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Clinical assessment of liver metabolism during hypothermic oxygenated machine perfusion using microdialysis

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SUMMARY

1. INTRODUCTION

Hypothermic oxygenated machine perfusion (HOPE) is gaining increasing interest in the transplant community as a tool to reduce ischemia-reperfusion injury and to improve outcomes of liver transplantation (LT). In clinical studies, HOPE use has been associated with improved outcomes with grafts from donors after circulatory death (DCD)[1–3], extended-criteria donors after brain death (DBD)[4,5], and steatotic grafts[6]. The protective mechanism of HOPE appears to be manifold, being related to adenosine-triphosphate replenishment, immunomodulation[7], better preservation of endothelial cells glycocalyx, peri-biliary vascular plexus, and peribiliary glands[8,9], and, more importantly, modulation of mitochondrial respiration. As shown in 2013 by Schlegel et al.[10] in their pivotal study, HOPE progressively decreases the rate of mitochondrial respiration and determines an oxidized state in mitochondria, which limits the production of reactive oxygen species (ROS) by reverse electron transfer upon organ reperfusion[11,12], thus resulting in diminished mitochondrial, nuclear, hepatocyte and sinusoidal injury.

Little is known, however, about other aspects of liver metabolism during HOPE. In particular, changes of liver metabolism occurring in the transition between the phase of static cold storage (SCS) and HOPE are largely unknown.

Microdialysis (MD) is a technique by which interstitial fluid (microdialysate) can be sampled from a variety of tissues to measure the concentration of metabolites like glucose, lactate, pyruvate and glutamate, which diffuse into the extracellular space, and has been extensively used in a variety of settings, especially for brain sampling in critically ill patients.[13–17] In LT, microdialysis has been used to assess liver metabolism during the phases of liver retrieval, cold preservation and after graft implantation[18–22], and explored as a tool for early detection of ischemic complications and acute rejection.[23–29] As applied to machine perfusion, MD has the potential of allowing continuous

monitoring of liver metabolism throughout SCS and HOPE, overcoming one limitation of perfusate analysis, which cannot assess metabolism during SCS.

In a study from our group, MD was used to assess metabolism of lungs perfused ex-vivo and emerged as a potential tool to discriminate lung function after transplantation.[30] MD has been occasionally used in the setting of kidney machine perfusion[31–33] but, to the best of our knowledge, its use in HOPE-treated livers has not been reported.

The aim of this study was to assess time course of liver metabolism biomarkers during SCS and HOPE using microdialysis and to explore association between MD metabolites and graft function after liver transplantation.

2. END-ISCHEMIC HYPOTHERMIC OXYGENATED MACHINE PERFUSION

2.1 HOPE

End-ischemic hypothermic oxygenated machine perfusion (HOPE) consists in the treatment of liver allograft at the end of static cold preservation by a period of hypothermic machine perfusion using an oxygenated perfusate. In most studies, the preservation fluid used for HOPE is a variant of Belzer solution MPS® (Bridge to Life Europe Ltd. London, UK) characterized by an "extracellular" ions composition and by a lower content of starch to decrease viscosity. It is a sterile, isotonic non-pyrogenic solution with an Osmolarity of 300mOsm and pH of 7.4 at room temperature. Composition and characteristics of MPS® solution are reported in Figure 1.

CONSTITUENT	CONCENTRATION
Adenine (free base)	5 (mmol/L)
Calcium Chloride (dihydrate)	0.5 (mmol/L)
Glucose	10 (mmol/L)
Glutathione (reduced)	3 (mmol/L)
HEPES (free acid)	10 (mmol/L)
Hydroxyethyl Starch	50 (g/L)
Magnesium Gluconate (anhydrous)	5 (mmol/L)
Mannitol	30 (mmol/L)
Potassium Phosphate (monobasic)	25 (mmol/L)
Ribose, D(–)	5 (mmol/L)
Sodium Gluconate	80 (mmol/L)
Sodium Hydroxide	0.7 (g/L)
Sterile Water for Injection	To 1 L final volume

Figure 1. Composition of Belzer MPS® solution.

Oxygen pressure is maintained at ~600 mmHg through active oxygenation by device oxygenators and external supply. Perfusion can be performed through

portal vein only or through both portal vein and hepatic artery (dual HOPE or D-HOPE). D-HOPE is the technique we use at the Turin Transplant Center. However, it has been demonstrated that satisfactory liver perfusion can be obtained through portal vein perfusion only and, at present, there is no evidence of the superiority of one technique over the other.[34] In this document, HOPE will be used as an acronym for D-HOPE as well.

