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Sequential use of normothermic regional and ex-situ machine perfusion in DCD liver transplant

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List of abbreviations:

AKI: acute kidney injury

ALT: alanine aminotransferase

AST: aspartate aminotransferase

BMI: body mass index

CIT: cold ischemia time

CNT: Italian national transplant center

CS: cold storage

CVA: cardio-vascular accident

DBD: donation after brain death

DCD: donation after cardio-circulatory death

cDCD: controlled donation after cardio-circulatory death

uDCD: uncontrolled donation after cardio-circulatory death

DHOPE: dual hypothermic oxygenated perfusion

D-MELD: donor age*Model for end-stage liver disease

EAD: early allograft dysfunction

ECMO: extracorporeal membrane oxygenation

EKG: electrocardiogram

FFP: fresh frozen plasma

GGT: gamma-glutamyl-transferase

HAT: hepatic artery thrombosis

HBV: hepatitis B virus

HCC: hepatocellular carcinoma

HCV: hepatitis C virus

H&E: hematoxylin and eosin

ICU: intensive care unit

IL: interleukin

INR: international normalized ratio

IQR: interquartile range

IRI: ischemia reperfusion injury

ISO: Italian score for organ allocation

ITBL: ischemic type biliary lesion

KDIGO: Kidney disease: improving global outcome

LF: low flow

LT: liver transplantation

LUCAS: Lund University cardiopulmonary assist system

MAP: mean arterial pressure

MELD: Model for end-stage liver disease

MP: machine perfusion

NMP: normothermic machine perfusion

NF: no flow

NRP: normothermic regional perfusion

OR: operation room

PAS: periodic acid/Schiff

PNF: primary non function

POD: post-operative day

PRS: post-reperfusion syndrome

TNF: tumor necrosis factor

WIT: warm ischemia time

f-WIT: functional warm ischemia time

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Abstract

In Italy, 20 minutes of continuous, flat-line electrocardiogram are required for declaration of death. In the setting of organ donation after cardiocirculatory death (DCD), prolonged warm ischemia time prompted introduction of abdominal normothermic regional perfusion (NRP) followed by post-procurement, ex-situ machine perfusion.

This was a retrospective review of DCD liver transplantations performed at two centers using sequential NRP and ex-situ machine perfusion. From January 2018 to April 2019, 34 DCD donors were evaluated. Three (8.8%) were discarded before NRP, 11 (32.4%) based on NRP parameters (n=1, 3.0%), liver macroscopic appearance at procurement and/or biopsy results (n=9, 26.5%), or severe macroangiopathy at back table evaluation (N=1, 3.0%). Twenty grafts (58.8%) (12 uncontrolled DCD, 8 controlled DCD) were considered eligible for LT, procured and perfused ex-situ (9 normothermic and 11 dual hypothermic machine perfusion). Eighteen (52.9%; 11 uncontrolled) were eventually transplanted.

Median (IQR) no-flow time was 32.5 (30-39) minutes, while median functional-warm ischemia time was 52.5 (47-74) minutes (controlled DCD) and median low-flow time 112 (105-129) minutes (uncontrolled DCD). There was no primary non-function, while post-reperfusion syndrome occurred in 8 (44%) recipients. Early allograft dysfunction happened in 5 (28%) patients, while acute kidney injury in 5 (28%). After a median follow up of 15.1 (9.5-22.3) months, one case of ischemic-type biliary lesion and one patient death were reported.

DCD liver transplantation is feasible even with the 20-minute no-touch rule. Strict normothermic regional perfusion and ex-situ machine perfusion selection criteria are needed to optimize post-operative results.

Introduction

The ongoing mismatch between patients waitlisted for liver transplantation (LT) and graft availability has prompted use of donation after circulatory death (DCD)[1]. However, prolonged warm ischemia time (WIT) may increase rates of graft dysfunction and post-transplant complications[2,3]. In Italy, 20 minutes of continuous, flat-line electrocardiogram (EKG) are required for declaration of death for both controlled (cDCD) and uncontrolled (uDCD) donors[4]. This long WIT has led to implementation of abdominal normothermic regional perfusion (NRP) immediately after death declaration[5]. NRP with extracorporeal membrane oxygenation (ECMO) allows to restore blood circulation, regenerate cellular energy substrates, and reduce organ damage, thus converting urgent organ recovery into an elective procurement procedure[6].

Use of machine perfusion (MP) has expanded in the last few years due to its capacity to preserve grafts in quasi-physiological conditions before implantation, reduce cold storage (CS)-related injuries, and assess graft function prior to transplantation. In addition, MP can be combined with organ repair and reconditioning with the potential to expand the organ donor pool beyond the currently accepted criteria[7]. However, there is no consensus on the optimal temperature of MP and if either dual-hypothermic (DHOPE) or normothermic machine perfusion (NMP) should be preferred after NRP. By limiting ischemia-reperfusion injury (IRI) and damage to cholangiocytes during preservation[8], MP may reduce the risk of primary non-function (PNF), early allograft dysfunction (EAD), and biliary complications[9].

Herein we report the experience of two centers on the sequential use of NRP and ex-situ, end-ischemic MP in DCD LT with prolonged WIT, focusing our attention on graft selection pathway and criteria.

Materials and methods

This study included all consecutive DCD LT performed at the transplant centers of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico in Milan and at the University of Pisa Medical School Hospital from January 2018 to April 2019. All procedures were performed using sequential NRP and ex-situ DHOPE or NMP.

Inclusion criteria

Recipients older than 18 years and with a laboratory MELD score <25 were considered eligible for a DCD graft at both institutions. Patients were evaluated as per the Italian National Transplant Agency (Centro Nazionale Trapianti, [CNT]) guidelines[10]. Recipients were required to provide an informed consent at the time of waitlisting and once a potential DCD graft was available.

Donor selection

uDCDs were potential donors who suffered out-of-hospital cardiac arrest, underwent cardiopulmonary resuscitation onsite, and were transferred to hospital under mechanical chest compression (LUCAS - Lund University cardiopulmonary assist system), Chest Compression System–Jolife AB/Physio-Control, Lund, Sweden). Death was declared after 20 minutes of flat-line EKG[11]. Once family consent to organ donation was obtained, the donor's femoral vessels were cannulated and a Fogarty balloon was inflated in the supra-celiac aorta. The NRP circuit consisted of a pump for cardio-pulmonary bypass and a membrane oxygenator[12]. The no-flow (NF) time was the period from cardiac arrest to manual or mechanical chest compression in u-DCD or from cardiac arrest to the start of NRP in c-DCD, plus the time required for death declaration (20'); in u-DCD, the low-flow (LF) time was defined as the period of chest compression, whilst total WIT (t-WIT) was the time from out-of-hospital cardiac arrest to start of NRP (**Figure 1A**). In cDCD, f-WIT was defined as the time from

systolic blood pressure falling below 50 mmHg or oxygen saturation below 70% to start of NRP[4] (**Figure 1B**).

The same donor acceptance criteria during NRP were shared between the two groups. Potential uDCD and cDCD were considered eligible for procurement according to the following criteria: 1) no absolute contraindication as per CNT guidelines[10]; 2) ≤70 years; 3) witnessed cardiac arrest; 4) NRP flow >2.0 L/min; 5) acceptable gross appearance at procurement surgery; and at least two of the following: a) t-WIT ≤170 minutes for uDCD or f-WIT ≤120 minutes for cDCD; b) ALT <1000 UI/L and c) downward lactate trend during NRP. Liver biopsy at procurement was mandatory and grafts were discarded if any of the following was present: macro-vescicular steatosis >30%; fibrosis >1 as per Ishak's score[13]; and severe macroangiopathy (as per arteriolar tickening >60%). Micro-vescicolar steatosis was evaluated but not considered for graft viability assessment.

