Original Study

ANNALS OF SURGERY OPEN

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Transcriptional Profiling Underscores the Role of Preprocurement Allograft Metabolism and Innate Immune Status on Outcomes in Human Liver Transplantation

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Objective: The adverse effects of ischemia-reperfusion injury (IRI) remain a principal barrier to a successful outcome after lifesaving orthotopic liver transplantation (OLT). Gene expression during different phases of IRI is dynamic and modified by individual exposures, making it attractive for identifying potential therapeutic targets for improving the number of suitable organs for transplantation and patient outcomes. However, data remain limited on the functional landscape of gene expression during liver graft IRI, spanning procurement to reperfusion and recovery. Therefore, we sought to characterize transcriptomic profiles of IRI during multiple phases in human OLT.

Methods: We conducted clinical data analyses, histologic evaluation, and RNA sequencing of 17 consecutive human primary OLT. We performed liver allograft biopsies at 4 time points: baseline (B, before donor cross-clamp), at the end of cold ischemia (CI), during early reperfusion (ER, after revascularization), and during late reperfusion (LR). Data were generated and then recipients grouped by post-OLT outcomes categories: immediate allograft function (IAF; n = 11) versus early allograft dysfunction (EAD; n = 6) groups.

Results: We observed that CI (vs B) modified a transcriptomic landscape enriched for a metabolic and immune process. Expression levels of hallmark inflammatory response genes were higher transitioning from CI to ER and decreased from ER to LR. IAF group predominantly showed higher bile and fatty acid metabolism activity during LR compared with EAD group, while EAD group maintained more immunomodulatory activities. Throughout all time points, EAD specimens exhibited decreased metabolic activity in both bile and fatty acid pathways.

Conclusions: We report transcriptomic profiles of human liver allograft IRI from prepreservation in the donor to posttransplantation in the recipient. Immunomodulatory and metabolic landscapes across ER and LR phases were different between IAF and EAD allografts. Our study also highlights marker genes for these biological processes that we plan to explore as novel therapeutic targets or surrogate markers for severe allograft injury in clinical OLT.

Keywords: human liver transplantation ischemia, reperfusion injury, transcriptomics

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Disclosure: The authors declare that they have nothing to disclose. This study was supported in part by The Maggie Z. Schultz We Care Award for Medical Innovation and Research of the Medical College of Wisconsin Department of Surgery (FP00015937) and The J. Scott Harkness Organ Transplantation Research and Education Fund to J.C.H.

SDC Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.annalsofsurgery.com).

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Annals of Surgery Open (2024) 2:e444

Received: 31 October 2023; Accepted 21 April 2024

Published online 13 May 2024

DOI: 10.1097/AS9.0000000000000444

INTRODUCTION

Patient survival outcomes after orthotopic liver transplantation (OLT) depend upon many interrelated predictive factors, including the degree of ischemia and reperfusion injury (IRI) and recipient and donor allograft status.[1–4] Hepatic IRI, an inherent event in the process of organ transplantation, begins with the interruption of blood flow to the liver (ischemia) that leads to disorders of the cellular respiratory chain, accelerated glycolysis, electrolyte disturbances, microcirculatory dysfunction, and impairment of cell function. Paradoxically, the return of blood flow after a period of ischemia (reperfusion) results in further cellular damage, which triggers the inflammatory cascade and cell death.[5]

For organ donors, the state of allograft health before organ procurement plays a critical role in the allograft's functional recovery from IRI and the recipient's prognosis after transplantation. While innovations in liver resuscitation and reconditioning (ie, liver machine perfusion) provide a platform for intervention toward converting high-risk organs into livers suitable for transplantation, the focus has been primarily on replenishing substrate deficits in various environments (ie, normothermic, hypothermic, and subnormothermic). Moreover, studies have been on molecular mechanisms after cold organ preservation, and data on liver allograft health before organ donation and responses to different stages of IRI are limited.[6–9] Recent advances in transcriptomics may provide insight into gene expression patterns of the liver allograft health before IRI and how the transcriptional responses play a role in understanding the pathophysiology of IRI and the development of potential therapeutic targets.[10–12] We have previously reported

an association between hepatic graft function procured after circulatory death and the transcriptomic profiles of liver ischemia and reperfusion in a porcine experimental model.[12] Therefore, this study aims to investigate the transcriptional responses of human liver allograft health during all phases of OLT and explore its potential clinical applications.