At our Center we use LiverAssist® (OrganAssist, Groningen, The Netherlands) device for HOPE. The device consists in two parallel pressure-controlled circuits made up of a centrifugal pump, an oxygenator and a filter. Each circuit serves either the portal vein or the hepatic artery. The liver graft effluent is collected in a common reservoir, which is connected by two separate lines to the centrifugal pumps. The only difference between the two circuits is that portal vein pump provides a continuous flow, whereas hepatic artery pump generates a pulsatile flow with an average pressure and a frequence of 60 cycles per minute. Pressure is set at 3-5 mmHg in the portal vein circuit and 25 mmHg (systolic 30 mmHg; diastolic 20 mmHg) in the hepatic artery circuits. These values have been shown to represent a good balance between organ perfusion and avoidance of endothelial injury.[35] Pressure, flow and temperature in each circuit are constantly monitored. The device can operate in a temperature range of 8°C – 37°C. Temperature is maintained by circulating water through a parallel circuit that is connected to a heat exchanger and to both oxygenators. During HOPE, temperature is set at ~10 °C. A picture and a scheme of the LiverAssist® device are presented in Figure 2.

There is a growing body of evidence, both in the laboratory and clinical setting, that the use of HOPE results in a reduction of ischemia-reperfusion injury.[2,6,7,36–42] The mechanisms by which this result is achieved are multiple. Better preservation of the endothelium is due in part to an improved preservation of endothelial cells glycocalix by low continuous shear stress.[43] Continuous perfusion also achieves a better perfusion of peri-biliary vascular plexus, resulting in a better preservation of biliary epithelium and, which is

probably more important, of peribiliary glands that are key players in biliary regeneration process after liver transplantation.[8] However, it has been postulated that the main mechanism by which HOPE protects from ischemia-reperfusion injury is related to its ability of delivering oxygen at a cellular level, thereby improving mitochondrial respiration and limiting the production of reactive oxygen species by reverse electron transfer upon organ reperfusion.[12,42]