Grafts were eventually shipped to the transplant center, prepared at the back table, and perfused ex-situ using the LiverAssist® (OrganAssist®, Groeningen, The Netherlands) device. D-HOPE or NMP were performed based on surgeons' preference or need for further viability assessment. In case of NMP, grafts were considered eligible for transplant if the following three conditions were fulfilled: 1) a downward trend in lactate in the perfusate, irrespective of its baseline and final value, 2) acceptable gross appearance with uniform vascularization and 3) stable flows. Perfusate transaminases, bile production and quality were evaluated but not considered for viability assessment.

Graft allocation policy

Grafts were allocated to patients with laboratory MELD score <25 based on the Italian score for organ allocation as previously described[13].

Normothermic regional perfusion

Veno-arterial ECMO cannulation (ECMO-machine, Maquet, Rastatt, Germany) was performed under echocardiographic guidance. Transesophageal echocardiography was used to ensure the thoracic aorta was completely occluded by the aortic balloon. NRP was set to achieve a pump flow >2L/min. A continuous pressure of 60-65 mmHg in the femoral arterial cannula was maintained together with normothermic conditions. Bicarbonate was administered to keep pH 7.35-7.45; a hematocrit >25% was targeted, and oxygen saturation before membrane lung was kept at >70%. Heparin was administered at a dosage of 20.000 IU. Blood samples from ECMO were obtained immediately after start of NRP, and hourly thereafter to measure pH, lactate, transaminases and creatinine levels.

Donor Surgery

All donors were procured with simultaneous aortic and portal flush. Donors received additional 30.000 IU heparin before cross-clamping if activated clotted time >250 seconds. Grafts were stored at 4°C, shipped for back-table preparation, and then perfused ex-situ at the transplant center.

Hypothermic machine perfusion

Grafts were perfused in an operation room (OR) next to the transplant OR under medical supervision as described elsewhere[11]. The perfusion system was primed with 4L of Belzer MPS® UW Machine Perfusion Solution (Bridge for Life Ltd, Columbia, SC, USA). The arterial and portal pressures were set at 25 mmHg with a pulsatile flow and at 3-4 mmHg with a continuous flow, respectively. The oxygen flow was set at 0.25 L/min. The target liver temperature was between 4 to 10°C. Graft temperature was measured with a custom-made probe applied to the liver surface, while the perfusate temperature was monitored using built-in probes. DHOPE was run until completion of recipient hepatectomy. During hypothermic perfusion no electrolytes, glucose or drug administration was required. Before

transplantation, the graft was further flushed from both the hepatic artery and portal vein with 1000 mL of Ringer's lactate.

Normothermic machine perfusion

Grafts were perfused at 37°C in an OR next to the transplant OR and under medical supervision using a blood-based perfusate as described elsewhere[14]. Initial perfusate temperature was set at 20°C and raised by 1°C every 2 minutes. Oxygenation was provided by an anesthesia ventilator initially set at 4L/min with 30% FiO2, and later adjusted based on perfusate pH, pO2 and pCO2. Blood gas analyses were drawn every 20 minutes during the first hour and every 30 minutes thereafter with the aim to maintain a physiological pH and ionogram, and a pO2 between 200 and 250 mmHg. Perfusate glucose, transaminases and lactate were measured during NMP and so were bile production and quality (pH, sodium, glycemia, lactate, HCO3). At the end of recipient hepatectomy, grafts were flushed from both the hepatic artery and portal vein with 1 L of cold Servator® each (re-cooling time). Liver graft weight was recorded before and after NMP.

Perfusate evaluation

Transaminases levels were normalized according to graft weight. Perfusate glucose, transaminases, lactate, interleukin (IL) 6, IL10, tumor necrosis factor α (TNF-α) concentration, and bile production (NMP) were collected every hour. Release ratio was calculated according to the formula $(C_{time2} - C_{time1})/C_{time1}$ and uptake ratio as $(C_{time1} - C_{time2})/C_{time1}$ (where C is the concentration of a considered metabolite/marker, and time2 is the time point which follows time1) [15,16].

Cytokine concentrations were tested after the procedure by enzyme-linked immunosorbent assay sandwich assay (R&D Systems, Minneapolis, MN). Bile pH was measured using a GEM4000 analyzer (Instrumentation Laboratory, Bedford, MA).

Histopathology

Liver biopsies were collected at procurement during NRP; end of back-table; end of MP, and end of LT, and were fixed and stained with H&E for standard histopathological analysis and with periodic acid/Schiff (PAS) to detect changes in glycogen cell content. Histopathological reports were classified according to Brockman and co.[17]

Recipients

All LT recipients were evaluated in the pre-transplant setting and followed-up after surgery according to each center's institutional policy. LT were performed using conventional or piggy-back technique with or without veno-venous bypass based on surgeon's preference. A T-tube was routinely used for duct-to-duct biliary anastomosis and removed three months post-transplantation under cholangiography. Post-transplant ischemic-type biliary lesion (ITBL) was defined as any non-anastomotic stenosis associated with symptoms or signs and requiring an endoscopic or surgical procedure in the absence of vascular complications. Patients were followed-up for ITBL at month 1 and 3, and then every 6 months. EAD was defined as per Olthoff et al.[18], while post-reperfusion syndrome (PRS) was defined as per Aggarwal et al.[19]. Acute kidney injury (AKI) was defined according to KDIGO (Kidney disease: improving global outcome) criteria[20]. Immunosuppression consisted of anti-CD25 induction, tacrolimus in association with antimetabolites (mycophenolic acid), and steroids for non-HCV-RNA positive patients. Time to return to normal was the number of post-operative days (POD) required to reach transaminases values <50 UI/L, and total bilirubin <1.5 mg/dl, and INR<1.2.

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 or Mann-Whitney test were used to compare continuous variables across indipendent

samples and paired data. One-way analysis of variance (ANOVA) for repeated measures, followed by Tukey's multiple comparison test, was applied to evaluate differences at each time points. For categorical variables, we used a Fisher exact test. A P value <0.05 was considered statistically significant.

All descriptive and inferential analyses were performed with the SPSS software (IBM, Chicago, IL, USA). Patient and graft survival were censored as of December 31, 2019.

