



What Is Hot and New in Basic Science in Liver Transplantation in 2018? Report of the Basic Science Committee of the International Liver Transplantation Society

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The 2018 Joint International Congress of the International Liver Transplantation Society (ILTS), European Liver and Intestine Transplant Association, and Liver Intensive Care Group of Europe meeting was held in Lisbon, Portugal, in May 2018. It was attended by 1200 delegates from 58 countries (Figure 1). Ten percent of the 759 abstracts were Basic Science or Translational Research and were published in *Transplantation*.¹ In addition, all oral presentations are available to the ILTS members at the ILTS website.²

The ILTS Basic Science Committee has selected 33 as representing the most interesting and innovative research covering the key areas of ischemia/reperfusion injury (IRI)/

organ preservation, organ bioengineering, biliary injury/regeneration, transplant immunobiology, biomarkers, and acute kidney injury after liver transplant.

IRI AND ORGAN PRESERVATION

Perfused liver preservation continues to be an extensively investigated area, with a focus on the role of normothermic machine perfusion (NMP) for the preservation of marginal and donation after circulatory death (DCD) liver grafts. Boteon et al³ assessed 46 discarded human liver grafts using lactate clearance as marker of viability. The authors compared NMP alone with a combination of hypothermic perfusion followed by normothermic ex vivo perfusion. They found that 52% of all discarded grafts were considered viable during NMP, as determined by lactate clearance, with viability being better for livers treated with combined cold plus warm ex vivo perfusion (71%) versus NMP alone (41%).³

Steatotic liver grafts remain to be the limiting factor for graft utilization and poor graft function postliver transplantation, as previously demonstrated.⁴ A novel defatting technique during 24-hour NMP was also investigated by Boteon et al⁵ in 6 discarded human steatotic grafts. Using a specific defatting cocktail in the perfusate, the authors were able to decrease macrosteatosis by 18% as determined by histology and, at the same time, increase perfusate triglyceride and cholesterol levels. Bile production was also improved by the treatment.

RNA interference is a natural process of posttranscriptional gene regulation that was the subject of the Nobel Prize in Medicine in 2006.⁶ Moore et al⁷ studied the utilization of gene silencing during NMP preservation. The authors used short interfering RNA (siRNA) to silence the antiapoptotic gene p53 associated to nanoparticles in both an in vivo rat model of liver clamping and a blood reperfusion model of DCD rat liver after machine perfusion preservation using siRNA + nanoparticles added to the perfusate. They showed that markers of liver inflammation and lipid peroxidation were inhibited, and siRNA was taken up by the liver during NMP.⁷

Small-for-size syndrome is a relatively common problem after living donor liver transplant (LDLT). It has been shown previously that small-for-size liver grafts (SFG) are associated with suppressed mitochondrial biogenesis and increased transforming growth factor (TGF)- β . Liu et al,⁸ using a rodent liver transplant model, showed that

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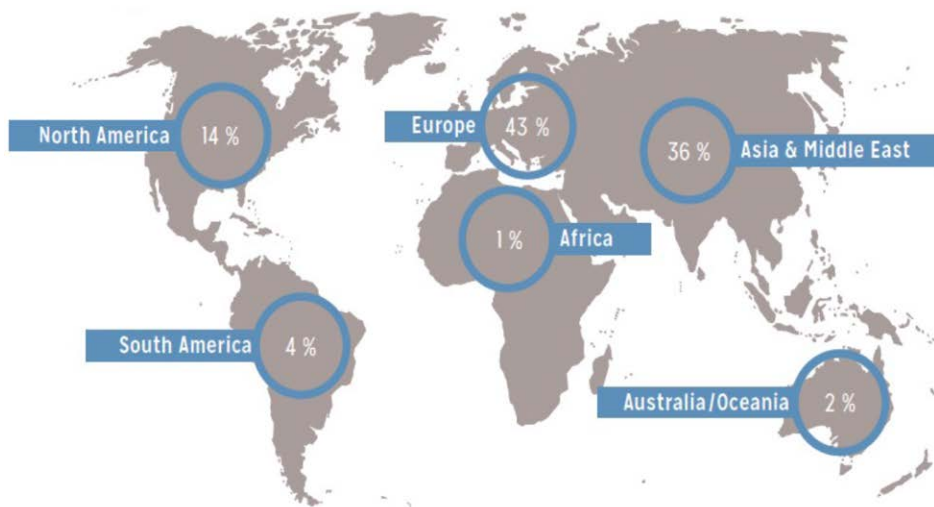