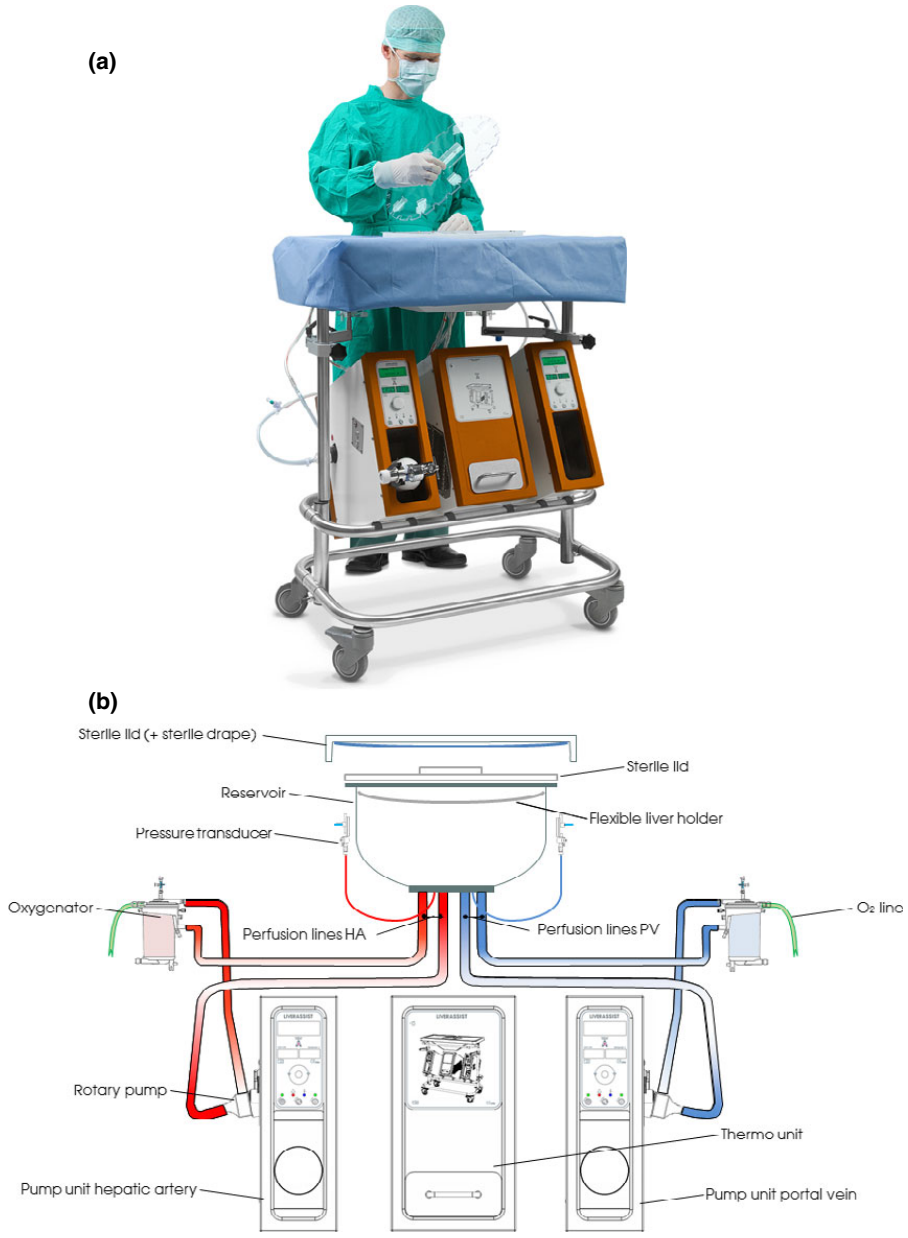

Figure 2. Picture (a) and scheme (b) of LiverAssist® device.

2.2 Ischemia-reperfusion injury

The concept of ischemia-reperfusion injury has been recently reappraised and can be applied to different organs and conditions.[11] It appears that ischemia primes tissues to subsequent damage that actually happens upon reperfusion. The very first event that is observed upon reperfusion is an abrupt overproduction ("burst") of ROS, which is responsible for mitochondrial damage, induction of mitochondrial permeability transition, disruption of ATP production and release of danger-associated molecular patter (as mitochondrial DNA – mtDNA) that can initiate inflammatory response. This will result in hepatocyte apoptosis or necrosis and alteration of the histological structure that will be replaced by fibrotic tissue, resulting in a poorly functioning graft. Although there are sources of ROS production other than mitochondria, a growing body of literature has demonstrated that the very first event in the induction of pathological ischemia-reperfusion injury is a burst of mitochondria-derived superoxide (O_2^-), which is highly unstable and leads to the production of other ROS, like hydrogen peroxide (H_2O_2). Reverse electron transfer (RET) through mitochondrial complex I is the main mechanism of superoxide production as a result of the high protonmotive force that is created upon reperfusion. To create this force, two condition needs to be met. First, ATP synthase must be inhibited, as it is the case during ischemia as a result of ADP depletion. Second, an electron store is required, which is represented by succinate that accumulates in the cytosol and in the extra-cellular space. Succinate really acts as an electron sink and may serve as the electron store that is required to drive superoxide production by RET through complex I. Upon reperfusion, succinate is rapidly oxidized to fumarate by succinate dehydrogenase, reducing CoQ pool and generating the electron force to pump protons across mitochondrial inner membrane and generating a high delta P that, as long as ATP synthase is inhibited by low ADP levels, drives RET and superoxide production. In vivo, succinate levels return to baseline after 5 minutes of reperfusion.[44,45]

Now, our question is: does hypothermic machine perfusion alter this process? Actually, what HOPE does is providing oxygen to liver cells under hypothermia. Does this prevent succinate accumulation or ROS production by RET upon reperfusion?

Concerning the protective mechanism of HOPE, what we do know is that NADH is consumed during HOPE during the first 60-90 minutes of HOPE, after which a highly oxidized state is achieved and NADH consumption slows down (Figure 3).[10]