METHODS

Patients

We performed a prospective study of all patients ≥18 years of age who provided written informed consent for the study and subsequently received OLT at Froedtert & Medical College of Wisconsin from September 2020 to June 2021. This study only included patients who received a primary OLT and excluded retransplantation of the liver. All patients received whole-liver allografts from brain-dead donors. The Medical College of Wisconsin Institutional Review Board approved this study (PRO00028513).

Surgical Technique

The surgical procedure for OLT was performed in the standard manner, with the replacement of the recipient's inferior vena cava.[13,14] As previously described, a planned reoperation and staged biliary reconstruction after OLT (SBRALT) is performed in high acuity patients who exhibited the following after hepatic graft reperfusion before biliary reconstruction: hemodynamic instability requiring high doses of vasopressor agents, severe coagulopathy requiring massive transfusion of blood products, metabolic acidosis, hypothermia, the additional need for extensive lysis of intra-abdominal adhesions, and significant visceral edema and bowel distention. SBRALT is performed 1 to 6 days after OLT once the patient has achieved hemodynamic stability, improvement of hepatic graft function, and reduction/resolution of visceral edema or bowel distention.[15,16]

Immunosuppression

The primary induction immunosuppression regimen consisted of basiliximab (Simulect, Novartis Pharmaceuticals Corporation, East Hanover, NJ) and steroids. Maintenance immunosuppression was a calcineurin inhibitor minimization regimen with tacrolimus (Prograf, Astellas Pharmaceutical Inc., Northbrook, IL), everolimus (Zortress, Novartis Pharmaceutical Corporation, East Hanover, NJ), and steroids.

Clinical Transplant Parameters

We collected the deceased donor and recipient demographics and clinical data. The model for end-stage liver disease (MELD) score was calculated based on the recipient's international normalized ratio and serum levels of bilirubin, sodium, and creatinine at the time of OLT.[17,18] We captured the patient's physiologic MELD scores at the time of OLT.

After transplantation, early allograft dysfunction (EAD) was defined as a serum bilirubin level of 10 mg/dL or higher on posttransplant day 7, international normalized ratio of 1.6 or higher on posttransplant day 7, or serum aminotransferase (alanine aminotransferase or aspartate aminotransferase) levels of 2000 IU/L or higher within the first 7 days after transplant.[19] The patients were divided into immediate allograft function (IAF) and EAD groups. The median follow-up was 11 months.

Liver Biopsy and Tissue Storage

Liver wedge specimens of approximately 2 cm^3 at the lateral section were obtained at 4 different time points: baseline (B, at the donor hospital before cross-clamp), post cold ischemia (CI, during bench procedure before transplantation), early reperfusion (ER, after portal and arterial reperfusion), and late reperfusion (LR, at the time of planned staged biliary reconstruction or abdominal wall closure) with median of 2 days (interquartile range, 1–3 days, maximum of 4 days) post OLT. The liver tissues were immediately submerged in RNAlater solution (Thermo Fisher Scientific) for 24 to 72 hours at 4 °C. Subsequently, the RNAlater solution was removed, and samples were stored at −80°C until the entire cohort of patient samples was collected.

Preparation for Transcriptomic Profiling

RNA extraction and library preparation were completed by the Mellowes Center for Genomic Science and Precision Medicine at the Medical College of Wisconsin, RRID:SCR_022926. For RNA isolation, tissue was pulverized (2 min, 30 Hz) with a cold 7 mm stainless steel bead in the TissueLyser (Qiagen). To complete cell lysis, QIAzol (Qiagen) was added, and the tissue was beaten again (1 min, 30 Hz). The isolation proceeded according to the RNeasy Plus Universal Kit (Qiagen) with on-column DNA digestion. Total RNA was quantified, and integrity was assessed (via Agilent fragment analyzer) with libraries prepared with 1 µg of RNA according to manufacturer's protocols (Illumina's TruSeq stranded mRNA library kit). Sequencing was completed on the Illumina NovaSeq600 with paired-end 100 base pair reads generating >50 million reads per sample. Samples were checked for quality using the RNA Integrity Number and only samples with RNA Integrity Number > 5 were analyzed. Sequencing reads were processed similarly to our previous studies[12] through the MAP-RSeq Workflow[20] with differential expression analysis completed using edgeR v 3.8.6[21] with the GENCODE v32 (based on Ensembl v98) reference transcriptome. Genes with a false discovery rate of less than 5% and an absolute fold change (FC) ≥2 were considered to be differentially expressed.

Bioinformatic Analyses

We utilized Hallmark gene sets and canonical pathways from the MSigDB resource.[22,23] For each comparison, pathway enrichment statistics were either calculated using the hypergeometric test[24] or weighted using GSEA on log2 FC values. The expressions of pathways as a whole were quantified using the geometric mean of the genes within each pathway and per specimen.