FIGURE 1. Distribution of delegates to the 2018 Joint ILTS-ELITA-LICAGE Meeting. This figure was regenerated with permission by the ILTS. ELITA, European Liver and Intestine Transplantation Association; ILTS, International Liver Transplant Society; LICAGE, Liver Intensive Care Group of Europe.

mitochondrial biogenesis occurred in a 50% size graft; however, there was significant evidence that mitochondrial biogenesis was blunted, and there was an increase in TGF- β and miR23a in a 30% size graft. When SD208 (TGF- β inhibitor) was injected, there was a decrease in both factors, with subsequent increase in mitochondrial activity. In addition, there was significant liver regeneration as opposed to suppression when the SD208 was administered. They concluded that TGF- β inhibitors could potentially be used in the treatment of SFSG dysfunction.⁸

Gu et al,⁹ using a mouse model of hepatotoxic drug-induced liver injury and analyzing patient samples, demonstrated that CHI3LI (a prototypic chitinase-like protein that has been retained over species and evolutionary time) protects livers against liver injury. Knockout of CHI3LI resulted in exacerbating liver injury. The CHI3LI levels were decreased in patients after liver injury. High levels of CHI3LI are associated with the improvement of liver function followed by medical treatment. CHI3LI-mediated hepato-protection by inhibiting Th17 and promoting Foxp3 + Tregs in a Stat3-dependent manner. Their findings established the regulatory role of CHI3LI in liver injury and may provide novel therapeutic targets in liver injury and liver failure.

Bontha et al¹⁰ performed a detailed analysis before implantation and after reperfusion allograft biopsies obtained from deceased donor kidney transplant recipients ($n = 30$) and liver transplant recipients ($n = 30$) using 120 gene expression (Gene Chips) arrays. They identified common intra-allograft inflammatory responses to IRI between kidney and liver transplantation. It was also observed that 40% to 50% of the differentially expressed genes were similar in both organs and these genes were mainly involved in inflammatory pathways and transcription regulation. Approximately 28% of the common genes were transcription factors, and 12% were cytokines and growth factors. Among the top common pathways were interleukin (IL)-6 signaling pathway, NF- κ B signaling, HMGB1 signaling, and production of NO and ROS in macrophages.¹⁰ This study revealed common and specific pathways affected in IRI between 2 different transplanted organs.

These approaches may lead to the discovery of new therapeutic targets, improve common therapeutic interventions during IRI, and help to identify organ-specific pathways that can be specifically targeted to minimize injury. It was recently demonstrated that 1 molecular pathway responsible for the formation of nitric oxide by the endothelial cells is dependent on the stimulation of the glypican-1, a proteoglycan present in the glycocalyx. Lopez et al¹¹ preserved Zucker rat fatty livers for 24 hours in static cold storage in IGL-1 ($n = 5$), UW ($n = 5$) or HTK ($n = 5$), and measured Glycocalyx proteoglycans, demonstrating that they were better preserved in IGL-1 compared to HTK and UW. The protective mechanisms of glypican-1 through the formation of nitric oxide in fatty livers may be due to its better preservation of the endothelial glycocalyx components during static cold storage.¹¹

It is well established that bile production and composition correlates with transplant outcome. Kollmann et al¹² investigated bile composition in a model of porcine liver transplantation as a marker of bile duct injury and function. The authors compared heart-beating donation with 30- and 60-minute ischemia in DCD liver transplantation. Bile production during NMP was significantly higher in heart-beating donation versus DCD liver transplantation. Prolonged warm ischemia was associated with increased bile CO₂ and bile glucose levels. In addition, bile cholesterol was lower during NMP in DCD grafts with prolonged warm ischemic injury.¹² Real-time assessment of the bile composition during NMP might help determine algorithms for graft viability and help with decision-making over acceptance of marginal liver grafts.

