Pilot, Open, Randomized, Prospective Trial for Normothermic Machine Perfusion Evaluation in Liver Transplantation From Older Donors

Davide Ghinolfi [iD] ,[1] Erion Rreka,[1] Vincenzo De Tata,[3] Maria Franzini,[3] Daniele Pezzati,[1] Vanna Fierabracci,[3] Matilde Masini,[3] Andrea Cacciatoinsilla,[4] Maria Lucia Bindi,[2] Lorella Marselli,[6] Valentina Mazzotti,[5] Riccardo Morganti,[5] Piero Marchetti,[6] Giandomenico Biancofiore,[2] Daniela Campani,[4] Aldo Paolicchi,[3] and Paolo De Simone [iD] [1]

[1]Hepatobiliary Surgery and Liver Transplantation Unit; [2]Department of Anesthesia, Medical School Hospital; [3]Department of Translational Research and New Technologies in Medicine and Surgery; [4]Department of Pathology; [5]Department of Statistics; and [6]Department of Endocrinology and Metabolism in Organ Transplantation Unit, University of Pisa, Pisa, Italy

Ex situ normothermic machine perfusion (NMP) might minimize ischemia/reperfusion injury (IRI) of liver grafts. In this study, 20 primary liver transplantation recipients of older grafts (≥70 years) were randomized 1:1 to NMP or cold storage (CS) groups. The primary study endpoint was to evaluate graft and patient survival at 6 months posttransplantation. The secondary endpoint was to evaluate liver and bile duct biopsies; IRI by means of peak transaminases within 7 days after surgery; and incidence of biliary complications at month 6. Liver and bile duct biopsies were collected at bench surgery, end of ex situ NMP, and end of transplant surgery. Interleukin (IL) 6, IL10, and tumor necrosis factor α (TNF-α) perfusate concentrations were tested during NMP. All grafts were successfully transplanted. Median (interquartile range) posttransplant aspartate aminotransferase peak was 709 (371-1575) IU/L for NMP and 574 (377-1162) IU/L for CS ($P = 0.597$). There was 1 hepatic artery thrombosis in the NMP group and 1 death in the CS group. In NMP, we observed high TNF-α perfusate levels, and these were inversely correlated with lactate ($P < 0.001$). Electron microscopy showed decreased mitochondrial volume density and steatosis and an increased volume density of autophagic vacuoles at the end of transplantation in NMP versus CS patients ($P < 0.001$). Use of NMP with older liver grafts is associated with histological evidence of reduced IRI, although the clinical benefit remains to be demonstrated.

The use of very old donors in liver transplantation (LT) is showing favorable results,[1] but this practice is not universally implemented[2] because of concerns about

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; BP, biliary pole; BT, back table; CEAVNO, Comitato Etico di Area Vasta Nord-Ovest; CIT, cold ischemia time; CNT, Italian National Transplant Agency; CS, cold storage; CVA, cardiovascular accident; DBD, donation after brain death; DCD, donation after circulatory death; DGF, delayed graft function; D-MELD, donor age × Model for End-Stage Liver Disease; EAD, early allograft dysfunction; EM, electron microscopy; FFP, fresh frozen plasma; HAT, hepatic artery thrombosis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IC, ischemic cholangiopathy; ICU, intensive care unit; IL, interleukin; IQR, interquartile range; IRI, ischemia/reperfusion

a higher risk for primary nonfunction (PNF), delayed graft function (DGF),[3] and worse longterm graft survival.[4] In our recent series of octogenarian donors, we reported favorable overall longterm results, and we found that hepatitis C virus (HCV) recurrence and ischemic-type biliary lesions (ITBLs) were 2 independent causes of graft loss in this population.[5] Although availability of direct antiviral agents is reducing the impact of donor age on HCV recurrence,[6] prevention of ischemia/reperfusion injury (IRI) is pivotal to the practice of elderly donor LT and for donation after circulatory death (DCD) donors.[7,8] Even though the concept of the ideal donor is well defined,[4,9] the definition of extended criteria donors remains controversial. Increased donor age contributes to a higher risk

of medical and surgical complications and worse IRI after LT.[10,11]

Machine perfusion (MP) has been expanded in the last few years. The potential benefits of MP are to maintain the organ in a more physiological condition before implantation, to reduce damages caused by cold storage (CS), and to assess organ function prior to transplantation. MP yields an exciting perspective for organ repair and reconditioning prior to transplantation, which may expand the pool of donor organs beyond the currently accepted criteria.[12] Ex situ MP may also have the potential to limit IRI as well as the damage to cholangiocytes induced by preservation.[13] Herein, we report the results of a pilot study evaluating the safety and the efficacy of normothermic machine perfusion (NMP) for LT from older donors.

Patients and Methods

STUDY DESIGN AND SETTING

This was a pilot, open, single-center, randomized, and prospective trial evaluating the safety and efficacy of NMP for organ preservation in whole-size, primary, adult LT from older (≥70 years) donation after brain death (DBD) donors (ClinicalTrials.gov identifier

NCT02940600; trial name, CEFEMA). Informed consent was obtained from each patient before enrollment, and the study was carried out in compliance with the ethical guidelines of the Declaration of Helsinki. Approval from the local ethical committee was obtained before the start of the study (the study protocol is available from our local ethical committee, Comitato Etico di Area Vasta Nord-Ovest [CEAVNO], protocol #687). Because of the exploratory characteristics of the present study, no sample size calculation was performed. Ten recipients receiving grafts preserved with standard CS (4°C) were compared 1:1 with recipients receiving grafts preserved with ex situ NMP. The primary study endpoint was to evaluate graft and patient survival at month 6 after transplantation. The secondary endpoint was to evaluate IRI by means of peak transaminases within 7 days after surgery, incidence of biliary complications at month 6, and evaluation of liver and bile duct histology.

INCLUSION CRITERIA

Patients were evaluated as per Italian National Transplant Agency (CNT) guidelines. All potential recipients of a DBD, whole-size, primary, ABO-compatible LT, older than 18 years old were considered eligible for the study. Recipients were enrolled by transplant hepatologists during pretransplant evaluation, informed about the study, and asked to sign the consent form at the time of wait listing. Recipients with a Model for End-Stage Liver Disease (MELD) score ≥24 at transplantation were not considered for the study. Once selected for transplantation, patients were asked to confirm their consent to the study and eventually randomized to the CS or the NMP group. Block randomization was with sealed envelopes prepared by a third party and opened once the graft was deemed fit for transplantation. The flow of participants through each stage is shown in Supporting Fig. 1.