Results

From January 2018 thru April 2019, 34 DCD were evaluated for liver transplant. Donor characteristics and reason for discard are summarized in **Table 1**. Three (8.8%) were discarded before NRP because of donor age (n=1, 3.0%), timing (n=1) and technical failure of the NRP (n=1, iliac artery dissection at cannulation), while 11 (32.4%) were discarded based on NRP parameters (n=1, 3.0%), transaminases or lactates perfusate levels, liver macroscopic appearance at procurement and/or biopsy results (n=9, 26.5%), or severe macroangiopathy at back table evaluation (N=1, 3.0%). Twenty grafts (59%) (12 uDCD, 8 cDCD) were considered eligible for LT, procured and perfused using MP. The characteristics of grafts considered acceptable at NRP are summarized in **Table 2.**

Eleven out of twenty grafts (55%) were perfused ex-situ using D-HOPE, 9 (45%) using NMP. No liver was discarded during HMP, while two uDCD grafts were discarded during NMP: the first showed proper lactate clearance but it was not used due to recipient's cardiac arrest at anesthesia induction; the other, with a prolonged f-WIT and a liver biopsy showing 25% necrosis, was characterized by persistent increased lactate concentration in the perfusate. Eighteen grafts were eventually transplanted, (10 uDCD, 8 cDCD). A flow chart describing the whole selection process is shown in **Figure 2.**

Normothermic regional

Timing and characteristics of acceptable grafts at NRP are summarized in **Table 2**. Interestingly, grafts considered acceptable were characterized by a trend towards a lower lactate last levels (5.9 vs 9.6 mmol/L, p=0.078) and improved lactate uptake ratio (0.36 vs 0.05, p=0.042), even if warm ischemic times were comparable. Vascular flows, lactate and ALT levels during NRP are shown in **figure 3**. A case-by-case description of blood flows and lactate is provided in supplemental materials (**Supplemental material figure 1 and 2**).

Ex-situ machine perfusion

Twenty grafts were perfused ex-situ. Two were discarded as described above and were not included in the description of results in this paragraph. The characteristics of the 18 transplanted grafts are reported in **Table 3**.

1. *Hemodynamic parameters*

During ex-situ perfusion all grafts had stable vascular flows within 1 hour. DHOPE arterial and portal flows ranged from 43 to 108 mL/min and from 130 to 350 mL/min, while NMP arterial and portal flows ranged from 270 to 502 mL/min and from 980 to 1400 mL/min respectively. (**Supplemental material figure 3)**.

2. Perfusate evaluation

a) Metabolic parameters

The lactate trends during ex-situ MP were different between DHOPE and NMP (**Figure 4A, supplemental materials figure 4**). While they progressively increased during DHOPE (p=0.007) up to a median terminal value of 3.4 (3.1-3.9) mmol/L, a rapid decrease (p=0.002) was observed in NMP up to a median terminal value of 1.5 (1-2.8) mmol/L (DHOPE vs NMP p=0.003). Only one case of NMP showed a terminal value of 6.5 mmol/L, after a downstream trend from 11.5 mmol/L. Lactate release ratio was 0.4 (0.32-0.6)) in DHOPE and -0.8 ((-0.5)-(-0.9)) in NMP (p<0.001). Consistently, terminal perfusate pH was 7.1 (7.01-7.1) in DHOPE,

and 7.33 (7.21-7.4) in NMP (p=0.008). Glucose concentration showed different trends between the two types of MP (**Figure 4B**) (p=0.018). Bile production and characteristics during NMP (DHOPE grafts did not produced bile) are summarized in **Table 4**.

b) Hepatocellular damage markers

Perfusate peak values of ALT and AST during DHOPE and NMP were 0.280 (0.141-0.352) IU/L/g vs 0.601 (0.527-1.157) U/L/g (p=0.008) (**Figure 4C**), and 0.210 (0.151-0.303) IU/L/g vs 0.869 (0.594-0.917) IU/L/g (p=0.032) respectively. ALT release ratio during MP is showed in **Figure 4D**.

c) Inflammatory markers

Inflammatory markers in the perfusate of NMP (n=6) and DHOPE (n=9) were analyzed. In all cases, cytokines concentration increased (IL-6, p=0.001; IL-10, p=0.013; TNF-α, p<0.001) during MP. Perfusate release ratio of IL-6, IL-10 and TNF-α from the first to the third hour of MP are shown in **figure 5**. IL-6 (p=0.018) had a steeper increase during DHOPE, while TNF-α (p=0.003) during NMP. Interestingly, IL-10 (p=0.745) resulted equally expressed in the two groups.

Histology

Liver graft biopsies showed progressive increase of injury scores from procurement through transplantation (p=0.014) **(Figure 6A**). However, no differences between the study groups were observed. Consistently, the glycogen tissue content (PAS staining) decreased progressively in both groups (p=0.022) (**Figure 6B**). Injury scores and glycogen content tended to be lower at the end of DHOPE, although the difference was not statistically different (p=0.117 and p=0.086, respectively). After reperfusion, both groups showed comparable injury scores and glycogen content.

Intra and post-transplant results

A total of 18 grafts were transplanted. Intra and post-operative results are summarized in **Table 5.** During surgery, recipients required a median of 7 (3-20) units (U) of fresh frozen plasma (FFP), higher in DHOPE recipients than in NMP (NMP, n=4U (2-8) vs DHOPE, n=8U (3-13), p=0.037). PRS occurred in 8 (44%) patients; 6 (86%) in NMP cases and in 2 (18%) DHOPE cases (p=0.020).

The incidence of EAD was 28%, and there was no difference between grafts perfused ex-situ with NMP vs D-HOPE (NMP 43% vs DHOPE 18%, p=0.549). Post-LT transaminases peak was 1147 IU/L (877-1865) for AST and 698 IU/L (598-1344) for ALT, and it was comparable between NMP and D-HOPE. Consistently, post-operative data on transaminases, bilirubin and gamma-glutamyl-transferase (GGT) blood serum levels were similar in the two groups (**Figure 7).** AKI developed in 5 (28%) patients, 4 in the DHOPE (36%) and one (14%) in the NMP group (p=0.631). Interestingly, patients affected by AKI received DCD grafts with higher transaminases during NRP/DHOPE and with DHOPE duration >4h. Two of these patients needed continuous venous-venous hemodialysis.

The number of days needed to return to normal bilirubin, AST and ALT serum levels were 15.5 (13-39.5), 8 (5.2-11), and 16 (13-23) respectively, without difference based on type of ex-situ perfusion.

One patient died (NMP group). He developed portal vein thrombosis on POD#7 successfully treated with surgical portal thrombectomy. Few days later, he showed positive blood and bile culture for New Delhi metallo-β-lactamase-1 E. coli without fever or white blood cells elevation, and on POD#32, suddenly developed severe abdominal pain due to a rupture of a hepatic artery psudoaneurysm with major intra-abdominal hematoma. He underwent urgent hepatectomy with temporary porto-caval shunt and was re-transplanted the following day. The patient died 10 days later for sudden cardiac arrest.

Three months after surgery, all patients had normal trans t-tube cholangiography except in one case (NMP), who developed ITBL amenable to endoscopic treatment. During the follow up two patients (1 NMP and 1 DHOPE) had a single episode of cholangitis characterized by fever and increased transaminases and bilirubin, which resolved after hospital admission and medical treatment. No biliary tract abnormalities were shown at the MRI. Two other patients (HMP) developed persistent ascites which resolved few months after transplant. One of these patients required ERCP for t-tube removal. One patient (NMP) was admitted 18 months after LT for upper gastrointestinal (GI) bleeding episode which was treated endoscopically. GI endoscopy showed F1 esophageal varices.

Discussion

The current manuscript was able to confirm that transplantation of DCD liver grafts with over-extended WIT is feasible with sequential combination of in-situ NRP and ex-situ, end-ischemic MP–with acceptable results. Similar approaches have been already proposed as single case reports[21] or limited case series[4], but the aim of our work is to provide the basis for a future standardization of this procedure. Graft functional recovery was achieved in all cases with no PNF. Only one case of ITBL amenable to endoscopic treatment and one patient death due to sepsis were diagnosed and patients had limited re-admissions or other type of medical or surgical complications. In our opinion, the strict graft selection (acceptance rate 56%), if compared to previously reported series[22], was pivotal to achieve favorable post-LT results.