Guo et al¹³ developed a new model of procurement graft preservation with NMP in pigs, called ischemia-free liver transplantation (IFLT). In this preservation method, there is no cold ischemia, and the organ is continuously perfused and oxygenated from retrieval until the time of transplant. Ten pigs were subjected to IFLT (6-h NMP) or conventional liver transplantation (6-h cold storage). The posttransplant graft function was better with minimal histological changes after IFLT. There were fewer apoptotic hepatocytes, less sinusoidal endothelial cell injury, and

proinflammatory cytokines (IL-1 β , IL-6, and tumor necrosis factor α) release.¹³ The same group showed the first series of IFLT in 6 human transplants using IFLT with a 30-day graft survival of 100%.¹⁴

Matrix metalloproteinase-9 (MMP-9) facilitates leukocyte transmigration across vascular barriers in hepatic IRI. Tissue inhibitor of metalloproteinases-1 (TIMP-1), the endogenous MMP-9 regulator, is insufficiently expressed in liver to inhibit an elevated MMP-9 activity postreperfusion. Duarte et al¹⁵ showed that gene therapy with viral vector (rAAV8/TIMP1-treated C57/BL6 mice) improved liver preservation histologically and led to significantly lower serum AST and ALT levels at 6 and 24 hours post-IRI. TIMP-1 gene transfer to TIMP1^{-/-} mice restored TIMP-1 expression in liver and enhanced the 7-day survival rate from 50% to 100% post-IRI. This study demonstrated that TIMP-1 acts as an endogenous hepatoprotective factor and may be useful for treatment of clinical liver IRI.¹⁵

ORGAN BIOENGINEERING/BIOPRINTING/ BIOARTIFICIAL LIVER

Bioprinting and biofabrication seek to produce tissue constructs from cells for tissue implants.^{16,17} Li et al¹⁸ using a novel scaffold-free 3-dimensional (3D) bioprinting technology sought to bioprint the liver using genetically engineered porcine cells. Optimization of 3D bioprinting was based on (1) porcine hepatocyte isolation; (2) spheroid diameter, roundness, smoothness, durability, stability, and viability; and (3) the ratio and combination of different cell types (fibroblasts, liver-derived cells [CD31⁺], and hepatocytes). The investigators also developed a biocompatible, 3D-printed bioreactor capable of containing, culturing, perfusing, and observing biofabricated tissues aseptically in real time. Using a combination of porcine fetal fibroblasts and liver-derived cells, spheroids were generated after 2 to 3 days of plating and successfully used to bioprint 3 preliminary 3D liver constructs. The 3D liver construct was perfused continuously for 1 week using this novel bioreactor. The authors therefore successfully bioprinted a scaffold-free 3D Bioprinted Porcine Liver Model, as a proof-of-concept for human liver bioprinting, and were able to continuously perfuse 3D-bioprinted livers for 1 week.^{17,18}

The concept of using allo- or xeno-organ scaffolds to recreate organs with stem cells has the potential to increase the pool of organs and avoid organ rejection.¹⁹ Willemse et al²⁰ developed a protocol to completely remove of cellular debris from cadaveric pig livers and use using the decellularized pig liver matrix as a scaffold for further recellularization with human liver-derived organoids (40 million HepG2 cells or human-derived organoids). After the inoculation of the liver scaffolds and incubation, histological analysis was done and cells were found in the parenchymal areas of the liver segments showing that recellularization of porcine liver scaffold with human organoids is feasible with HepG2 cells.²⁰ Although the success was shown with HepG2 cells, the challenge will be to repeat the same experiment with primary liver hepatocytes.

Artificial extracorporeal liver supportive devices could support patients with potentially reversible liver failure as bridging therapy to recovery or transplantation.²¹ However, randomized clinical trials have not demonstrated

clear beneficial effects.²² The spheroid reservoir bioartificial liver is an experimental hepatic replacement therapy to achieve ammonia detoxification. Chen et al²³ assessed spheroid reservoir bioartificial liver in posthepatectomy pigs. Pigs underwent intracranial pressure (ICP) monitor placement followed by 85% hepatectomy, confirmed by CT volumetric measurement. They were then randomized into standard therapy, bioartificial liver with 0g of hepatocytes, and bioartificial liver with 200g of hepatocyte spheroids. In the standard therapy group (0g), all animals had grade IV hepatic encephalopathy or ICP >20 with herniation, while 5 out of 6 animals in the 200g group survived up to 90 hours, with decreased ICP and INR, and evidence of liver regeneration on CT.²³