DONOR SELECTION

Deceased donor data were obtained from clinical charts. Eligibility to liver donation was evaluated as per our institutional policy and according to the CNT guidelines.[14] The included variables were as follows: age; sex; body weight; height; body mass index (BMI); cause of death; liver and renal function tests at procurement; history of comorbidities (diabetes mellitus, hypertension, cardiovascular disease, renal disease,

hemodialysis, and dyslipidemia); use of inotropic agents; history of cardiac arrests or hemodynamic instability; intensive care unit (ICU) stay; location of procurement; serologic status with regard to hepatitis B virus (HBV) infection and HCV, and cultures when applicable. For the purpose of current analysis, donor hemodynamic instability was defined as any donor experiencing a clinically documented cardiac arrest or requiring noradrenaline or more than 1 vasopressor to maintain a mean arterial pressure (MAP) >60 mm Hg. Donors were classified as affected by diabetes mellitus, dyslipidemia, or hypertension if on medication and regardless of the onset date. The donor evaluation policy in use at our center has been published previously.[5] Once accepted for procurement, a liver graft biopsy was performed on demand depending on surgical evaluation at procurement. A graft was discarded in the presence of any of the following: macrovesicular steatosis >30%; necrosis >5%; fibrosis ≥2 as per Ishak's score[15]; severe microangiopathy (as per arteriolar thickening >60%), and macroangiopathy with impossibility to perform arterial anastomosis. All grafts were considered transplantable before patient randomization.

RECIPIENTS

All LT recipients were evaluated in the pretransplant setting and followed up after surgery according to our institutional policies. Data included in the current analysis were as follows: demographics (age, sex, body weight, height, and BMI); indication to LT; MELD score; donor age × Model for End-Stage Liver Disease (D-MELD)[16]; posttransplant surgical complications (according to the modified Clavien-Dindo[17] classification system); and graft and patient survival. For the purpose of this study, patients were followed for 6 months after LT. A posttransplant biliary complication was any abnormality in the biliary tree associated with symptoms or signs and requiring an endoscopic or surgical procedure. ITBL was any donor biliary tract with nonanastomotic stenosis requiring an endoscopic or radiological procedure in the absence of vascular complications. Early allograft dysfunction (EAD) was defined by the presence of 1 or more of the following: total bilirubin ≥10 mg/dL or international normalized ratio ≥1.6 on day 7, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) >2000 IU/L within the first 7 days, whereas postreperfusion syndrome (PRS) was defined as a

decrease in MAP >30% below the baseline value, lasting for at least 1 minute, and occurring during the first 5 minutes after reperfusion of the liver graft. Immunosuppression consisted of anti-CD25 induction, tacrolimus in association with antimetabolites (mycophenolic acid), and steroids for non-HCV-positive patients.

GRAFT ALLOCATION POLICY

Grafts were allocated based on the Italian score for organ allocation and have been previously described.[18]

SURGERY

All donors were procured with aortic and portal flush with Celsior solution (Genzyme-Sanofi, Milan, Italy) and en bloc liver and pancreas procurement. Grafts were stored at 4°C and shipped to our institution for back-table preparation and then perfused on the machine or preserved at 4°C until reperfusion in the recipient based on randomization. All transplants were performed using the conventional technique with vena cava replacement and venovenous bypass. A T-tube was used for duct-to-duct biliary anastomosis. The tube was removed 3 months after transplantation after percutaneous cholangiography.

NORMOTHERMIC MACHINE PERFUSION

Once randomized in the NMP group, grafts were reperfused at 37°C using the LiverAssist device (OrganAssist, Groningen, the Netherlands). According to the article by Karangwa et al.,[19] the procedure can be classified as post-CS NMP because the CS period was longer than 3 hours. The NMP procedure was performed in an operation room (OR) next to the transplant OR and under medical supervision. According to vessel size, a 10- or 12-Fr cannula was inserted at the origin of the celiac trunk, and the gastroduodenal, left gastric, and splenic arteries were tied. A blood-based perfusate (Supporting Table 1) was used. The initial perfusate temperature was set at 20°C and then raised by 1°C every 2 minutes until 37°C. Oxygenation was provided by an anesthesia ventilator and was initially set at 4 L/minute with 30% fraction of inspired oxygen. It was later adjusted based on perfusate pH, partial pressure of oxygen (PO_2), and partial pressure of carbon dioxide. A blood gas

analysis was performed every 20 minutes during the first hour and every 30 minutes thereafter. Our target was to maintain a physiological pH and ionogram, and a PO_2 between 200 and 250 mm Hg. Perfusate glucose, transaminases, lactate, interleukin (IL) 6, IL10, tumor necrosis factor α (TNF-α) concentration, and bile production were collected every hour. Cytokine concentrations were tested after the procedure by enzyme-linked immunosorbent assay sandwich assay and are reported as pg/mL (R&D Systems, Minneapolis, MN). Bile pH was measured using a GEM4000 analyzer (Instrumentation Laboratory, Bedford, MA). At the end of hepatectomy, grafts were flushed with cold Celsior solution both from the hepatic artery and the portal vein (2 L each; recooling time). The weight of the liver grafts was measured before and after NMP.

HISTOPATHOLOGY

Liver biopsies were collected at the end of back-table surgery (time point [T]1); at the end of NMP (NMP group only; T2); and at the end of transplantation (T4). They were fixed and stained with hematoxylineosin for standard histopathological analysis and with periodic acid–Schiff to detect changes in glycogen cell content. For the sake of comparison, changes in glycogen content were also assessed biochemically on homogenates of liver. Glycogen was measured on liver homogenates obtained by mechanical disruption using the Dounce homogenizer and was then quantified using a couple-enzyme assay (glucose oxidase and horseradish peroxidase), which produces a colorimetric product detected at 570 nm (Glycogen assay kit MAK016, Sigma-Aldrich, St. Louis, MO). The background signal generated by glucose was evaluated for each sample. Data were expressed as μg of glycogen per mg of tissue.

Bile duct biopsies were obtained at the end of back table (BT; T1); end of NMP (NMP group only; T2), and before biliary anastomosis (T3). They were evaluated as per op den Dries et al.[20]

ELECTRON MICROSCOPY

Liver biopsies for electron microscopy (EM) evaluation were obtained at the same time points (T1,2,4). Liver tissue samples were fixed with 2.5% (vol/vol) glutaraldehyde in 0.1 mmol/L cacodylate buffer (pH 7.4) for 1 hour at 4°C and then postfixed in 1% (vol/vol) cacodylate-buffered osmium tetroxide for 2 hours at room temperature. Samples were dehydrated in a graded series of ethanol, transferred to propylene oxide, and embedded in Epon-Araldite. The specimens acquired for EM were also used to obtain semithin (500-nm thick) sections for light microscopy analysis. Ultra-thin sections (60- to 80-nm thick) were cut with a diamond knife, placed on formvar/carbon-coated copper grids (200 mesh), and stained with uranyl acetate and lead citrate. Morphometric analyses were performed with stereologic techniques,[21] as previously described.[22] For the analysis of mitochondria, lipid droplets (LDs) and autophagic vacuoles micrographs were obtained at ×10,000 and analyzed by overlay with a grid (11 cm × 11 cm) composed of 169 or 121 points, respectively. The volume density of subcellular organelles was calculated according to the formula:

$$\text{volume density} = Pi/Pt,$$

where Pi is the total number of points within the subcellular component and Pt is the total number of points and expressed as mL/100 mL tissue (mL%).