Only 2.8% of LT were performed using DCD grafts in Italy in 2018[23]. Nevertheless, despite several concerns raised by the extremely prolonged WIT[24], both cDCD and uDCD programs were implemented thanks to two important devices: 1) LUCAS, which is able to provide mechanical chest compression with a MAP ranging from 20 to 50 mmHg[25,26] even when cardio-pulmonary resuscitation is difficult or impossible[27] and 2) NRP which is able to

counterbalance the detrimental effects of the prolonged time of flat-line EKG requested for declaration of death[28]

In this context, graft evaluation process should be separated in 4 different critical steps: donor evaluation, NRP evaluation, procurement and ex-situ perfusion. In our experience, donor age and NRP flow were considered categorical parameters. The first, even if not scientifically validated, was decided to counterbalance the acute ischemic damage with a (potentially) optimal regenerative capacity[29], while the second was set up to provide adequate graft perfusion during NRP, allow organ repair and perform an indirect assessment of graft damage. For these reasons, an NRP lasting a minimum of 3-4 hours is needed for proper graft evaluation. Moreover, NRP requiring continuous fluid administration and an increase in intra-abdominal pressures suggest a severe ischemic injury and a critically damaged liver.

Liver viability should then be based on multiple parameters such as timing, transaminases, lactate, macroscopic evaluation and liver biopsy, however, the weight of each of them and their interaction are far to be clearly understood. Many Authors suggested lactates levels could be used to assess liver metabolic residual activity[30]. Accordingly, in our analysis, accepted grafts showed improved lactates uptake ratio and a trend towards lower terminal values. It is important to underline that as lactate absolute values might be influenced by several factors (e.g. small bowel and/or limb ischemia), the lactate downtrend could be better used to evaluate liver quality. Conversely, transaminases as viability criteria during NRP are matter of concern for many reasons: they should be normalized according to NRP blood volume and liver weight, their values can be influenced by the ischemic damage of other organs (e.g. muscles) and, furthermore, the relation between the acute damage expressed by transaminases levels and liver quality and synthetic capacity is still a matter of debate[30]. We believe the identification of a transaminases cut-off value deserves further investigations and it should be kept in consideration only together with other relevant parameters.

Even if organ direct inspection cannot be easily standardized and surgeon's experience plays a fundamental role, it is a key step in the decision-making process. Accordingly, in our experience, graft appearance at procurement was the most important reason for organ discard. The role of liver biopsy is still uncertain and relies more in the assessment of chronic liver disease than of cardiac arrest-induced acute injuries. Indeed, necrosis can be hardly identified on frozen sections[31,32].

The role of ex-situ MP is controversial as well. Several studies showed ex-situ NMP and DHOPE have the potential to minimize IRI and promote graft and bile duct protection.[33,34] However, the underlying mechanisms are still unclear,[35] and there is no consensus on type of technique (hypo vs normothermic) to adopt[30,36].

Even if markers of graft viability during DHOPE are going to be clinically introduced,[37] NMP offers the potentiality of ex-situ graft evaluation.[36] While we decided to rely on lactate clearance, rather than a specific lactate value as suggested by Mergental et al.[38] to asses liver graft metabolic ability (e.g.: one graft was successfully transplanted with a terminal lactate value of 6.5 mmol/L), the evaluation of cholangiocellular injuries is more challenging. Recently, Matton et al.[39] stated that biliary pH, bicarbonate, and glucose may predict ITBL. Our preliminary experience using DCD grafts fully supports this hypothesis, as the only graft who developed ITBL showed bile parameters exceeding Matton's criteria.[39] Some criticisms were raised by our recent published experience[14] with very old DBD grafts (median age 81 years) preserved with end-ischemic NMP, which would have prompted discarding 7 out of 10 livers as per Matton's criteria. It might be speculated that bile composition is influenced by graft quality, residual synthetic capacity and regenerative potential, while bile production during NMP is more related to the severity of hepatocellular damage (the two cases with highest transaminase peaks and histology-proven necrosis did not produce bile).

The limited number of cases considered in our work and the differences in the two populations do not allow drawing definitive conclusions on pro and cons of the two different techniques of ex-situ MP (NMP vs DHOPE). Nevertheless, the analysis of liver histology and perfusate cytokines concentration deserve some considerations. While, NMP grafts show higher transaminase peak immediately after reperfusion, liver biopsies display a more pronounced glycogen consumption and higher glucose level during D-HOPE. At the same time, our preliminary analysis seems to confirm that the severity of IRI and the biological phenomena elicited by MP directly affect post-LT events[40]. In fact, NMP upregulates the release of TNF-α which has been recognized as a crucial factor for PRS.[41,42] Consistently, IL-6 overproduction, as shown during DHOPE, has been associated with a higher risk of AKI development.[43] Interestingly, IL-10, which is involved in the reduction of IRI inflammation associated injury[44], is equally produced by NMP and DHOPE.

Based on this preliminary experience, a new operative flowchart for DCD grafts management and evaluation introducing selective use of a) NRP alone or b) sequential NRP + ex-situ DHOPE or c) sequential NRP + ex-situ NMP has been proposed and will be matter of future re-evaluation (**figure 8**).

This work has several limitations related to the limited sample size and its retrospective design. Nevertheless, it offers a novel prospective on utilization of DCD grafts with overextended WIT when NRP and strict selection criteria are implemented. It is our opinion there should be no preclusion to evaluate and use such donors, even if a higher incidence of intra- and post-operative complications might be expected. Sequential use of NRP and ex-situ MP might be the safest way for graft evaluation and protection from severe IRI in DCD LT. Further studies should evaluate if clinical and NRP data may guide clinicians on a selective use of ex-situ machine perfusion before LT.

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Figure Legends

Figure 1. Timing of uncontrolled (uDCD) and controlled (cDCD) DCD donation in Italy

Figure 2. Donor grafts selection flow chart

Figure 3: Main characteristics of normothermic regional perfusion. Lactate concentration (A), lactate uptake ratio (B), alanine transferase (ALT, C) and blood flows (p=0.486) (D). In figure B the graft that was discarded due to atherosclerosis was not considered because it was considered not suitable due to non-functional parameters. P evaluated using repeated measures ANOVA.

Figure 4: Lactate (A), glucose (B), transaminases (ALT) (C) and transaminases (ALT) release ratio perfusate level during ex-situ machine perfusion. ALT, aspartate alanine transferase; °, p=0.003; *, p=0.018; §, p= 0.002; #, p=0.044

Figure 5: Interleukin (IL)-6, TNF-α and IL-10 release ratio in perfusate during ex-situ machine perfusion. *, p=0.018; °, p=0.043; §. p=0.745

Figure 6: Liver biopsies evaluation as per Brockman et al. and periodic acid/Schiff (PAS) to detect changes in glycogen cell content after NRP (end NRP), end of ex-situ perfusion (end MP), and end of liver transplantation (post-reperfusion). #, p=0.014; *, p=0.022

Figure 7: Post-operative transaminases (A, p=0.583; B, p=0.335), bilirubin (p=0.629) (C), and gamma glutamine transferase (GGT) (p=0.317) levels (D)

Figure 8: DCD evaluation flowchart

Supporting information statement

Additional supporting information may be found online in the Supporting Information section at the end of the article

Table 1. Potential DCD donors' characteristics during the study period.