Statistical Analysis

The data are expressed as means with standard errors. $P < 0.05$ was considered significant, and the difference among the groups was measured by the Mann-Whitney test using GraphPad Prism v7.04 (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Donor and Recipient Characteristics

Among the 17 OLT patients, 11 (65%) experienced IAF while 6 (35%) developed EAD. Recipients' age, MELD score, and etiology of liver disease were comparable between those with IAF versus EAD (Table 1). Donor age and cause of death and allograft cold and warm ischemia durations were not significantly different between both groups. All patients in this study underwent SBRALT. Figure 1 shows the actual posttransplant day for LR liver allograft biopsy for each patient. The median (interquartile range) day for LR liver allograft biopsy was posttransplant day 2 (1–3) for the IAF group and 3 (2–3) for the EAD cohort (Table 1).

TABLE 1.

Donor and Recipient Characteristics

	IAF Group (n = 11)	EAD Group (n = 6)
Recipient		
Age, yr; median (IQR)	47 (40–59)	43 (38–54)
MELD score, median (IQR)	34 (25–38)	40 (24–44)
Late reperfusion biopsy (posttransplant day), median (IQR)	2 (1–3)	3 (2–3)
Underlying liver disease		
Alcohol liver disease, n (%)	8 (73)	4 (67)
Others, n (%)	3 (27)	2 (33)
Brain-dead donor		
Age, yr; median (IQR)	27 (20–32)	33 (23–41)
Cause of death		
Anoxia or CVA, n (%)	3 (27)	2 (33)
Trauma, n (%)	8 (73)	3 (50)
Liver graft ischemia duration		
Cold, min; median (IQR)	287 (266–313)	322 (282–351)
Recipient warm, min; median (IQR)	42 (36–44)	42 (38–47)
Posttransplant serum chemistry		
Total bilirubin (POD 7), mg/dL; median (IQR)	3.4 (2.7–5.2)	11.7 (11.0–17.1)
INR (POD 7), median (IQR)	1.1 (1.0–1.2)	1.2 (1.1–1.4)
AST (≤POD 7), U/L; median (IQR)	75 (34–234)	1038 (608–1730)
ALT (≤POD7), U/L; median (IQR)	96 (59–200)	477 (288–657)

ALT indicates alanine aminotransferase; AST, aspartate aminotransferase; CVA, cerebrovascular accident; INR, international normalized ratio; IQR, interquartile range; POD 7, post-OLT day 7.

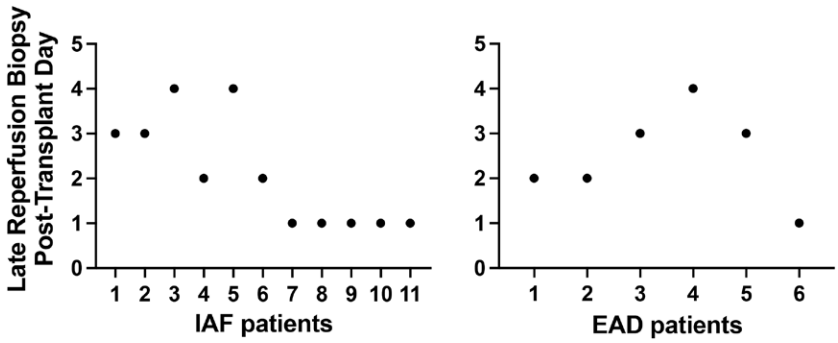

FIGURE 1. Timing of LR liver allograft biopsy per patient by group (IAF and EAD).

Posttransplant Outcomes

Among posttransplant serum chemistry levels, the serum total bilirubin levels on post-OLT day 7 and peak aspartate aminotransferase levels during the first 7 days after OLT were significantly higher in the EAD Group. Overall survival rates were 95% (graft) and 100% (patient). There was no significant difference in survival between the 2 groups.