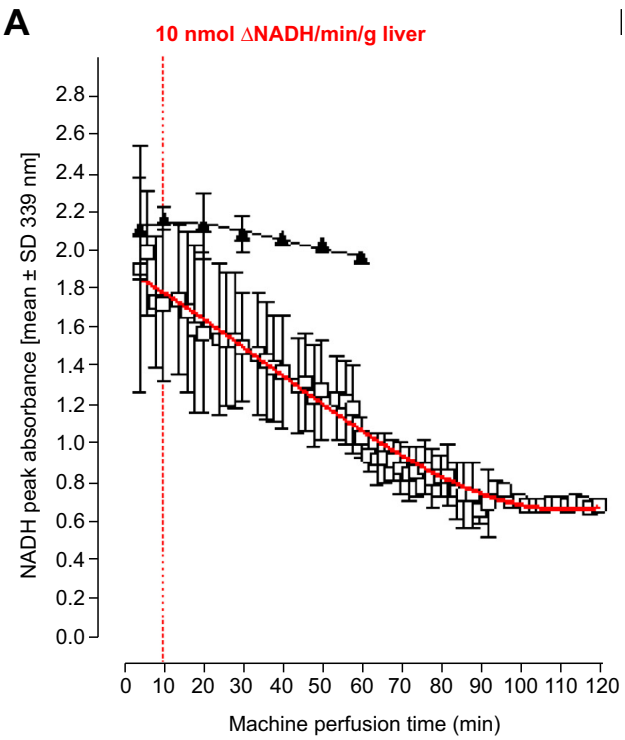

Figure 3. NADH consumption during HOPE. (From Schlegel et al. J Hepatol 2013)

During HOPE, hepatic ATP increases by several folds and, as a result of its decreased metabolism under hypothermia, accumulates in the cell reducing the "oxygen debt" due to hypoxia.[2] Hepatic succinate content, as evaluated on pre-transplant liver biopsies in a rat model of HOPE and orthotopic liver transplantation using fatty liver grafts, is reduced after HOPE treatment as compared to grafts preserved by static cold storage.[6] Overall, these findings suggest that HOPE restores mitochondrial phosphorylation and redox state in hypothermic conditions, reducing the burst of ROS at warm reperfusion.[12]

However, the evidence supporting the theory about HOPE mechanism of action is scarce and largely inductive.

3. MICRODIALYSIS

Microdialysis (MD) is a technique that allows investigating the concentration of solutes in the interstitial fluid. The microdialysis probe is designed to mimic a blood capillary and consists of a shaft with a semipermeable hollow fiber membrane at its tip, which is connected to inlet and outlet tubing. The probe is continuously perfused with an aqueous solution (perfusate) that closely resembles the ionic composition of the surrounding tissue fluid at a low flow rate of approximately 0.1-5µL/min. Once inserted into the tissue or fluid of interest, small solutes can cross the semipermeable membrane by passive diffusion. The direction of the analyte flow is determined by the respective concentration gradient and allows the usage of microdialysis probes as sampling as well as delivery tools. The solution leaving the probe (dialysate) equilibrates with the interstitial fluid and can be collected at certain time intervals to determine levels of metabolites, citokines or other molecules (Figure 4).

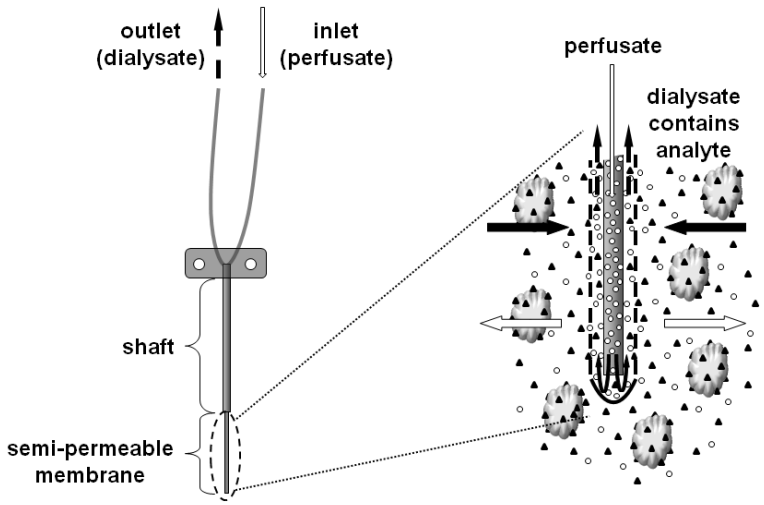

Figure 4. Schematic illustration of a microdialysis probe.

Different MD catheters have membranes with pores of different size allowing the passage of molecules of different molecular weights. Thus, the choice of the catheter depends upon the size of molecules the level of which is to

be determined in the dialysate. The molecular weight cutoff of commercially available microdialysis probes covers a wide range of approximately 6-100kD.