Donor				Timing				NRP						Histology			Result		Ex-situ
Donor (#)	Center	Donor type	Age (years)	F-WIT (min.)	T-WIT (min.)	LF-time (min.)	NF-time (min.)	NRP time (min.)	AST peak (IU/L)	ALT peak (IU/L)	Lactate peak (mmol/L)	Lactate last (mmol/L)	Flow last (L/min)	Necrosis (%)	Macro-steatosis (%)	Fibrosis (stage)	Accepted/ transplanted (Y/N)	Discard reason	NMP/ HMP
1	Pisa	Uncontrolled	47	/	160	130	30	345	348	422	17	12.4	3.9	0	10	0	Y/Y		NMP
2	Pisa	Uncontrolled	42	/	174	132	42	428	2100	1795	23	14	4.2	0	40	0	N/N	increased transaminases and excessive steatosis	
3	Pisa	Uncontrolled	62	/	132	112	22	326	455	307	16	7.4	3.1	0	60	1	N/N	excessive steatosis	
4	Pisa	Uncontrolled	40	/	156	111	45	415	291	217	20	14.3	3.5	0	5	1	Y/Y		NMP
5	Pisa	Controlled	56	20	/	/	20	130	1998	1922	16	12.2	2.5	/	/	/	N/N	increased transaminases and macroscopic appearance	
6	Pisa	Controlled	62	25	/	/	25	431	93	252	8.3	0.9	3	0	10	1	Y/Y		NMP
7	Pisa	Uncontrolled	54	/	149	122	27	453	594	456	17	13.5	3	0	5	0	N/N	severe graft hypoperfusion at procurement	
8	Pisa	Uncontrolled	51	/	145	125	20	255	2653	2377	12.3	24	2	0	<5	1	N/N	increased transaminases, lactates and macroscopic appearance	
9	Pisa	Uncontrolled	60	/	170	133	37	366	1955	1009	16	5.5	3	/	/	/	N/N	increased transaminases and macroscopic appearance	
10	Pisa	Uncontrolled	55	/	169	136	33	299	137	146	17	8.8	3.5	0	5	1	Y/Y		NMP
11	Pisa	Uncontrolled	47	/	165	145	20	375	290	157	29	24.2	2	0	10	0	Y/Y		NMP
12	Pisa	Uncontrolled	58	/	203	173	30	452	108	491	8.8	7.6	3.5	0	0	0	Y/N	graft not transplanted due to recipient cardiac arrest at anesthesia induction	NMP
13	Pisa	Uncontrolled	38	/	30	/	30	/	/	/	/	/	/	/	/	/	N/N	NRP technical failure	
14	Pisa	Uncontrolled	59	/	150	127	23	400	628	708	12.8	22	2.9	0	5	1	N/N	Increased lactates and macroscopic appearance	
15	Pisa	Uncontrolled	35	/	141	113	28	376	691	251	19.1	2.6	2.5	0	20	1	Y/Y		NMP
16	Pisa	Uncontrolled	39	/	90	60	30	375	450	374	24	21	3.5	0	15	0	Y/Y		NMP
17	Milan	Controlled	66	97	/	/	37	317	1769	2067	7.2	7	1.7	/	/	/	N/N	NRP parameters, increased transaminases	
18	Milan	Controlled	59	116	/	/	46	240	730	1270	11	9.2	2	/	/	/	N/N	macroscopic appearance, increased transaminases	
19	Milan	Controlled	57	135	/	/	45	355	325	460	13	8.7	4	25	10	0	Y/N	discarded during NMP	NMP
20	Milan	Uncontrolled	60	/	170	127	43	375	2496	1547	11.8	7.1	2.7	0	5	0	Y/Y		HMP
21	Milan	Uncontrolled	62	/	140	105	35	473	2702	7286	4.4	3.3	3.2	10	20	0	Y/Y		HMP
22	Milan	Controlled	64	46	/	/	35	300	176	110	11.3	4	2.8	0	40	1	Y/Y		HMP
23	Milan	Controlled	47	80	/	/	28	380	272	114	7.7	2.7	3.1	0	20	0	Y/Y		HMP

24	Milan	Controlled	60	50	/	/	30	360	1511	940	8.2	4.9	3	0	5	0	Y/Y		HMP
25	Milan	Uncontrolled	68	/	139	106	33	310	373	1295	5	3.3	3	<5	5	0	Y/Y		HMP
26	Milan	Controlled	54	55	/	/	40	220	372	103	7.9	5.5	3	0	5	0	Y/Y		HMP
27	Milan	Uncontrolled	71	/	170	129	41	240	190	781	6.5	5.7	4	<5	45	1	N/N	excessive steatosis	
28	Milan	Controlled	65	83	/	/	32	120	197	276	12.5	9.6	2	/	/	/	N/N	severe macroangiopathy	
29	Milan	Controlled	61	82	/	/	32	300	2112	3052	12.3	6.4	3.5	<5	5	0	Y/Y		HMP
30	Milan	Controlled	56	142	/	/	97	/	/	/	/	/	/	/	/	/	N/N	prolonged no-flow and f-WIT	
31	Milan	Uncontrolled	69	/	145	96	49	320	1324	1187	14.5	4.5	2.9	0	5	0	Y/Y		HMP
32	Milan	Controlled	64	92	/	/	60	200	234	210	11.2	1.8	4.3	<5	0	1	Y/Y		HMP
33	Milan	Controlled	40	73	/	/	32	190	62	36	10.7	7.3	2.5	<5	0	0	Y/Y		HMP
34	Milan	Uncontrolled	82	/	102	65	47	/	/	/	/	/	/	<5	5	0	N/N	donor age	NMP

Table 2. Potential donors' characteristics and NRP parameters.

Variables	**Suitable grafts during NRP (n=20)**	**Unsuitable grafts during NRP (n=11)**	**p**
	Median (IQR) or (%)	Median (IQR) or (%)	
Donors			
Gender (male)	19 (95.0)	10 (90.9)	0.749
Age (years)	54.5 (45.8-58)	59 (55-63.5)	0.067
DCD donor type 2	11 (55)	7 (63.6)	0.932
Comorbidities			
Diabetes Mellitus	1 (5)	2 (18)	0.580
Dyslipidemia	2 (10)	3 (27)	0.459
Nephropathy	2 (19)	2 (18)	0.928
Arterial hypertension	8 (40)	5 (45)	0.932
Cardiopathy	10 (50)	5 (45)	0.894
Timing			
Functional WIT (controlled DCD) (minutes)	55 (48-81)	90 (67-102)	0.641
Low Flow time (uncontrolled DCD) (minutes)	113 (105-133)	127 (123-130)	0.527
No Flow time (minutes)	32 (30-41)	32 (22-39)	0.789
Total warm ischemia time (minutes)	156 (140-167)	150 (147-170)	0.789
Normothermic regional perfusion time (min)	357 (300-377)	317 (240-383)	0.199
NRP parameters			
Lactates (peak)	11.5 (8.3-17)	12.8 (11.6-16)	0.784
Lactates (last)	5.9 (3.3-8.7)	9.6 (7.2-13.8)	0.078
AST peak (IU/L)	336 (219-849)	730 (524-1976)	0.139
ALT peak (IU/L)	313 (154-1002)	1009 (582-1858)	0.646
Flow (last) (L/min)	3.1 (2.9-3.5)	2.9 (2-3.1)	0.112

Table 3. Liver transplant donors and recipients characteristics: overall and stratified by type of ex-situ perfusion.