Preprocurement Allograft Immuno-Metabolic Status Predicts EAD after Transplantation

Global gene expression profiles showed little change during the B and CI phases. On the other hand, significant and consistent global changes occurred across the cohort in the ER and LR phases (Fig. 2A). Using principal component analysis, samples formed clear spatial groups by IRI phases of OLT, yet with high interindividual heterogeneity (Fig. 2B). We quantified differentially expressed genes between sequential time points. From B to CI, there were 46 downregulated genes and 10 upregulated genes. From CI to ER, there were 20 downregulated and 515 upregulated. From ER to LR, there were 688 downregulated and 983 upregulated. Among these differentially expressed genes, we investigated key inflammatory markers including IL6, IL1B, and IL1A. These key markers were activated during ER and then returned toward baseline levels in LR (Fig. 2C). We also found that within each time point, levels of these same key inflammatory markers were generally higher for samples that progressed to EAD (Fig. 2D), marked by higher REL and LIF with lower IL1A, IL1B, JUN, and FOS. Early reperfusion (ER vs B) strongly activated immunomodulatory programs, marked by high LIF, JUN, FOS, and IL6. Notably, these data indicate the potential to identify transcriptomic differences among samples that progressed to EAD compared to those with IAF.

Subsequently, we characterized the time-dependent patterns within the transcriptomic signatures obtained above, and by EAD status. Within time point B and comparing samples with EAD compared to those with immediate function, there were 79 downregulated genes and 676 upregulated genes. Within CI, there were 70 upregulated and 225 downregulated; within ER, there were 97 upregulated and 264 downregulated; and within LR, there were 174 upregulated and 117 downregulated (Fig. 3A). The deconvolution of the transcriptional landscape into pathways showed differences in overall expression across surgical time points, which engaged gene expression networks that regulate cell cycle, inflammation, and metabolism (Fig. 3B). We next compared the expression changes across time points, with the expression changes within each time point, split across EAD status. Comparing the EAD versus IAF, within each time point, we found similar changes in these linked programs (Fig. 3C). When focusing on one specific pathway (eg, TNF) or when making considerations of expression on a per-gene basis, we detected highly intervariable individual differences. However, upon further analyses, we detected an emerging pattern of gene expression constituting the differential transcriptomic landscape for samples that progress to EAD (Fig. 3D). The differentially