STATISTICAL ANALYSIS

According to variables and their level of distribution, descriptive statistics are reported as medians (interquartile range [IQR]) and frequencies, as appropriate. Normality of distribution of quantitative variables was tested according to Kolmogorov-Smirnov. The 2-tailed Student t test was used to compare continuous variables across independent samples and paired data. For categorical variables, we used a 2-proportion z test. To determine the correlation between lactate, glucose, AST, TNF-α, IL6, and IL10 perfusate levels and between AST perfusate peak levels and posttransplant AST levels on postoperative day (POD) 1, a parametric correlation analysis was used (Pearson) and the results are expressed with r coefficient and P value. A P value <0.05 was considered statistically significant. All descriptive and inferential analyses were performed with the SPSS software (IBM, Chicago, IL).

Results

There were 20 patients who were enrolled in the study, and 10 were randomized in each group. The algorithm of participants through each stage is described in Supporting Fig. 1. Donor and recipient characteristics are summarized in Table 1. There were no differences between the 2 groups.

TABLE 1. Donor and Recipient Characteristics

Variables	NMP (n = 10)	CS Group (n = 10)	P Value
Recipient			
Sex, male	90%	80%	1.000
Age at transplant, years	57 (46-61)	55 (43-61)	0.649
Liver disease*			
HCV	60%	30%	0.369
HCC	20%	60%	0.171
HBV	30%	50%	0.648
Alcoholic cirrhosis	20%	30%	1.000
Biliary cirrhosis	10%	0%	1.000
Other	10%	20%	1.000
Biological MELD	12.5 (9-16)	9.5 (8-15)	0.425
D-MELD	1016 (711-1392)	778.5 (637-1200)	0.545
Donor			
Sex, male	70%	30%	0.180
Age, years	81 (77.5-87.2)	80 (72-87.2)	0.762
BMI, kg/m^2	25.5 (23.7-27.7)	24.7 (22-25.7)	0.384
ICU stay, days	2.0 (2.0-3.0)	2.5 (2.0-5)	0.754
Cause of death: CVA	100%	80%	0.456
Cause of death: trauma	0%	20%	0.456
Peak laboratory values during ICU stay			
AST, IU/L	24 (18-83)	38.5 (28-59)	0.405
ALT, IU/L	21.5 (14-27)	26.5 (23-61)	0.449
Total bilirubin, mg/dL	0.75 (0.55-0.96)	0.85 (0.78-1)	0.850
Na, mEq/L	153.5 (148-156)	153 (150-155)	0.790
Hemodynamic instability	10 (100)	9 (90)	1.000
Cardiac arrest	0 (0)	0 (0)	1.000
Use of norepinephrine	100%	80%	0.456
Diabetes mellitus	100%	90%	1.000
Dyslipidemia	30%	20%	1.000
Nephropathy	10%	10%	1.000
Arterial hypertension	70%	70%	1.000
Cardiopathy	40%	30%	1.000
Pretransplant liver biopsy	50%	40%	1.000
Transplantation			
CIT, minutes	246.5 (206-267)	394 (366-465)	0.0001
Normothermic perfusion time, minutes	250 (195-282)	NA	NA
Recooling time, minutes	20 (12-35)	NA	NA
Warm ischemia time, minutes	74 (70-82)	69 (62-78)	0.212
Total CIT, minutes	280 (242-297)	394 (366-465)	0.0001
Time from donor cross-clamping to graft reperfusion, minutes	522 (430-587)	462.5 (432-528)	0.406
Liver			
Graft weight (at end of back-table surgery), grams	1312.5 (1215-1450)	1362 (930-1450)	0.453
Graft weight (at end of NMP), grams	1382.5 (1250-1500)	NA	0.109[†]
Bile production	22.5 (20.0-38.7)	NA	

NOTE: Data are given as median (IQR) unless otherwise noted.
*Multiple diseases can be present in the same patient.
[†]Comparison between weight at end of BT and weight at end of NMP in the NMP group only.

TIMING

Median (IQR) cold ischemia time (CIT) was 394 (366-465) minutes in the CS group versus 246 (206-267) minutes in the NMP group (time from donor cross-clamping to reperfusion in the machine; $P < 0.0001$). Median (IQR) NMP time was 250 (196-282) minutes. The median (IQR) recooling time was 20 (12-35) minutes. Total CIT in the NMP group (CIT + recooling time) was 280 (242-297) minutes, 114 minutes shorter than the CS group ($P < 0.0001$), whereas the median (IQR) total time from cross-clamping to revascularization in the recipient was 462 (432-528) minutes in the CS group versus 522 (430-587) minutes in the NMP group ($P = 0.406$; Table 1).

PERFUSATE

All livers reached stable vascular flow at 2 hours (205-420 mL/minute for the artery; 1100-1700 mL/minute for the portal vein; Supporting Fig. 2). The perfusate peak AST level ranged from 219 to 3125 IU/L (Fig. 1A), and it showed correlation with glucose concentration (Table 2) but not with postreperfusion AST levels on POD 1 ($P = 0.092$; $r = 0.560$). Terminal perfusate pH ranged from 7.19 to 7.44 (Fig. 2A). A rapid decrease in lactates (Fig. 2B) was observed except in 1 patient with suboptimal portal perfusion at procurement. Three hours after the start of NMP, the perfusate lactate concentration started rising in all patients. The perfusate glucose concentration is shown in Fig. 2C. TNF-α
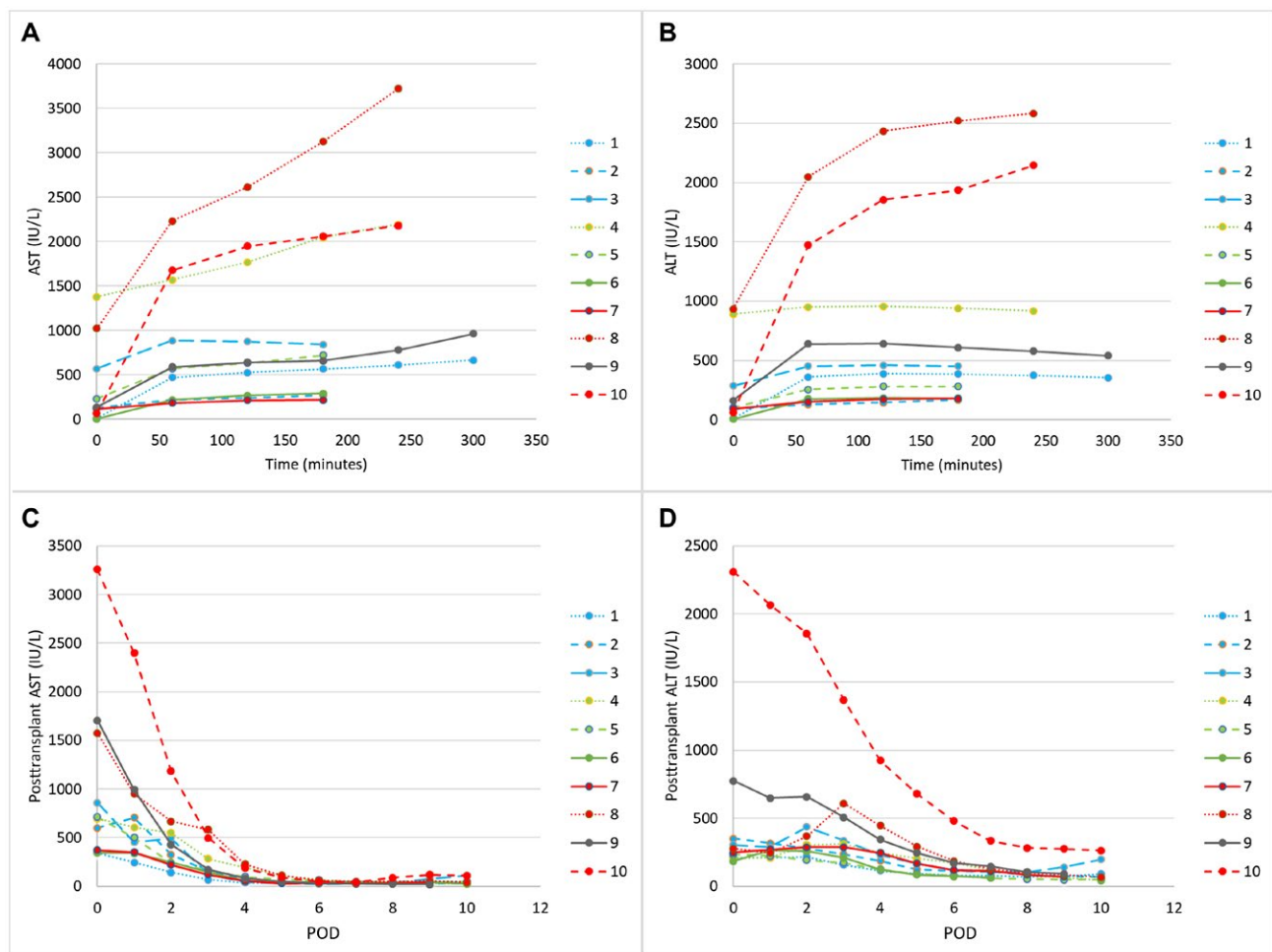