Pig livers would represent an unlimited source of hepatocytes for both bioengineered livers and direct hepatocyte replacement. Nelson et al²⁴ attempted to create a new model to study xenotransplantation of hepatocytes. Livers of fumarylacetoacetate hydrolase (FAH)-deficient pigs provide an advantage for the implantation of (FAH⁺) donor hepatocytes. However, the host immune response against nonautologous donor cells blocks this advantage. They hypothesized that the knockout of recombina-activating gene-2 will produce a severe combined immunodeficient (SCID) phenotype and may allow robust expansion of nonautologous hepatocytes. They produced 13 FAH/recombinase-activating gene-2 double-knockout piglets. They demonstrated variable growth curves from normal weight gain to moderate failure to thrive. The induction of an SCID phenotype may allow for consideration of xenotransplantation of hepatocytes in the future.²⁴

Hepatocellular Carcinoma Tumor Biology

It is suspected that hepatocellular carcinoma (HCC) recurrence is more frequent after LDLT than deceased donor liver transplantation²⁵ through SFSG providing a favorable immune microenvironment for tumor growth. Yang et al²⁶ aimed to investigate the role of CCR5(+) NK cells in tumor recurrence after liver transplantation. They discovered that decreased peripheral activated NK cells resulted in increased risk of HCC recurrence among LDLT recipients. Inhibition of NK cell recruitment by blocking PD-L1 in TLR4^{-/-} mice increased HCC recurrence. Therefore, the authors concluded that PD-L1/TLR4 signaling upregulated during liver graft injury directly induced the exhaustion of CCR5(+) NK cells, which further promoted HCC recurrence after transplantation.²⁶ These data have a great clinical relevance because targeting this pathway may decrease the incidence of HCC recurrence after LDLT. It is well known that the biology of the tumor represents a critical prognostic factor for HCC progression and recurrence posttransplant.²⁷ Baciú et al²⁸ aimed to determine a proteomic/transcriptomic signature of HCC recurrence in liver transplant patients with HCC within Milan criteria. Using high-throughput proteomics/transcriptomics and integrating the data, the authors found that 79 proteins were differentially expressed between recurrent and nonrecurrent cases. They also identified 3 proteins predictive of recurrence (ALDH1A1, Galectin-3, and Galectin-3-binding protein), independent of clinical risk factors, such as microvascular invasion, AFP, and tumor size/number.²⁸

Yeung et al²⁹ showed that a subpopulation of macrophages (M2) has been associated with poor

recurrence-free survival in HCC patients after hepatectomy. The authors analyzed M2 macrophages (expressed with a natural isoform of a key immune regulator) and its association with tumor recurrence in the context of living and deceased donor liver transplantation and liver graft injury in a rat transplant model. A 2.1-fold increase of intragraft M2 was found in liver grafts from living donors compared with deceased donors, and $\Delta 42PD-1$ was highly expressed in these cells associated with shorter recurrence-free survival in HCC recipients. In the rat transplant tumor model, liver injury was associated with accumulation of M2 macrophages and protumor cytokines secretion after transplantation and in larger tumor volume. They concluded that targeting this specific population represents a potential therapeutic strategy in attenuating HCC tumor recurrence.²⁹

Although circulating microRNAs (miRNAs) play important roles in tumorigenesis and metastasis, they include miRNAs passively released from apoptotic and necrotic cells. Circulating exosomal miRNAs are more specific markers in tumorigenesis than circulating miRNAs. Lam et al³⁰ quantified 754 exosomal miRNAs from preoperative serum of HCC patients. Of those, miRNA-1290 was found to have significant association with HCC recurrence and predicted the recurrence following tumor resection. High level of miRNA-1290 was an independent predictor of poor overall survival and disease-free survival. Authors concluded that exosomal miRNA-1290 might serve as a prognostic biomarker for HCC recurrence and a potential therapeutic target.³⁰

Li et al³¹ investigated the relationship between tumor stem cell marker epithelial cell adhesion molecule (EpCAM) in HCC tissue samples and HCC recurrence in patients undergoing liver transplantation. They showed that EpCAM expression, maximum tumor diameter, and microvascular invasion were significant predictors of survival and recurrence. EpCAM expression was also associated with tumor metastasis. Authors concluded that EpCAM can be a predictor for HCC distant recurrence and long-term survival of patients with HCC after transplantation.³¹

Injury Biomarkers (OMICS)