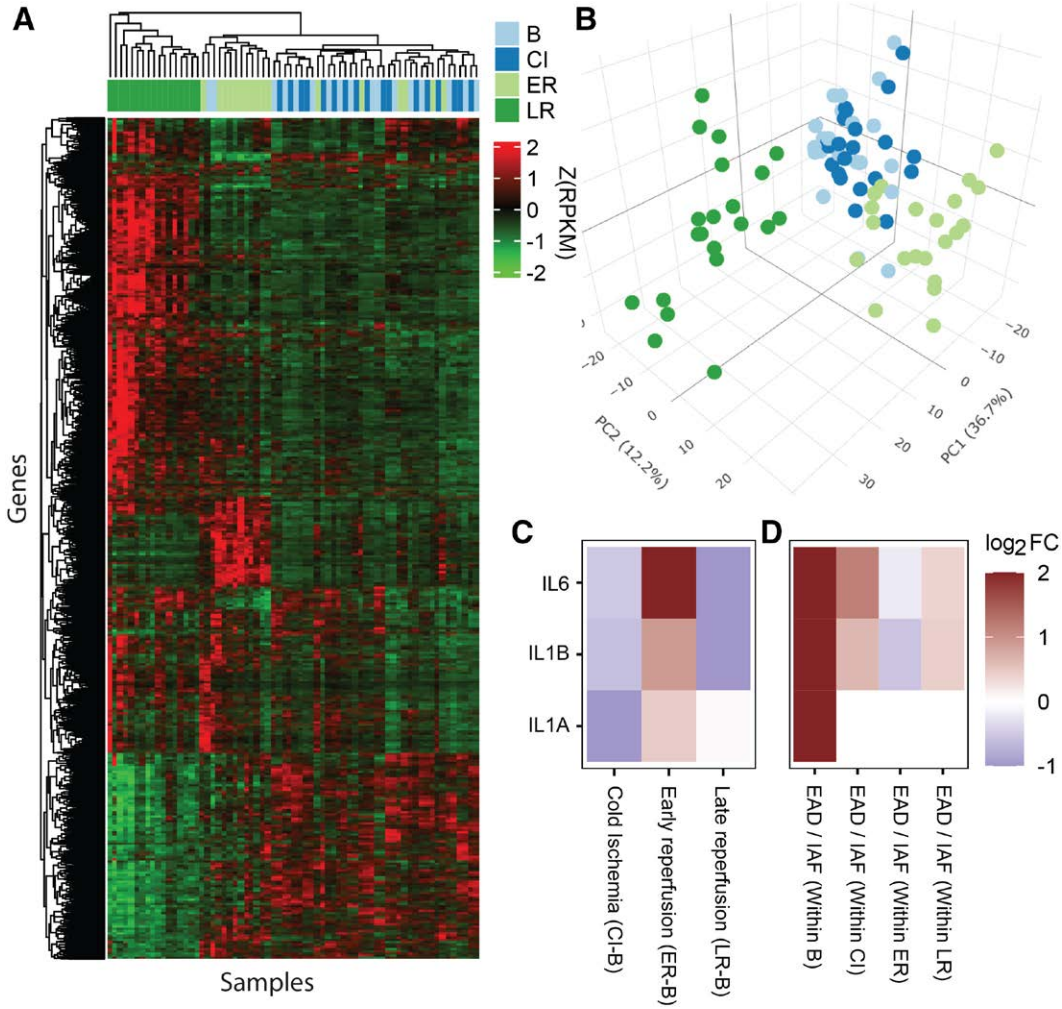

FIGURE 2. Allograft gene expression landscape reveals global and immunologic shifts associated with EAD. A, Normalized gene expression showed significant global changes across the phases of OLT, yet with B and CI displaying similar profiles, while ER and LR were more distinct. Here, we show the normalized expression levels across 4968 genes with FDR < 1×10^{-6} across the phases. Interindividual heterogeneity is visually evident, as are strong shifts consisting of many hundreds of genes changing their expression between OLT phases. B, Patient samples clustered according to transcriptomic landscapes and by OLT phases moving from B to CI, ER, and LR. Each point is a sample, shown in a principal component (PC) space, which summarizes the relative global similarities across the phases of OLT from graft preprocurement to posttransplantation. C, At each phase, we observed differences in immune landscapes, with marker genes such as *IL1A*, *IL1B*, and *IL6*. D, Within each phase, we observed differential activities between EAD and IAF samples, such as those exemplified by the same marker genes. FDR indicates false discovery rate.

expressed genes in each time point comparison and between patients with EAD and IAF can be summarized for 3 key pathways as follows: (1) Among the Hallmark gene sets for inflammatory response, we observed high expression levels moving from CI to ER, and lower expression from ER to LR, indicating that inflammatory processes are high after organ grafting but lower after recovery. Within all time points, inflammatory components were higher for patients experiencing EAD. (2) Similar patterns were shown by TNF signaling, but with EAD patients having lower expression after organ grafting as well as after recovery. (3) Fatty acid metabolism displayed a trend opposite to that of inflammation. Indeed, we observed lower expression levels of the metabolic gene network in patients experiencing EAD at all time points (Fig. 3D).

The pathway-level results above indicated the compelling hypothesis that there are linked immune and metabolic patterns that may distinguish graft function beginning at early time points. Therefore, augmenting pathway enrichment, we used the geometric mean of expression to quantify pathway-level expression for metabolic gene sets. We observed that bile acid metabolism, fatty acid metabolism, and oxidative phosphorylation were all higher at the majority of surgical time points for samples that had IAF compared to those that progressed to EAD (Fig. 4A). Within metabolic gene sets, we identified that key genes involved in critical biologic processes for the liver could act as potential biomarkers, such as ACSL4 or CYP1A1, as they showed higher levels for EAD samples even during the CI phase (Fig. 4B). Moreover, many key inflammatory genes were differentially expressed even during the CI phase (Fig. 4C). This network differs from TNF signaling downstream of NFkB, where genes are enriched early on during the course of a stimulus and not after recovery, with EAD patients having lower expression after organ grafting and after recovery (Supplemental Figure S1, see http://links.lww.com/AOSO/A351). Thus, combined, these observations reveal that inflammation and metabolism are the most critical functions being modified by the liver tissue conditions associated with the susceptibility of liver grafts to IRI and subsequent EAD.

DISCUSSION

In clinical OLT, hepatic allograft IRI remains a major barrier to expanding the donor pool and optimizing patient outcomes after OLT. An insight into the allograft metabolic health before