FIG. 1. Perfusate (A) AST and (B) ALT and posttransplant (C) AST and (D) ALT. Patients who developed EAD are shown with a red dotted line. The patient with HAT is shown by a continuous red line.

TABLE 2. Pearson's Correlation Analysis (*r*) Between Perfusate Lactate, Glycemia, AST Peak, TNF-α, IL10, IL6, and POD 1 AST Concentration in the NMP Group

	Lactate	Glycemia	AST peak	TNF-α	IL6	IL10
Glycemia						
r	0.073					
P value	0.493					
AST peak						
r	−0.194	0.704				
P value	0.213	<0.0001				
TNF-α						
r	−0.486	−0.19	−0.043			
P value	0.001	0.211	0.779			
IL6						
r	−0.600	0.390	0.367	0.380		
P value	<0.0001	0.008	0.013	0.008		
IL10						
r	−0.700	0.268	0.385	0.232	0.668	
P value	<0.0001	0.075	0.009	0.117	<0.0001	
POD 1 AST						
r	0.463	−0.310	0.011	0.270	−0.338	−0.290
P value	0.178	0.383	0.978	0.450	0.340	0.416

levels are shown in Fig. 2D, whereas IL6 and IL10 are shown in Supporting Fig. 3. Correlation was observed between TNF-α, IL6, IL10, and lactate (Table 2), and results are reported in Table 2. All grafts produced bile during NMP, ranging from 5 to 54 mL. The biochemical characteristics of bile at 2 hours (pH, glycemia, bicarbonate, and sodium level) are reported in Table 3.

GRAFT AND PATIENT SURVIVAL

One case of graft loss was observed in the NMP group due to hepatic artery thrombosis (HAT) on POD 9. The patient was retransplanted on POD 10 and is alive at a follow-up of 1 year. One patient in the CS group died on POD 31 from septic shock after readmission for intestinal occlusion.

POSTOPERATIVE RESULTS

The postoperative results are shown in Table 4. No PNF was observed in either group. There were 2 cases of EAD in NMP and 1 in CS, respectively (Fig. 1C,D). A total of 3 cases of PRS were observed in the NMP group versus 1 in the CS group. No differences were observed in terms of postoperative transaminases or bilirubin peak. No other vascular complication was observed. One patient was

diagnosed with biliary casts 1 month after T-tube removal and was successfully treated with endoscopic retrograde cholangiopancreatography.

HISTOLOGY

Liver biopsies (necrosis, steatosis, and periodic acid–Schiff–glycogen evaluation) and bile duct histology are reported in Table 5.

Intracellular glycogen increased after NMP, followed by substantial decrease at the end of surgery in both groups (Fig. 3), and the mean difference between BT and surgery was comparable in the 2 groups (−1.08 ± 1.88 μg/mL for NMP versus −0.78 ± 0.91 μg/mL for CS; *P* = 0.708).