Liver transplantation provides a complex biological system affected by unique donor and recipient biology, allograft injury, recipient response to injury, and the impact of immunosuppression.³² In current multi-omics era, complex systems are best studied through integration of genomics, transcriptomics, proteomics, metabolomics, and epigenomics. Cho et al³³ tested samples of 118 patients (n = 236 biopsies paired preimplantation and postreperfusion biopsies, and paired plasma samples collected at preimplantation, on postoperative days 1 and 2). They identified the characteristic expression of messenger RNA and miRNA profiles and associated it with donor quality and short-term outcomes. Discovering biomarkers of injury pathways that reflect in donor quality and graft function may also lead to targeted therapeutic interventions to avoid or stop tissue injury.³³ Fernandez et al,³⁴ using pre- and postreperfusion liver biopsies, investigated if epigenetic modifications predispose graft sensitivity to injury severity of IRI. They found that methylome/transcriptome correlations in severely injured grafts were associated with

apoptosis signaling activation, ubiquitin protein degradation, and cell cycle regulation. They identified 94 genes of liver damage, apoptosis, and cell cycle regulation in severely injured grafts at postreperfusion biopsy.³⁴

Wang et al³⁵ compared circular RNA (circRNA) sequencing of donor liver tissues between early allograft dysfunction (EAD) and non-EAD patients using Illumina HiSeq 3000 platform. A total of 431 circRNAs were identified differentially expressed between EAD and non-EAD donor liver tissues, of which 332 circRNAs were significantly upregulated and 99 circRNAs were downregulated. They showed that circRNA hosting genes were mainly involved in metabolic process, protein binding, and apoptosis, and certain circRNAs are potentially diagnostic biomarkers and therapeutic targets for EAD after liver transplantation.³⁵

Bontha et al³⁶ investigated the utility of cell-free DNA and messenger RNA panels postliver transplantation as biomarkers of severe IRI analyzing biopsies and plasma of 64 DDLT and 10 LDLT. Cell-free circulating DNA corresponded to 95% to the liver graft at the time postreperfusion and decreased to 5% on POD2. Circulating levels of miRNA-16, -155, and -146a significantly increased in DDLTs, while miRNA-122 significantly decreased in LDLTs after 48 hours. In addition, cell-free circulating DNA was found to be elevated postreperfusion and was associated with the degree of injury.³⁶

Pagano et al³⁷ analyzed the liver perfusate after whole graft washout in adult DBD with flow cytometry and the predicting role of NK cell subset on the biopsy-proven acute cellular rejection (ACR). The NK cell subset of liver perfusate was significantly associated with moderate-severe ACR, and it was associated with any grade of rejection, thus conceivably useful as a preoperative predictor of the risk of ACR.³⁷ Chruscinski et al³⁸ reported a novel biomarker gene set for the identification of tolerance in murine transplant models. An 8-gene expression panel, which consisted of the increased expression of 6 immunoregulatory genes and decreased expression of 2 proinflammatory genes, was found to be predictive of tolerance. In phase 2A single-center clinical study (LITMUS) (n = 54), they examined a panel of 8 target and 5 housekeeping gene expressions in the peripheral blood mononuclear cells. Of the 10 remaining patients, 5 have been weaned off of immunosuppression, 2 are undergoing withdrawal, and 3 developed ACR, which was easily reversed.³⁸ These data suggest that a combination of gene expression monitoring in peripheral blood mononuclear cell and liver allograft may identify operationally tolerant recipients.

Miscellaneous (Transplant Immunology, Biliary Injury/Regeneration, Microbiomes)

Kubal et al³⁹ showed that the development of de novo specific antibodies (dnDSA) has been identified as a risk factor for complications after liver transplantation. It is believed that HLA epitope/epitope mismatch is a potential risk factor for dnDSA formation. Eighty liver transplant patients were analyzed. Thirty-four percent developed dnDSA, and 11% developed ACR. Class II epitope/epitope mismatches were strongly associated with risk of class II dnDSA formation and rejection after liver transplant, and they concluded that these patients may benefit from closer monitoring.³⁹

The biliary tree can be damaged by ischemia during and after liver transplantation, in the setting of hepatic artery complications (especially in LDLT) or DCD liver transplantation. It has been previously shown that the biliary tree harbors stem cells that contribute to bile duct homeostasis and repair. Burka et al⁴⁰ aimed to confirm the presence, expand, and characterize biliary stem cells using 3D cultures of human bile duct organoids. They used human extrahepatic bile ducts (n = 32) collected from donor or explant patient livers during liver transplantation. They were able to demonstrate the presence of LGR5-positive stem/progenitor cells, which can be expanded long-term in 3D cultures. In the future, these organoids can potentially be used for bile duct regeneration in damaged grafts before liver transplantation.⁴⁰ This would be particularly interesting to be tested during machine perfusion preservation of DCD grafts.