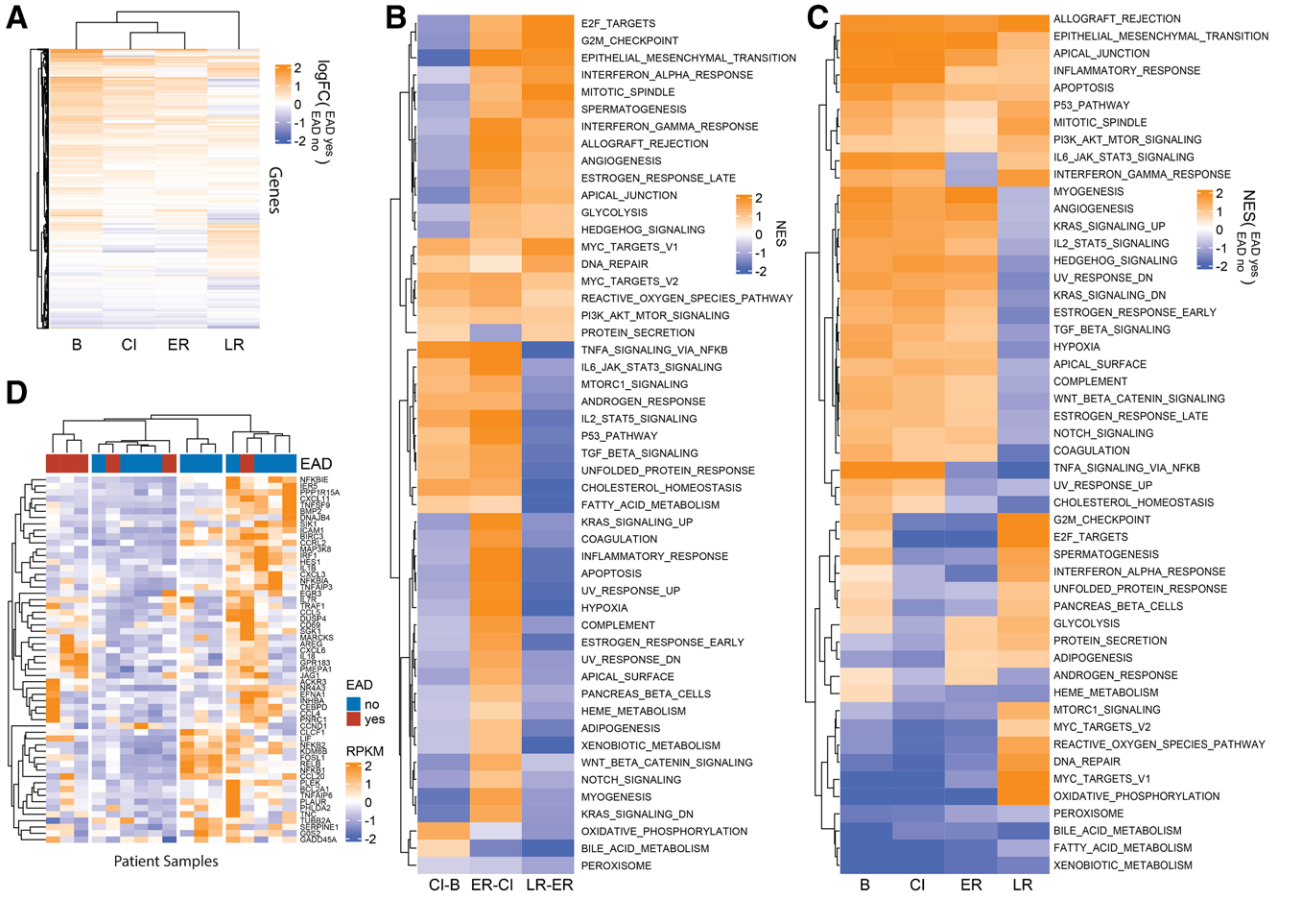

FIGURE 3. Pathway and gene-level features distinguish linked biologic programs that may influence EAD incidence. We used the normalized enrichment score (NES) to summarize the overall expression levels among key pathways and across OLT phases and using Hallmark cellular processes. A, Average global gene expression profiles differed within time points between samples from livers that progressed to EAD and those that did not (IAF). Relatively coherent differences were observed at early time points, with related but modified difference profiles at LR. B, Pathway enrichment statistics for differences between consecutive OLT phases for the graft. C, Pathway enrichment statistics for differences in graft samples that progressed to EAD compared to IAF. D, We selected genes from the TNF signaling via NFkB geneset by their magnitude of difference between EAD status at ER, as well as statistical significance across time comparisons, and show normalized gene expression levels for individual samples.

ischemic (organ procurement) insults, during organ preservation, and after transplantation is vital in helping predict the allograft's ability to withstand IRI. Consequently, data on preprocurement allograft metabolism and innate immune status can facilitate the development of customized organ resuscitative interventions for each phase of organ procurement and preservation.

Recent advances in transcriptomics can provide insights into precisely how cells respond to IRI during organ procurement, preservation, and transplantation. Previous studies restricted their scope to limited time points (eg, only post CI and ER injury)[6–9] or concentrated on normothermic liver perfusion,[25] lacking a comprehensive temporal profile. To our knowledge, our study is the first to investigate the transcriptomic profile from the deceased donor allograft over an extended period from the time before organ procurement to a median of 2 days after OLT.