ELECTRON MICROSCOPY

EM evaluation of liver samples showed similar ultrastructural alterations at the end of BT in both groups: mitochondrial swelling (Supporting Fig. 4); presence of several LDs in the cytoplasm of the hepatocytes (Supporting Fig. 5A,B); biliary poles (BPs) with the presence of dense bodies (Supporting Fig. 5C,D); small Disse spaces (Supporting Fig. 6A,B); and nonactivated Kupffer cells (Supporting Fig. 6C,D). Liver samples obtained after NMP showed diffuse and massive activation of autophagy, as demonstrated by the presence
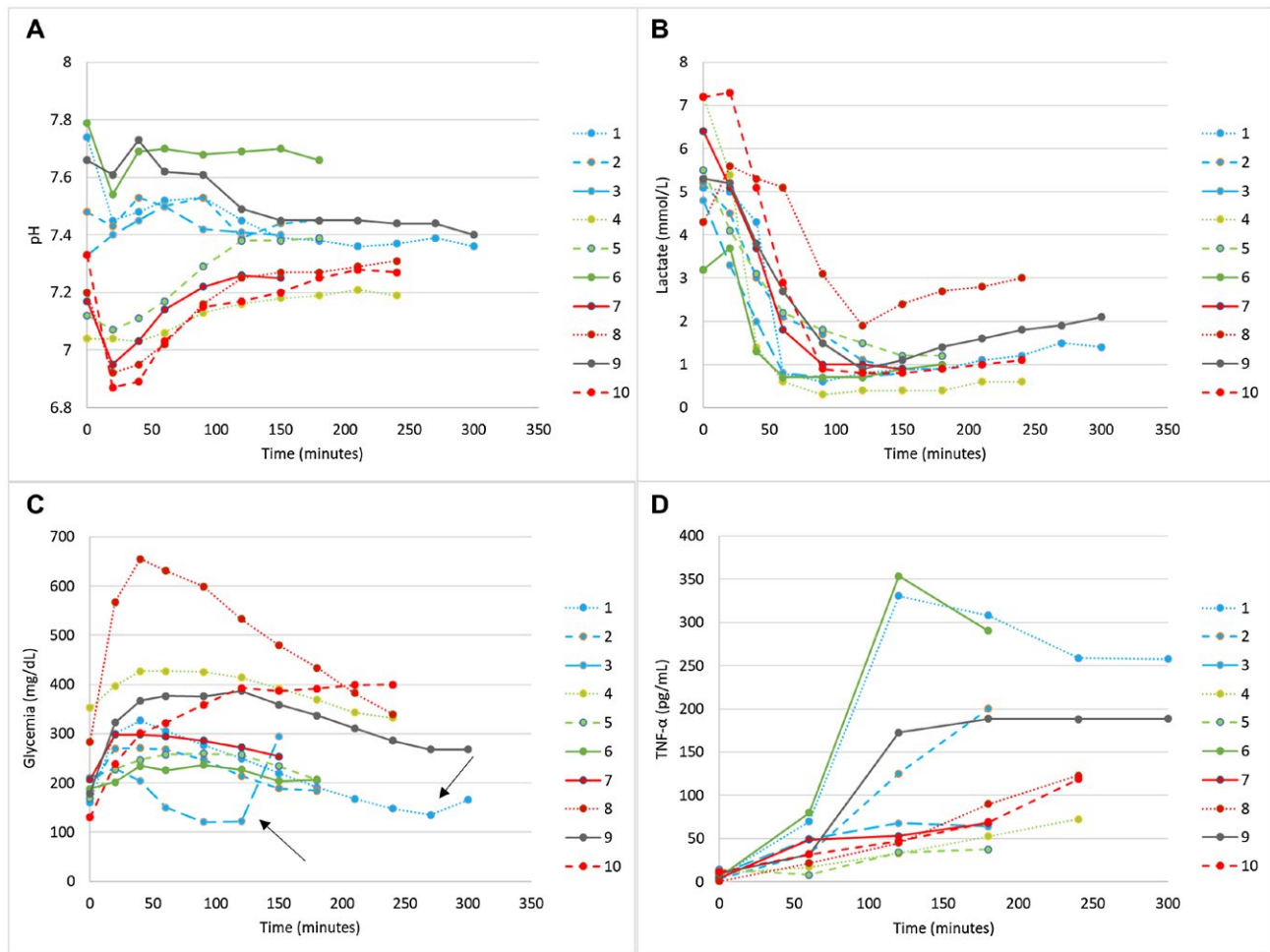
FIG. 2. (A) pH, (B) lactate, (C) glycemia, and (D) TNF-α perfusate concentration during NMP. The arrows indicate glucose administration.

of several autophagic vacuoles in the hepatocytes (Fig. 4A-E). Liver samples retrieved 2 hours after reperfusion showed significant differences between the 2 groups. In the CS group, progression of mitochondrial swelling (Supporting Fig. 7A,B); further LD accumulation (Supporting Fig. 7C,D); increase in dense bodies at the level of BPs (Supporting Fig. 8A); damaged endothelial cells (Supporting Fig. 8B), activated Kupffer cells (Supporting Fig. 8C), and neutrophil infiltration (Supporting Fig. 8D) were observed. On the other hand, 2 hours after implantation, hepatocytes of NMP livers showed normal-size mitochondria, a reduced number of LDs (Supporting Fig. 9A-C), and only a few dense bodies in the BPs (Supporting Fig. 9D), whereas no damaged vessel endothelium or neutrophil infiltration was found.

Mitochondrial volume density increased after transplantation in CS versus NMP patients (Fig. 5A). At the end of NMP, a significant reduction of volume density of intracellular LDs (Fig. 5B) was observed concurrent with an increase in volume density of autophagic vacuoles (Fig. 5C).

Discussion

LT has gradually emerged as the treatment of choice in patients with end-stage liver disease, but the growing gap between organ supply and demand has prompted expansion of allograft selection criteria.[23] Older, steatotic, and suboptimal liver grafts show reduced tolerance to cold ischemia preservation[24]
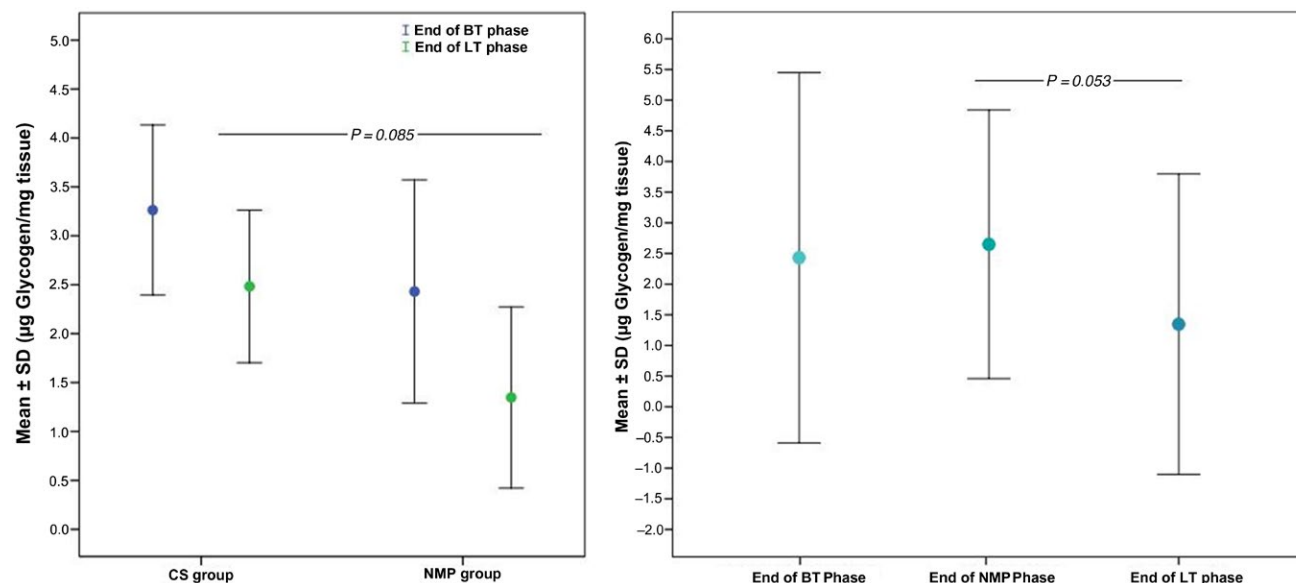
FIG. 3. Glycogen concentration measurement in the 2 groups at different time points.