Variables	Transplanted grafts (NRP+ex-situ MP) (N=18)		Transplanted grafts (NRP+NMP) (N=7)	Transplanted grafts (NRP+HMP) (N=11)	p
	Median (IQR) or N (%)		Median (IQR) or N (%)	Median (IQR) or N (%)	
Donor characteristics					
Gender (male)	17 (94)		7 (100)	10 (90.9)	0.815
Age (years)	53.5 (43-57.5)		47 (39-51)	55 (52-58)	0.076
BMI (kg/m^2)	26 (24-28)		27.8 (26.8-30.1)	25.5 (24-26)	0.266
DCD donor type 2	10 (56)		6 (85)	4 (36.4)	0.117
Comorbidities					
Diabetes Mellitus	1 (6)		0 (0)	1 (9.1)	0.751
Dyslipidemia	2 (12)		1 (14.3)	1 (9.1)	0.746
Nephropathy	2 (12)		1 (14.3)	1 (9.1)	0.746
Arterial hypertension	7 (39)		3 (42.9)	4 (36.4)	0.702
Cardiopathy	9 (50)		4 (57.1)	5 (45.5)	1.000
Timing					
Functional WIT (controlled DCD) (minutes) (n=18; 1; 7)	52.5(47-74)		25 (NA)	55 (50-80)	NA
Total warm ischemia time (minutes) (n=18; 6; 4)	150.5 (140-164)		158 (145-164)	142.5 (140-151)	0.920
Low Flow time (uncontrolled DCD) (minutes) (n=18; 6; 4)	112 (105-129)		112 (105-129)	105.5 (103-111)	0.665
No Flow time (minutes)	32.5 (30-39)		30 (26.5-31.5)	35 (32-41.5)	0.091
Normothermic regional perfusion time (min)	352.5 (300-376)		375 (360-396)	310 (260-367.5)	0.096
Pre ex-situ machine cold ischemia time (min)	312 (256-334)		248 (233-270)	330 (320-347)	0.0002
Ex-situ normo or hypothermic perfusion time (min)	240 (180-285)		195 (161-316)	240 (180-265)	0.725
Time from donor cross-clamping to graft reperfusion (min)	577 (535-639)		565 (429-649)	585 (550-622)	0.356
Grafts weight					
Graft weight (at end of back-table surgery)	1605 (1434-1767)		1550 (1447-1742)	1610 (1463-1734)	0.662
Graft weight (at end of MP)	1645 (1557-1817)		1600 (1542-1765)	1700 (1610-1798)	0.995
Recipients characteristics					
Gender (male)	17 (94)		7 (100)	10 (90.9)	0.815
Age at transplant (years)	61 (56.5-64)		60 (56-64)	61 (57-64)	0.642
HCC*	10 (56)		6 (85.7)	4 (36.4)	0.117
HCV*	6 (33)		3 (42.9)	3 (27.3)	0.864
HBV*	3 (17)		2 (28.6)	1 (9.1)	0.665
ETOH*	7 (39)		3 (42.9)	4 (36.4)	0.826
NASH*	2 (12)		0 (0)	2 (18.2)	0.669
Biological MELD	11.5 (9-16)		14 (10-16.5)	10 (9-15.5)	0.558
D-MELD	580.5 (513-796)		560 (446-826)	585 (545-751)	0.896

* multiple diseases are possible

Table 4. Bile production, pH, sodium level, glycemia, lactate and bicarbonate at 120 minutes during NMP.

Patient #	Bile production (ml)	pH	Sodium (mmol/L)	Glycemia (mg/dl)	Lactate (mmol/L)	HCO3- (mmol/L)
1	25	7.59	145	43	1.3	18.7
2	35	7.68	153	29	4.2	22.3
3	10	7.58	149	41	2.9	31.4
4*	40	7.43	143	86	1.7	15.7
5	0	/	/	/	/	/
6	25	7.89	149	91	2.4	33.5
7	0	/	/	/	/	/

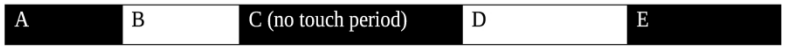
* This patient developed ITBL 5 months after LT. The patient is doing well with normal lab tests after two ERCP

Table 4. Post-operative results.

Variables	**NRP + ex-situ MP (n=18)**		**NRP + NMP (n=7)**	**NRP + HMP (n=11)**	**p**
			Median (IQR) or (%)	Median (IQR) or (%)	
Patient death (n)	1		1 (14)	0 (0)	0.815
PNF (n)	0		0	0 (0)	NA
Graft loss (n)	1		1 (14.3)	0 (0)	0.815
EAD (n)	5 (28)		3 (43)	2 (18)	0.549
Post reperfusion syndrome (n)	8 (44)		6 (85.7)	2 (18)	0.020
Intraoperative blood units (n)	5 (2-11)		4 (2-8)	7 (3-12)	0.172
Intraoperative FFP units (n)	7 (3-20)		6 (4-7)	10 (7-22)	0.037
AST peak (UI/L)	1147 (877-1865)		1553 (930-2509)	1125 (898-1549)	0.992
ALT peak (UI/L)	698 (598-1344)		832 (632-1157)	691 (574-1615)	0.625
Bilirubin peak (mg/dl)	6.1(3.2-8.6)		7.8 (5.1-9.7)	5.5 (3.1-7.7)	0.391
Vascular complications (n)	1		1 (14.3)	0 (0)	0.815
Biliary complications (n)	1		1 (14.3)	0 (0)	0.815
Acute kidney injury	5		1 (14)	4 (36)	0.631
Post-op. bleeding	2		0 (0)	2 (18)	0.669
Refractory ascites	2		0 (0)	2 (18)	0.669
Hospital stay (days)	15 (14-22)		16.5 (13-20)	15 (14-24)	0.882
Days to normal AST (n)	8 (5.2-11)		6.5 (6-9)	11 (6.5-12.5)	0.128
Days to normal ALT (n)	16 (13-23)		14 (10-16)	22 (12.5-32)	0.088
Days to normal bilirubin (n)	15.5 (13-39.5)		15 (14-51)	20 (8.5-34)	0.780
Bilirubin at 1 month (mg/dl)	0.97 (0.76-2.87)		1.00 (0.89-2.99)	0.92 (0.77-2.31)	0.219
Bilirubin at 3 months (mg/dl)	0.82 (0.58-1.59)		0.80 (0.60-1.02)	0.91 (0.71-1.77)	0.382
Bilirubin at 6 months (mg/dl)	0.92 (0.61-2.13)		1.00 (0.47-2.30)	0.87 (0.62-1.89)	0.143
GGT at 1 month (IU/L)	83 (45-234)		80 (53-244)	92 (47-221)	0.295
GGT at 3 months (IU/L)	41 (31-153)		38 (36-129)	54 (31-182)	0.428
GGT at 6 months (IU/L)	35 (25-241)		31 (26-338)	45 (33-195)	0.329
ALP at 1 month (IU/L)	97 (74-259)		95 (77-241)	102 (81-327)	0.637
ALP at 3 months (IU/L)	99 (80-129)		108 (82-140)	97 (83-117)	0.381
ALP at 6 months (IU/L)	135 (83-176)		174 (108-213)	98 (83-151)	0.077
Follow up (months)	15.1 (9.5-22.3)		14.1 (11.9-19.9)	16.0 (8.9-25.7)	0.581

Figure 1. Timing of uncontrolled (uDCD) and controlled (cDCD) DCD donation in Italy.