Visseren et al⁴¹ evaluated pretransplant colonic microbiome in 100 patients who underwent transplant for primary sclerosing cholangitis (PSC) by extracting bacterial DNA from samples of screening colonoscopy biopsies and performing 16S rRNA sequencing. They determined that those with recurrent PSC posttransplant (14% of the total population) had significantly more abundant *Microbacterium* (phylum, *Actinobacteria*) and *Thermicoccus* (phylum, *Firmicutes*). Results suggest a potential contribution of these bacteria to the pathogenesis of recurrent PSC.⁴¹ Thus, modulation of the gut microbiome would have a critical role in preventing or alleviating liver disease. While a sophisticated understanding of the role of the microbiomes in liver transplantation is in its infancy, the potential deserves more both basic and clinical research.⁴²

New targets to understand liver fibrosis are of key importance to prevent the development of cirrhosis. Liver fibrosis frequently results from chronic damage to the liver, which leads to accumulation of extracellular matrix proteins. miRNAs are reported to play a critical role in the development of liver fibrosis. Hu and Li⁴³ suggested that lower miR-152 expression might be involved in generation of liver fibrosis by promoting Gli3 expression, and overexpression of miR-152 could reduce the process of liver fibrosis. These findings will provide insight into miR-152 potential as an antifibrotic therapy through modulating Gli3.⁴³

Hessheimer et al⁴⁴ investigated the impact of changes in gene expression on coagulopathy in high-risk uncontrolled DCD (uDCD) liver transplantation using microarrays and RT-PCR. uDCD livers had significantly upregulated expression of genes that provoke fibrinolysis and inhibit coagulation, including TPA, urokinase plasminogen activator, urokinase plasminogen activator receptor, and thrombomodulin. There was also decreased expression of genes implicated in hemostasis von Willebrand factor gene and inhibition of fibrinolysis (α -2-macroglobulin). Interestingly, no fibrin microthrombi were detected, even in the patients who developed ischemic-type biliary lesions. Endogenous fibrinolysis is upregulated in uDCD livers at risk for ischemic cholangiopathy, and clinical DCD protocols using TPA to help prevent ischemic cholangiopathy should undergo critical appraisal.⁴⁴

Liver Xenotransplantation

With the development of the gene-editing technology CRISPR/Cas9, there is a renewed interest in liver

xenotransplantation.⁴⁵ It is known that platelet sequestration and coagulation cascade activation are associated with poor outcomes in multiple liver xenotransplant models. Cimen et al⁴⁶ ex vivo perfused livers from α 1,3-galactosyltransferase knockout (GalTKO), and human membrane cofactor (hCD46) pigs (group 1, n = 3) and GalTKO.hCD46 pigs also transgenic for human endothelial protein C receptor (hEPCR), thrombomodulin (hTBM), integrin associated protein (hCD47), and heme oxygenase 1 (HO-1) treated with DDAVP and clodronate liposomes (group 2, n = 4) with whole human blood. Transgenic expression of the hEPCR.hTBM.hCD47.HO-1 cassette, along with donor pig DDAVP and clodronate liposome pretreatment, was associated with improved liver xenograft survival in ex vivo perfusion model.⁴⁶

Xenograft vascular endothelium represents the initial site of recipient immune exposure to xenoantigens. Hassanein et al⁴⁷ successfully engrafted human umbilical vein endothelial cells into the deendothelialized rat livers. This method demonstrates the ability to manipulate a key component of the immune response to xenogeneic antigen, and biologically engineered liver xenografts after reendothelialization of the vascular tree with allogenic endothelial cells has the potential to evade the recipient's immune response.⁴⁷

CONCLUSIONS

One of the substantial tasks for ILTS, European Liver and Intestine Transplant Association, and Liver Intensive Care Group of Europe is to nurture and maintain vibrant basic and translational science in liver transplantation. The 2018 meeting has demonstrated that advances including gene-editing, the various omics technologies, bioengineering, bioprinting, and new approaches to organ preservation and revitalization are yielding progress. The field needs to research long-term goals, such as identifying patients who will most benefit from a transplant and through expanding the potential donor pool of organs for liver transplantation using ex vivo perfusion, xenotransplantation, organ and tissue engineering, and bioprinting. Basic science discoveries have yielded several areas that may help to transform the field of liver transplantation over the next decade.

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