TABLE 3. Bile pH, Sodium, Glycemia, Lactate, and Bicarbonate Levels 120 Minutes After the Initiation of NMP

Patient Number	pH	Sodium (mmol/L)	Glycemia (mg/dL)	Lactate (mmol/L)	HCO$_3$- (mmol/L)
1	7.39	142	207	1.1	12.1
2	7.37	154	69	1.7	13.3
3	7.43	143	67	3.1	15.3
4	7.67	153	133	1.9	27.7
5	7.52	148	186	2.7	14.7
6	6.88	152	151	1.1	6.7
7*	7.17	150	73	1.4	19.3
8†	7.00	140	497	2.7	7.9
9	7.75	157	166	1.2	11.1
10†	6.94	133	180	6.4	5.6

NOTE: Patient 6 had high perfusate peak AST and ALT levels (2195 and 958 IU/L, respectively) but had relatively low postoperative peak AST and ALT levels (695 and 313 IU/L, respectively).
*Patient developed HAT.
†Patients developed EAD.

and increased vulnerability to IRI.[25] One of the most promising technical innovations in the field of organ preservation has been the introduction of MP for liver allografts.[26] By delivering oxygen and nutrients at physiological temperature, NMP maintains aerobic metabolism preventing mitochondrial damage caused by adenosine triphosphate depletion.[27,28] The potential benefits of NMP are under investigation, and some clinical studies have already been published.[29,30] However, most of these have used historical matched cohorts and lack statistical power to appraise efficacy of this new technology. In their randomized study comparing conventional CS to NMP, Nasralla et al. showed improved post-LT transaminases peak but similar graft and patient survival rates.[31]

In the last few years, the progressive aging of donors has spurred development of extended criteria and identification of allocation strategies to optimize the outcome of LT from elderly donors,[5] due to a higher

TABLE 4. Posttransplant Outcomes in the 2 Groups

Variables	NMP Group (n = 10)	CS Group (n = 10)	P Value
Patient death	0	10	1.000
PNF	0	0	1.000
Graft loss	10	0	1.000
EAD, %	20	10	1.000
PRS, %	30	10	0.576
Intraoperative blood units	4.0 (2-7)	3.5 (0-6)	0.443
Intraoperative FFP units	5 (0-9)	3 (0-6)	0.357
AST peak, IU/L	709 (371-1575)	574 (377-1162)	0.597
ALT peak, IU/L	332 (263-610)	428 (303-616)	0.821
Bilirubin peak, mg/dL	5.5 (3.8-8.2)	5.0 (3.2-6.3)	0.364
Vascular complications	10	0	1.000
Biliary complications	10	0	1.000
Hospital stay, days	17.0 (14.0-22)	12 (11-15)	0.119

NOTE: Data are given as median (IQR) and percentage.

incidence of DGF[7] and ITBL[10] when compared with younger ones. We designed the current pilot study in order to evaluate the potential of NMP in reducing the negative impact of IRI on older liver grafts, given their vulnerability to perfusion injuries.

Even though in our series all liver grafts were accepted prior to use of MP, this experience shows that NMP is feasible. Given its limited sample size and the relatively low MELD score of enrolled patients, the study lacks the statistical power to detect any difference in patient and graft survival. No case of PNF was observed, and the incidence of EAD was comparable between the 2 groups, but NMP was not associated with any major advantage when compared with CS. The incidence of PRS, postoperative transaminases, major complications, and length of hospitalization were similar across groups. The risk of endothelial injury and vascular complications requires special consideration. Perfusion cannulas are to be inserted far away from the presumed anastomotic site—usually at the level of the donor celiac trunk—in order to minimize vessel injuries or dehydration. The dual thermic shock might promote damage to the liver endothelium and release of procoagulant factors such as factor VIII or von Willebrand factor. From a logistic point of view, NMP shows limited advantage. Even if successful cases of prolonged NMP have recently been reported,[32] in our experience progressive increase in lactates was observed approximately 3 hours after the start of perfusion. This increase might be indicative of suboptimal liver grafts[33] but could also be accounted for by progressive accumulation of toxic metabolites and proinflammatory mediators, with a negative impact on graft metabolism and reconditioning. The correlation between TNF-α and perfusate lactate is evidence that accumulation of proinflammatory and diabetogenic ILs are detrimental. Although intracellular glycogen concentrations improve after NMP, histologic samples collected at the end of surgery showed no advantage across groups. Future steps should aim at improving technologies in view of purifying the perfusate during NMP and avoiding the cold phase during graft procurement.[34]

A current advantage of NMP is that it allows dynamic evaluation of liver grafts. Lactate clearance, bile production, and graft inspection during perfusion are all key elements in evaluating graft quality, whereas glycemia and transaminases levels can be considered an indirect measure of graft injury. Our experience shows that a low bile pH (<7.1) is associated with higher perfusate or postoperative AST and ALT levels, and it should be considered as a marker of severe IRI. Others[35] postulated that ischemic cholangiopathy (IC) might be predicted by a bile pH <7.4. In agreement with Watson et al.,[35] we believe that bile production and pH is a complex process dependent on the integrity of many facets of liver function, but several other parameters may play a fundamental role in the development of IC such as the type of donor (DCD versus DBD), the warm ischemia time, or the procurement technique (single versus dual perfusion).[36]

The mechanisms underlying the beneficial effects of NMP on transplanted livers have limitedly been investigated, and scanty information can be expected by limited series. Nevertheless, histological analyses did not show major benefit in the NMP group whose biopsies are comparable to the CS group, apart from injuries to periluminal biliary glands. NMP might have the potential to detect IRI-induced biliary injuries earlier than other clinical variables. The degree of steatosis seems comparable between the 2 groups, whereas glycogen concentration showed a greater decrease in the NMP group at the end of LT.

Restoration and maintenance of mitochondrial function is a key factor for prevention of the negative effects of CS preservation.[27] Our observation that NMP was associated with a significant decrease in mitochondrial swelling is in keeping with this consideration. It has been demonstrated previously

TABLE 5. Liver and Bile Duct Histological Evaluation

	End BT			End of NMP		End LT		
	CS Group	NMP Group	P Value	NMP Group	P Value*	CS Group	NMP Group	P Value
Liver biopsy								
Steatosis			1.000					0.456
≤10%	9	10		10		8	10	
10%-30%	1	0		0		2	0	
>30%	0	0		0		0	0	
Glycogen variation			0.470		0.478			0.019
≤30%	0	1		0		2	8	
30%-60%	4	5		7		5	2	
>60%	6	4		3		3	0	
Necrosis			1.000		0.348			0.865
<5%	9	8		5		6	5	
5%-10%	1	2		5		2	3	
>10%	0	0		0		2	2	
Bile duct biopsy injury score (n)								
Biliary epithelial injury			<0.0001		0.002			1.000 without Grade 1
Grade 0	0	0		0		0	1	
Grade 1	0	9		1		0	0	
Grade 2	10	1		9		10	9	
Mural stroma necrosis			0.626		0.846			0.065
Grade 0	7	7		3		6	2	
Grade 1	3	3		2		3	1	
Grade 2	0	0		2		0	3	
Grade 3	0	0		3		1	4	
Peribiliary vascular			1.000		0.171			0.717
Grade 0	9	10		7		7	7	
Grade 1	1	0		2		2	1	
Grade 2	0	0		1		1	2	
Grade 3	0	0		0		0	0	
Thrombosis					1.000			
Grade 0	10	10		9		10	10	
Grade 1	0	0		1		0	0	
Intramural bleeding					1.000			1.000
Grade 0	10	10		9		5	6	
Grade 1	0	0		1		5	4	
Grade 2	0	0		0		0	0	
Periluminal PBG injury			0.895		0.036			0.648
Grade 0	1	1		2		0	0	
Grade 1	4	5		0		5	3	
Grade 2	5	4		8		5	7	
Deep PBG injury			0.871		1.000			0.325
Grade 0	1	1		1		1	1	
Grade 1	7	6		6		9	7	
Grade 2	2	3		3		0	2	

TABLE 5. *Continued*

	End BT			End of NMP		End LT		
	CS Group	NMP Group	*P* Value	NMP Group	*P* Value*	CS Group	NMP Group	*P* Value
Inflammation			0.039		0.451			0.314
Grade 0	10	5		3		7	4	
Grade 1	0	5		6		3	5	
Grade 2	0	0		1		0	1	

*Comparison between end of BT and end of NMP in the NMP group.

