP | Reduce liver injury                                                        |
|                       | Yu et al. (2019)97          | mcc950                  | Porcine LT HMP | Reduce liver injury                                                        |
| Vasodilators          | Hara et al. (2016)98        | Prostaglandin E1        | Rat LT NMP | Reduce liver injury and improve bile production, energy status, and rat survival |
|                       | Nassar et al. (2014)99      | Prostacyclin analog (epoprostenol) | Porcine LT NMP | High bile production and good histopathology                           |
|                       | Echeverri et al. (2018)100  | Endothelin1 antagonist (BQ123), epoprostenol, verapamil | Porcine LT NMP | High hepatic artery flow and reduce hepatocyte injury                   |
| Defatting             | Nagrath et al. (2009)101    | A cocktail*              | Rat NMP        | Decrease the intracellular lipid content of liver by 50% during 3 h perfusion |
|                       | Boteon et al. (2019)102     | A cocktail* + L-carnitine | Human NMP      | Decrease liver triglycerides by 30% and macrosteatosis by 40% over 6 h perfusion |

Cocktail*, a combination of peroxisome proliferator activated receptor α ligand GW7647, peroxisome proliferator activated receptor δ ligand GW501516, pregnane X Receptor ligand hypericin, the constitutive androstane receptor ligand, the glucagon mimetic and cAMP activator forskolin, and the insulin-mimetic adipokine visfatin. DCS, dendritic cells; EVs, extracellular vesicles; HCV, hepatitis C virus; HMP, hypothermic machine perfusion; HOPE, hypothermic oxygenated perfusion; IRI, ischemia-reperfusion injury; LT, liver transplantation; MSCs, mesenchymal stem cells; NMP, normothermic machine perfusion; siRNA, small interfering RNA; Tregs, regulatory T lymphocytes.

domain leucine-rich repeat containing family pyrin domain containing 3 inflammasome, as an addition to the HMP perfusate in a porcine LT model. They found that Mcc950 significantly reduced inflammatory cytokines and histological injury, and prolonged long-term survival after LT.
and found that the use of prostacyclin analog led to high bile production and good histopathology. Furthermore, Echeverri et al. compared the effects of endothelin1 antagonist (BQ123), prostacyclin analog (epoprostenol) and calcium channel antagonist (verapamil) to treat hepatic artery vasospasm induced by IRI in a porcine LT model. They demonstrated that grafts with BQ123 and verapamil treatment had higher hepatic artery flow and less hepatocyte injury compared with those treated with epoprostenol.

Defatting agents

Moderate to severe (>30%) macrosteatosis is a well-known risk factor for poor graft quality, making it necessary to defat prior to LT.14 Nagrath et al.101 treated rat fatty livers with a combination of six defatting agents normothermically and showed that the treatment could decrease the intracellular lipid content of rat liver by 50% after 3 h perfusion. Furthermore, Boteon et al. assessed the efficacy of the above six agents combined with additional L-carnitine in defatting human livers with severe steatosis. They found that this method reduced liver triglycerides and macrosteatosis by 38% and 40% over 6 h NMP, enhanced metabolic parameters including increased urea and bile production, and downregulated biomarkers of liver injury (e.g., lower ALT and reduced inflammatory cytokines).

Other agents

In addition to the above agents, human atrial natriuretic peptide (hANP), heavy water, marine worm super hemoglobin (M101), glycine, relaxin, and polyethylene glycols have been found to alleviate liver injury.111–116 Nigmet et al. added hANP, a protective cardiovascular hormone for vascular endothelia, to SCS perfusate to preserve rat livers, showing that hANP supplementation decreased transamidinase release, increased bile production, and protected sinusoidal architecture. In a porcine LT model, Alix et al. added M101 to SCS perfusate and demonstrated that M101 significantly reduced blood levels of ALT, AST, and tumor necrosis factor α in recipients 3 days following LT. Moreover, Gassner et al. used glycine, a simple amino acid that protected sinusoidal cells and hepatocytes, as an addition to NMP rat liver perfusate. They found less sinusoidal dilatation and tissue damage in the treated group.

Conclusions and perspectives

This review summarized and updated biomarkers in donor blood, liver tissue or graft perfusate to evaluate early graft injury (e.g., EAD, and PNF) and ITBL, and to identify potential therapies for graft repairment during the era of ECMO. We focused on studies using human liver grafts and investigations of potential biomarkers involved in anti- or pro-inflammatory processes, which in turn shape immunity, regulate graft IRI, and further influence the development of EAD, PNF, or ITBL following LT. Given that relevant mechanisms of some molecules are lacking, further prospective studies and experiments are urgently needed to clearly understand their roles.

Although various biomarkers with available prognostic and diagnostic value in graft quality assessment have been widely explored, few are currently used in clinical practice. Current challenges associated with biomarker discovery research are as follows. Firstly, the sample sizes of these studies were small and mainly limited to single centers, suggesting that large multicenter cohorts or prospective randomized clinical trials are greatly necessary. Another problem is that the studies lack standardized endpoints and control groups.117 Graft quality is commonly considered to be associated with early graft dysfunction or ITBL, yet other complications after LT (e.g., ACR, metabolic disorders, and graft steatosis or fibrosis) are still a matter of substantial debate. Therefore, we primarily summarized biomarkers predictive of EAD, PNF, and ITBL. Current studies mainly focus on finding biomarkers related to early graft injury, do not have prolonged follow-up and overlook long-term complications like ITBL. Importantly, the measurement of biomarkers should be rapid and easy and have high predictive specificity and sensitivity for graft quality. However, detection of potential biomarkers is costly and time consuming. Moreover, biomarkers need to be stable and measurable during graft procurement, preservation, and implantation.

Despite the availability of liver biopsies for histological assessment and quantification of liver injury during LT, they are invasive and only represent specific parts of the grafts. On the contrary, perfusates can be collected in large volumes and contain markers from the whole graft. In recent years, MP has constantly advanced, and it use in evaluation of graft viability has gradually increased. Nevertheless, different regions or centers have their own standards to determine graft quality.79,118 More clear international guidelines that could guide the decision for organ usage, discard, or restoration prior to LT are recommended. In addition, we believe that MP could provide a platform for graft preconditioning, making it convenient to explore novel strategies for graft repair. Although high cost and the technical complexity limit wide usage of MP at its current stage, recently completed and ongoing clinical trials will make it an indispensable part of LT.72,73

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Conflict of interest

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Author contributions

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