A. Uncontrolled



A+C= No Flow time (NF-time); NF-time is the period from witnessed cardiac arrest to the start of CPR + the period of flat line EKG registration (minimum 20 minutes); **max allowed: 45 minutes**

B= Low Flow time (LF-time); LF-time is the period from the start of mechanical or chest compression to the EKG registration

A+B+C= Total WIT (t-WIT); max allowed: 170'

B. Controlled

B= No Flow time (NF-time); after care withdrawal, NF-time is the period from cardiac arrest (no cardiac output) to the start of NRP; max allowed 45 minutes

Functional-WIT (f-WIT) after care withdrawal, f-WIT is the period from systolic blood pressure <50 mmHg or oxygen saturation <70% to the cardiac arrest; max allowed 120 minutes

Abbreviations: Cardiac arrest (CA), manual and/or mechanical chest compression (CPR), electrocardiogram (EKG), normothermic regional perfusion (NRP), no flow time (NF-time), low flow time (LF-time), functional warm ischemia time (f-WIT), care withdrawal and agonic phase (CW)

Figure 2. Donor grafts selection flow chart

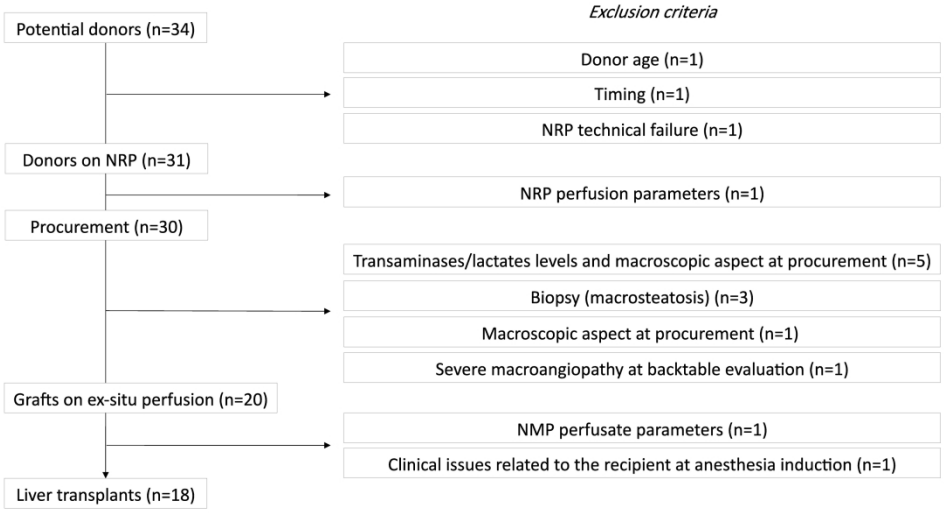
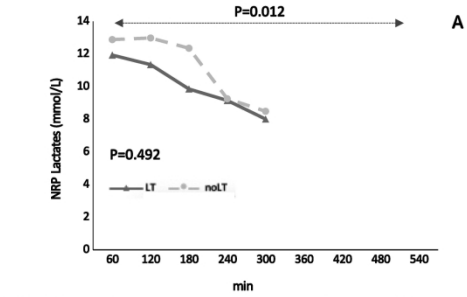
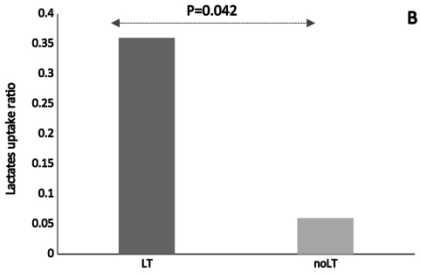
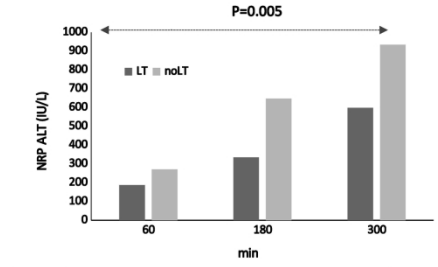


Fig. 3. Main characteristics of normothermic regional perfusion. Lactate concentration (A), lactate uptake ratio (B), alanine transferase (ALT, C) and blood flows (p=0.486) (D). In figure B the graft that was discarded due to atherosclerosis was not considered because it was considered not suitable due to non-functional parameters. P evaluated using repeated measures ANOVA.

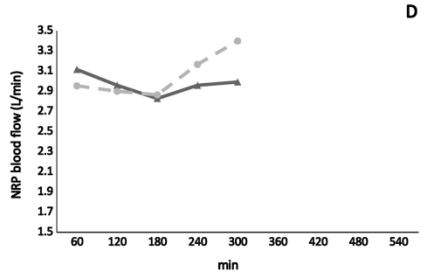


Tot grafts, n	31	31	29	22	21
LT grafts, n	18	18	18	14	13
noLT grafts, n	13	13	11	8	8





Tot grafts, n	28	22	12
LT grafts, n	16	15	8
noLT grafts, n	12	7	4



Tot grafts, n	31	31	29	22	21
LT grafts, n	18	18	18	14	13
noLT grafts, n	13	13	11	8	8

Fig. 4. Lactate (A), glucose (B), transaminases (ALT) (C) and transaminases (ALT) release ratio perfusate level during ex-situ machine perfusion. ALT, aspartate alanine transferase; °, p=0.003; *, p=0.018; §, p= 0.002; #, p=0.044

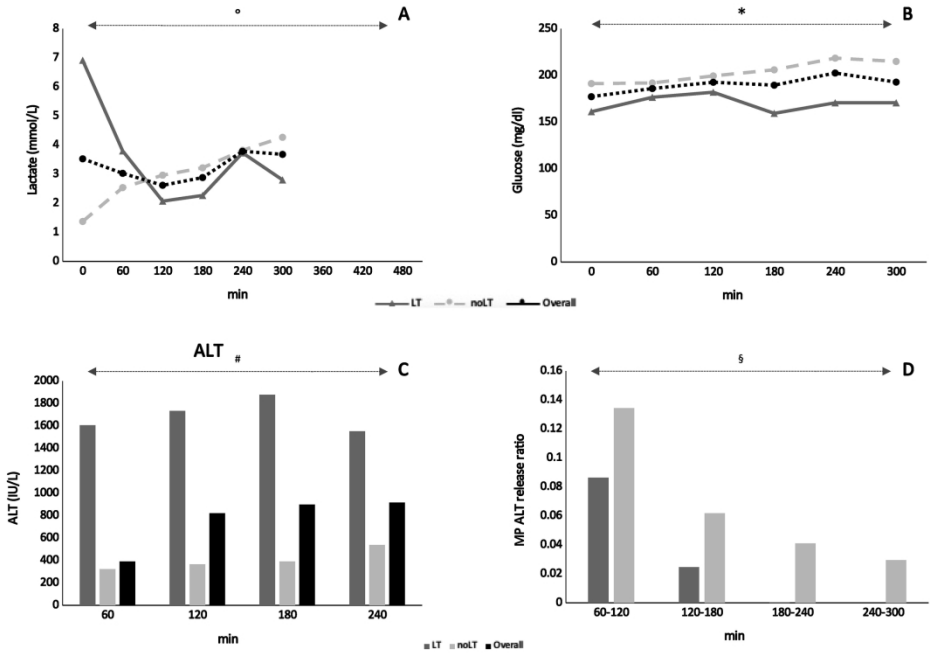


Fig. 5. Interleukin (IL)-6, TNF-α and IL-10 release ratio in perfusate during ex-situ machine perfusion.
*, p=0.018; °, p=0.043; §. p=0.745