FIG. 4. (A) Electron micrographs of liver samples after normothermic perfusion showing a diffuse and relevant activation of autophagy. Scale bar corresponds to 1 μm. Electron micrographs of liver samples after normothermic perfusion showing (B) autophagic vacuoles containing ER membranes, (C) degraded mitochondria, or (D,E) unrecognizable degraded material. Scale bars correspond to 0.45 μm.
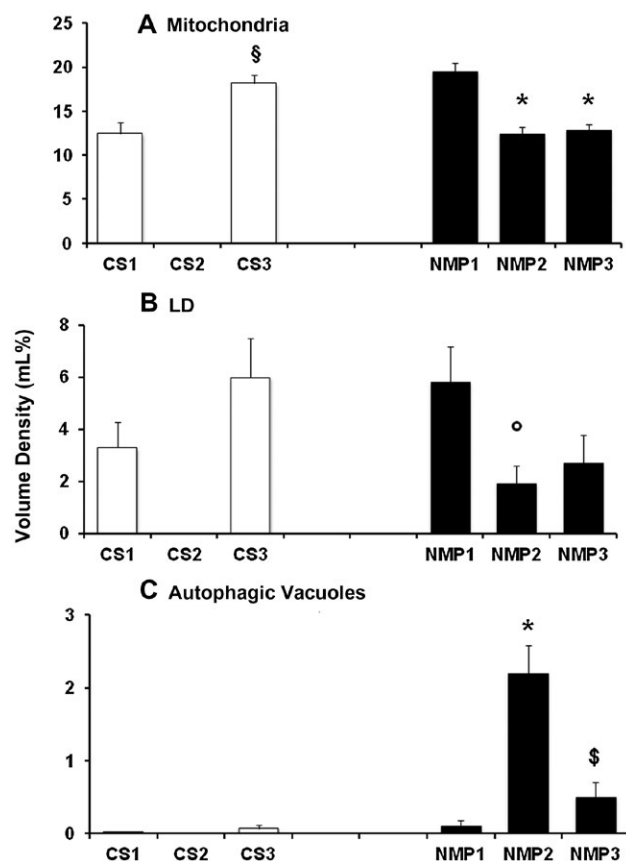
FIG. 5. Quantitative morphometric analysis of volume density of (A) mitochondria, (B) LDs, and (C) autophagic vacuoles in control and perfused livers at different time points: end of BT (CS1, NMP1), end of NMP (NMP2), and end of LT (CS3, NMP3). Data are expressed as mean ± standard error of the mean of n = 10 cases for each point. Statistical analysis (post hoc Student t test): §$P < 0.01$ versus CS1; *$P < 0.01$ versus NMP1; °$P < 0.05$ versus NMP1; $P < 0.01$ versus NMP2.

that NMP can contribute to reduction of macrovesicular liver steatosis.[37] In agreement with this finding, we observed a significant reduction in the volume density of liver droplets in perfused hepatocytes. Ultrastructural modifications similar to those observed in the present study (ie, mitigation of deleterious alterations in sinusoidal microvasculature and hepatocellular mitochondria, both related to steatosis) were previously described in steatotic livers treated with subnormothermic MP.[38] Another relevant observation of our study was activation of autophagy in the liver. Autophagy is a physiologically preserved and tightly regulated process that involves the degradation of cellular components through the lysosomal machinery.[39] A major role of autophagy is

to derive nutrients from endogenous sources for survival purposes and under conditions such as starvation or deprivation of growth factors.[40] Recently, the induction of autophagy has emerged as a new potential strategy to improve liver function after IRI.[41,42] Overall, the experimental evidence reported so far suggests that activation of autophagy may improve IRI by providing cells with energy derived from lysosomal degradation of cellular components.[42] NMP could possibly activate cell repair programs through degradation of the organelles damaged during CS.

Despite its limitations and the limited sample size, the present study shows that NMP with older liver grafts is associated with histological evidence of reduced IRI, although the clinical benefit remains to be demonstrated. Further studies are favored to better highlight the physiologic mechanisms activated by NMP and how to optimize the currently available technology.

REFERENCES

1) Dasari BVM, Schlegel A, Mergental H, Perera MTPR. The use of old donors in liver transplantation. Best Pract Res Clin Gastroenterol 2017;31:211-217.

2) Lai JC, Covinsky K, Feng S. The octogenarian donor: can the liver be "younger than stated age"? Am J Transplant 2014;14:1962-1963.

3) Cameron AM, Ghobrial RM, Yersiz H, Farmer DG, Lipshutz GS, Gordon SA, et al. Optimal utilization of donor grafts with extended criteria: a single-center experience in over 1000 liver transplants. Ann Surg 2006;243:748-753.

4) Feng S, Goodrich NP, Bragg-Gresham JL, Dykstra DM, Punch JD, DebRoy MA, et al. Characteristics associated with liver graft failure: the concept of a donor risk index. Am J Transplant 2006;6:783-790.

5) Ghinolfi D, Marti J, De Simone P, Lai Q, Pezzati D, Coletti L, et al. Use of octogenarian donors for liver transplantation: a survival analysis. Am J Transplant 2014;14:2062-2071.

6) Charlton M, Gane E, Manns MP, Brown RS Jr, Curry MP, Kwo PY, et al. Sofosbuvir and ribavirin for treatment of compensated recurrent hepatitis C virus infection after liver transplantation. Gastroenterology 2015;48:108-117.

7) Ghinolfi D, Lai Q, Pezzati D, De Simone P, Rreka E, Filipponi F. Use of elderly donors in liver transplantation: a paired-match analysis at a single center. Ann Surg 2018;268:325-331.

8) Detry O, Deroover A, Meurisse N, Hans MF, Delwaide J, Lauwick S, et al. Donor age as a risk factor in donation after circulatory death liver transplantation in a controlled withdrawal protocol programme. Br J Surg 2014;101:784-792.

9) Pezzati D, Ghinolfi D, De Simone P, Balzano E, Filipponi F. Strategies to optimize the use of marginal donors in liver transplantation. World J Hepatol 2015;7:2636-2647.

10) Ghinolfi D, De Simone P, Lai Q, Pezzati D, Coletti L, Balzano E, et al. Risk analysis of ischemic-type biliary lesions after liver transplant using octogenarian donors. Liver Transpl 2016;22:588-598.