We detected an emerging pattern of gene expression constituting the differential transcriptomic landscape for hepatic allografts that developed EAD (Fig. 3D). Interestingly, the transcriptomic profile preprocurement of the EAD group significantly differs from that of the IAF group despite sharing similar donor clinical characteristics. Transcriptomic data acquired across various phases of IRI can facilitate the development of innovative diagnostic and therapeutic strategies by identifying key upstream regulators linked to adverse outcomes. Our study indicates that optimal timing for specific interventions may vary across the 4 pivotal time points during transplantation based on specific immediate tissue responses at various phases of IRI.

Across all time points, transcriptomic levels of genes associated with inflammation were significantly increased in the EAD group compared with the IAF group during all OLT phases. Furthermore, these cytokine gene expression levels were significantly higher in the liver graft tissue of the EAD group compared with the IAF group at B even before procurement. This suggests that allografts at risk for EAD can be identified before OLT or even before procurement. Limited preischemia data in existing literature necessitate future validation of our findings. However, our data suggest that despite similar clinical parameters, variance in inflammatory responses may persist. Existing diagnostic tools fall short in accounting for the disparities in posttransplant outcomes among ostensibly similar risk categories. Employing a transcriptomic analysis could assist in differentiating these groups and identifying cases that warrant thorough examination.

Inflammatory cytokines and their associated signals also play a role in regulating oxidative stress, as well as fatty acid and bile acid metabolism during liver injury.[26–29] Indeed, hepatic lipid and bile metabolism are associated with the pathophysiology of hepatic IRI.[30–32] In this regard, the IAF group had higher activity in bile and fatty acid metabolism, whereas the EAD group maintained more immunomodulatory activities and showed lower metabolic activity in bile and fatty acid pathways.