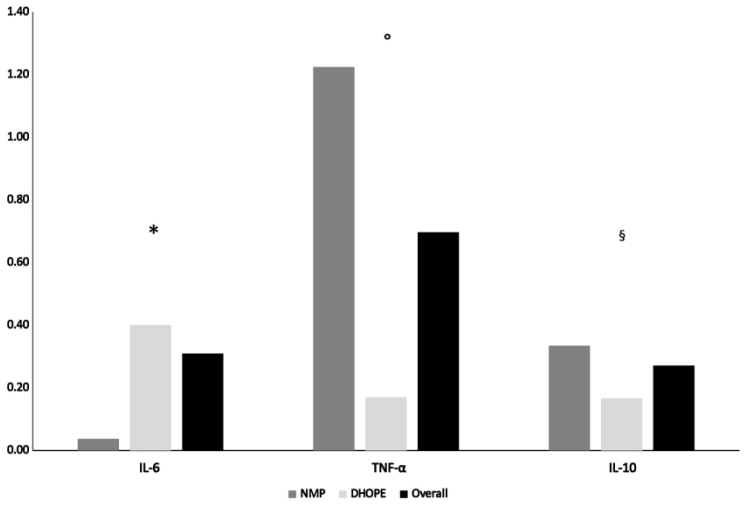


Fig. 6. Liver biopsies evaluation as per Brockman et al. and periodic acid/Schiff (PAS) to detect changes in glycogen cell content after NRP (end NRP), end of ex-situ perfusion (end MP), and end of liver transplantation (post-reperfusion). #, p=0.014; *, p=0.022

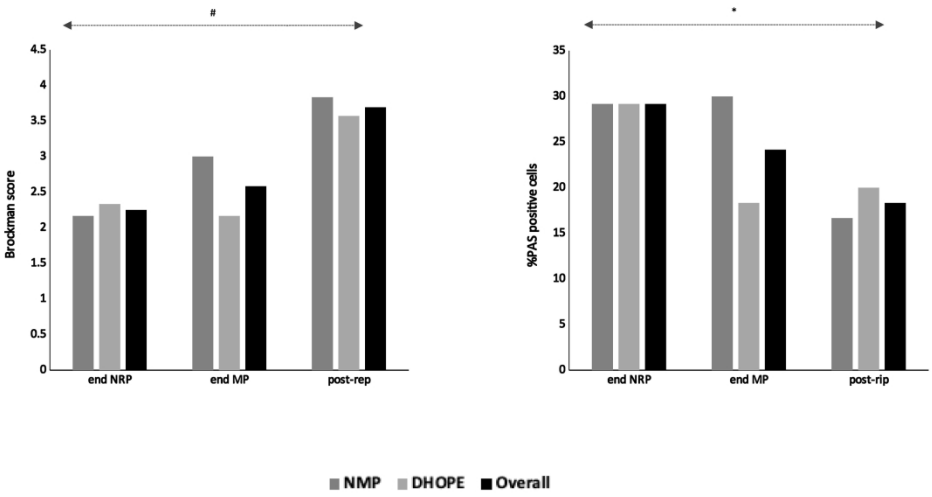


Fig. 7. Post-operative transaminases (A, p=0.583; B, p=0.335), bilirubin (p=0.629) (C), and gamma glutamine transferase (GGT) (p=0.317) levels (D)

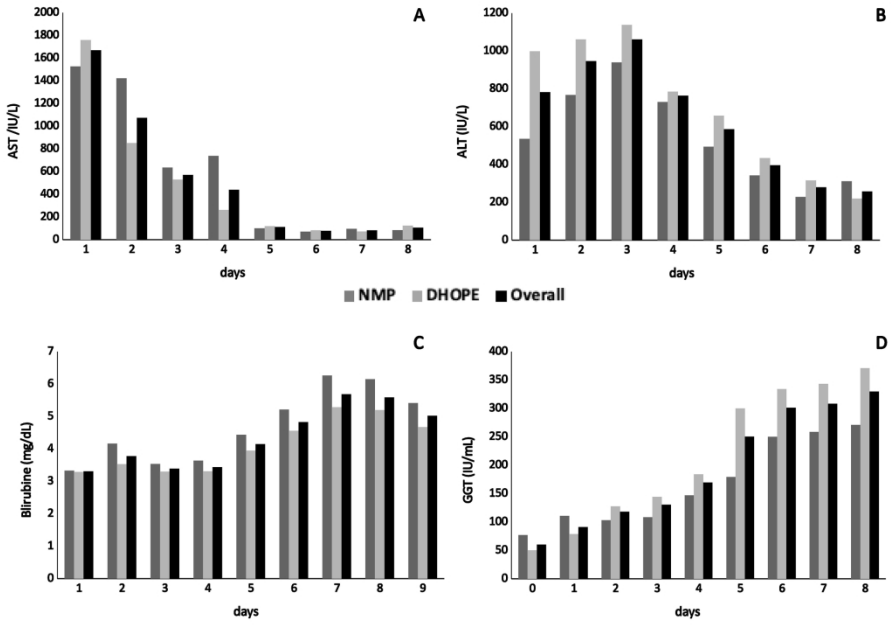
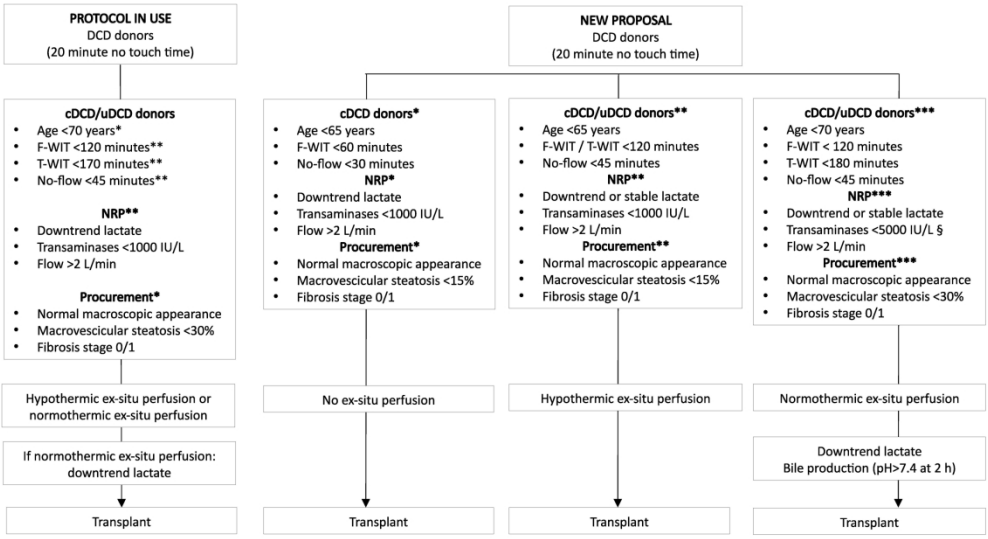


Fig. 8. DCD evaluation flowchart



* All parameters to be fullfilled
** At least 2 criteria to be fullfilled
*** Graft to be discarded if these parameters are not fullfilled
§ unless severe rabdomiolisis is present