11) Lai Q, Melandro F, Levi Sandri GB, Mennini G, Corradini SG, Merli M, et al. Use of elderly donors for liver transplantation: has the limit been reached? J Gastrointestin Liver Dis 2011;20:383-387.

12) Gilbo N, Catalano G, Salizzoni M, Romagnoli R. Liver graft preconditioning, preservation and reconditioning. Dig Liver Dis 2016;48:1265-1274.

13) Liu Q, Nassar A, Farias K, Buccini L, Baldwin W, Mangino M, et al. Sanguineous normothermic machine perfusion improves hemodynamics and biliary epithelial regeneration in donation after cardiac death porcine livers. Liver Transpl 2014;20:987-999.

14) Nanni Costa A, Grossi P, Gianelli Castiglione A. Grigioni WF; for Italian Transplant Research Network. Quality and safety in the Italian donor evaluation process. Transplantation 2008;27(suppl):S52-S56.

15) Ishak K, Baptista A, Bianchi L, Callea F, De Groote J, Gudat F, et al. Histological grading and staging of chronic hepatitis. J Hepatol 1995;22:696-699.

16) Halldorson JB, Bakthavatsalam R, Fix O, Reyes JD, Perkins JD. D-MELD, a simple predictor of post liver transplant mortality for optimization of donor/recipient matching. Am J Transplant 2009;9:318-326.

17) Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. Ann Surg 2004;240:205-213.

18) Cillo U, Burra P, Mazzaferro V, Belli L, Pinna AD, Spada M, et al. for I-BELT (Italian Board of Experts in the Field of Liver Transplantation). A multistep, consensus-based approach to organ allocation in liver transplantation: toward a "blended principle model." Am J Transplant 2015;15:2552-2561.

19) Karangwa SA, Dutkowski P, Fontes P, Friend PJ, Guarrera JV, Markmann JF, et al. Machine perfusion of donor livers for transplantation: a proposal for standardized nomenclature and reporting guidelines. Am J Transplant 2016;16:2932-2942.

20) op den Dries S, Westerkamp AC, Karimian N, Gouw AS, Bruinsma BG, Markmann JF, et al. Injury to peribiliary glands and vascular plexus before liver transplantation predicts formation of non-anastomotic biliary strictures. J Hepatol 2014;60:1172-1179.

21) Weibel ER. Stereological Methods. Vol. 1. Practical Methods for Biological Morphometry. New York, NY: Academic Press; 1979.

22) Masini M, Martino L, Marselli L, Bugliani M, Boggi U, Filipponi F, et al. Ultrastructural alterations of pancreatic beta cells in human diabetes mellitus. Diabetes Metab Res Rev 2017;33:e2894.

23) Starzl TE, Fung JJ. Themes of liver transplantation. Hepatology 2010;51:1869-1884.

24) Reddy S, Zilvetti M, Brockmann J, McLaren A, Friend P. Liver transplantation from non-heart-beating donors: current status and future prospects. Liver Transpl 2004;10:1223-1232.

25) de Rougemont O, Lehmann K, Clavien PA. Preconditioning, organ preservation, and postconditioning to prevent ischemia-reperfusion injury to the liver. Liver Transpl 2009;15:1172-1182.

26) Marecki H, Bozorgzadeh A, Porte RJ, Leuvenink HG, Uygun K, Martins PN. Liver ex situ machine perfusion preservation: a review of the methodology and results of large animal studies and clinical trials. Liver Transpl 2017;23:679-695.

27) Brockmann J, Reddy S, Coussios C, Pigott D, Guirriero D, Hughes D, et al. Normothermic perfusion: a new paradigm for organ preservation. Ann Surg 2009;250:1-6.

28) Friend PJ. Advances in normothermic perfusion of the liver. Liver Transpl 2017;23(suppl 1):S50-S51.

29) Ravikumar R, Jassem W, Mergental H, Heaton N, Mirza D, Perera MT, et al. Liver transplantation after ex vivo normothermic machine preservation: a phase 1 (first-in-man) clinical trial. Am J Transplant 2016;16:1779-1787.

30) Bral M, Gala-Lopez B, Bigam D, Kneteman N, Malcolm A, Livingstone S, et al. Preliminary single-center Canadian experience of human normothermic ex vivo liver perfusion: results of a clinical trial. Am J Transplant 2017;17:1071-1080.

31) Nasralla D, Coussios CC, Mergental H, Akhtar MZ, Butler AJ, Ceresa CDL, et al. for Consortium for Organ Preservation in Europe. A randomized trial of normothermic preservation in liver transplantation. Nature 2018;557:50-56.

32) Watson CJ, Randle LV, Kosmoliaptsis V, Gibbs P, Allison M, Butler AJ. 26-hour storage of a declined liver before successful transplantation using ex vivo normothermic perfusion. Ann Surg 2017;265:e1-e2.

33) Sutton ME, op den Dries S, Karimian N, Weeder PD, de Boer MT, Wiersema-Buist J, , et al. Criteria for viability assessment of discarded human donor livers during ex vivo normothermic machine perfusion. PLoS ONE 2014;9:e110642.

34) He X, Guo Z, Zhao Q, Ju W, Wang D, Wu L, et al. The first case of ischemia-free organ transplantation in humans: a proof of concept. Am J Transplant 2018;18:737-744.

35) Watson CJE, Kosmoliaptsis V, Randle LV, Gimson AE, Brais R, Klinck JR, et al. Normothermic perfusion in the assessment and preservation of declined livers before transplantation: hyperoxia and vasoplegia-important lessons from the first 12 cases. Transplantation 2017;101:1084-1098.

36) Ghinolfi D, Tincani G, Rreka E, Roffi N, Coletti L, Balzano E, et al. Dual aortic and portal perfusion at procurement prevents ischemic-type biliary lesions in liver transplantation when using octogenarian donors: a retrospective cohort study. Transpl Int 2019;32:193-205.

37) Banan B, Watson R, Xu M, Lin Y, Chapman W. Development of a normothermic extracorporeal liver perfusion system toward improving viability and function of human extended criteria donor livers. Liver Transpl 2016;22:979-993.

38) Okamura Y, Hata K, Tanaka H, Hirao H, Kubota T, Inamoto O, et al. Impact of subnormothermic machine perfusion preservation in severely steatotic rat livers: a detailed assessment in an isolated setting. Am J Transplant 2017;17:1204-1205.

39) Kim KH, Lee MS. Autophagy–a key player in cellular and body metabolism. Nat Rev Endocrinol 2014;10:322-337.

40) Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. J Pathol 2010;221:3-12.

41) Wang JH, Behrns KE, Leeuwenburgh C, Kim JS. Critical role of autophagy in ischemia/reperfusion injury to aged livers. Autophagy 2012;8:140-141.

42) Cursio R, Colosetti P, Gugenheim J. Autophagy and liver ischemia-reperfusion injury. Biomed Res Int 2015;2015:417590.

ORIGINAL ARTICLE | 449