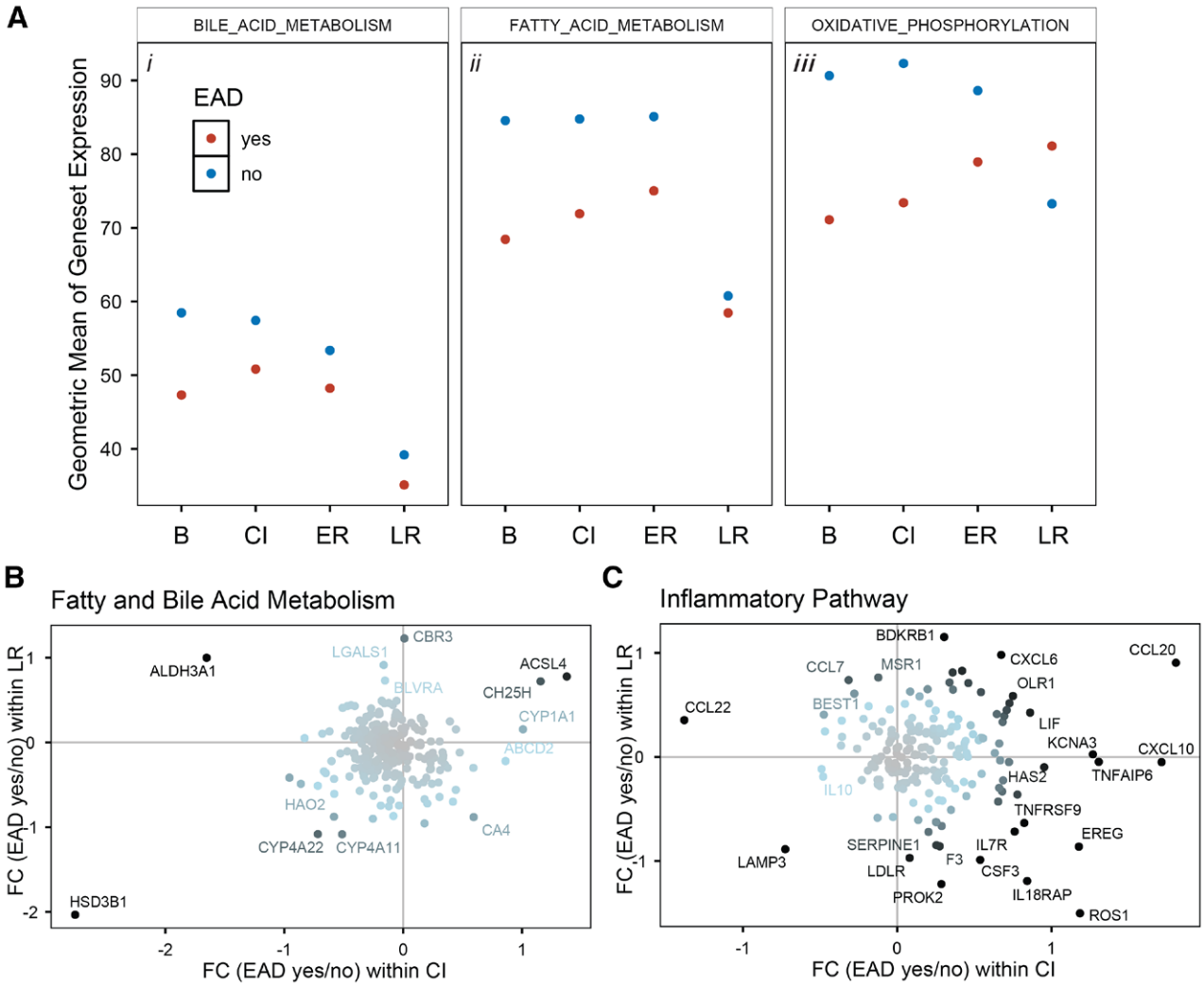

FIGURE 4. Inflammatory and metabolic pathway activities concurrently differ among patients with EAD. A, The differentially expressed gene in each time point comparison and between patients with EAD versus IAF is summarized for 3 key metabolic pathways. Namely, samples with EAD had a lower expression of (i) bile acid metabolism, (ii) fatty acid metabolism, and (iii) oxidative phosphorylation across time points. A scatterplot of genes in the pathways of (B) fatty and bile acid metabolism and (C) inflammatory pathways, comparing FC between time points CI and LR, highlights the genes with the greatest differences. Color is a linear scale from the origin to emphasize the genes with the largest FC.

We also noted that the overall expression levels of cellular processes at a specific phase of OLT did not correspond to its relative expression levels according to the occurrence of EAD. For example, genes associated with TNF signaling via NFkB were activated during ER but suppressed in the EAD group compared with the IAF group. This implies that the activation or suppression of certain gene pathways at a specific phase of OLT cannot be used to predict its role in injurious or reparative signals. Importantly, the patterns of relative expression among key cellular pathways between EAD and IAF were dependent on the OLT phases. This suggests that the potential diagnostic or therapeutic use of a target associated with a specific gene pathway can depend on the timing of its application during the OLT phases. However, inflammatory response and apoptosis pathways were consistently more activated in the EAD group, while bile acid and fatty acid metabolism pathways were suppressed, potentially representing their consistent role in the pathophysiology of hepatic IRI.

There are limitations to this study. First, the sample size is relatively small, which inherently limits its comprehensive analysis across various groups and temporal intervals. Second, the timing of the LR biopsy varied based on the patients' clinical stability. While the number of early (posttransplant day 1 or 2) and late (posttransplant day 3-4) LR biopsies were equally distributed among the EAD patients, and all patients received the same immunosuppressive regimen, the difference in the timing of LR between IAF and EAD may influence the gene expression analysis of the LR phase. As such, an area of future study would be to differentiate gene expression of recovering liver graft from improving patient milieu. Third, given the high acuity of the recipients, data in this study should be viewed in the context of patients with high MELD scores who received high-quality livers yet exhibit diverse transcriptomic expression patterns. Finally, this study focused on capturing transcriptomic shifts to understand the initial cellular reactions to IRI. While this offers a detailed picture of the liver tissue's adaptive mechanisms, it may not directly reflect the functional impact. This caution applies to transcriptomic studies at large.

In conclusion, our results suggest that transcriptomic expressions for genes associated with inflammation are increased and those for metabolism are suppressed in EAD during the OLT process. The significant differences in gene expression in key signal pathways were identified from the pretransplant phases. Further research is necessary to identify key upstream targets for treatment and clinically applicable surrogate markers to increase the success of OLT and avoid unnecessary organ discard.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the contribution of Alicia Martin, MS, MBA, to this manuscript.

Study concept and design, model development, acquisition of data, analysis and interpretation of data, drafting of manuscript, and critical revision of manuscript: J.K.; Study concept and design, acquisition of data, analysis and interpretation of data, and critical revision of manuscript: M.T.Z.; Acquisition of data, analysis and interpretation of data, drafting of manuscript and critical revision of manuscript, particularly in transcriptomic data analysis: A.M., G.L., R.A.U.; Study concept and design, model development, acquisition of data, analysis and interpretation of data, drafting of manuscript, critical revision of manuscript, and final approval of the version to be published: J.C.H.

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