MD has been used to monitor liver graft metabolism and interstitial cytokines concentration in the early postoperative period after liver transplantation[18,29] and has been shown to have some discriminatory ability in the early detection of vascular complications and rejection.[25,26]

MD has been rarely used to evaluate organs during machine perfusion too. Baicu et al.[31] used microdialysis during kidney hypothermic machine perfusion to assess changes in metabolism after supplementing the perfusate with fructose-1,6-diphosphate. Findings of this experiment were not really surprising (fructose added to the perfusate enhanced pyruvate production), but showed how microdyalisis can be effectively employed to study metabolism in a hypothermically perfused organ. Hamaoui et al.[33] compared cortical and medullar lactate concentrations in porcine kidney preserved by either static cold storage or hypothermic machine perfusion and subsequent rewarming, showing different patterns of lactate metabolism. This study set the stage to the possibility of graft metabolism monitoring during both static cold storage and hypothermic machine perfusion. In a recent study, Mazzeo et al.[30] showed that MD metabolites level predicted unfavorable outcome of ex-vivo lung perfusion (defined as lung deemed not suitable for transplantation or transplanted and presenting initial graft dysfunction) and was more reliable than metabolites level on perfusate.

The objectives of this study are confirming HOPE putative mechanism of action by analyzing interstitial space metabolites concentration using microdialysis and gathering further knowledge about the timing and dynamic of processes happening at a cellular level during HOPE, with the potential to identify viability markers.

4. LIVER TRANSPLANTATION AND D-HOPE

The unmet gap between organ demand and supply in recent years has pushed the liver transplant community to make increasing use of so-called extended criteria or marginal donors (ECD). Although criteria to define marginality are arbitrary, extended criteria grafts are most frequently those with moderate or severe graft steatosis or from donors of an advanced age or after circulatory death. Utilizing these grafts has been associated with an increased rate of primary non-function, early allograft dysfunction, graft cholangiopathy and with reduced graft survival.[38,46–48] In this regard, various machine perfusion techniques have emerged as valuable alternatives to static cold storage, allowing a safer utilization of ECDs.[6,38–40,49]

The term "machine perfusion" covers a variety of techniques characterized by different settings, timing and technology. The potential benefits and indications of each of these factors are still under investigation.[6,38,49]

Amongst these, end-ischemic hypothermic oxygenated machine perfusion (HOPE) has been associated with improved post-transplant outcomes, expecially with grafts from DCD donors.

At our Center we use HOPE for all grafts from DCD donors and for specific brainstem death ECD donors that we identified as: donor age ≥80 years; donor body mass index (BMI) ≥30 kg/m2 associated with apparent severe graft steatosis as assessed by the retrieval surgeon; logistic issues determining expected ischemia time ≥10 hours.[4]

5. AIM OF THE STUDY

The aim of this study was to assess time course of liver metabolism biomarkers during static cold storage and HOPE using microdialysis and to explore association between MD metabolites and graft function after liver transplantation.

6. PATIENTS AND METHODS

6.1 Study design

This was a prospective, open-label observational pilot study on 10 consecutive grafts treated with dual-HOPE (i.e. double cannulation of both portal vein and hepatic artery – D-HOPE) in the period October 2019 – January 2020 at our Institution. The study was approved by the local ethics committee (resolution nr. 739 of June 10th, 2019). Patients signed a consent form for receiving an organ treated with machine perfusion and for participating in the study. All study procedures complied with the Declaration of Helsinki and the Declaration of Istanbul (https://www.wma.net). The study was supported by grant no. S1618_L2_ MAZA_01 from Compagnia San Paolo, Torino.

Our machine perfusion and LT protocols have been previously described.[4,50,51] Use of D-HOPE was systematic for grafts from DCD donors and evaluated on a case-by-case basis for grafts from DBD donors, mainly based on donor age and steatosis.[4] End-ischemic D-HOPE using LiverAssist® (OrganAssit, Groningen, The Netherlands) primed with 3 L of Belzer MPS® fluid (Bridge to Life Europe Ltd. London, UK) was continued for a minimum of 90 minutes during recipient hepatectomy. For this study, liver graft was weighed before and after machine perfusion to detect swelling during machine perfusion.

During these procedures, MD was used to sample extracellular fluid during backtable preparation and subsequent machine perfusion. Study design and timing of sample collection are summarized in Figure 5. Briefly, liver was perfused with Celsior® (IGL, Lisseu, France) at retrieval and transported in SCS at our centre. The liver was unpacked upon arrival and, before the start of backtable preparation, a 61 hepatic microdialysis catheter® with membrane length = 30 mm and membrane cutoff ~ 20kDa (M Dialysis AB, Stockholm,

Sweden) was sterilely inserted at a ~ 4 cm depth by the mean of a splitable introducer into liver segment 6 and secured by a 5/0 Prolene suture.

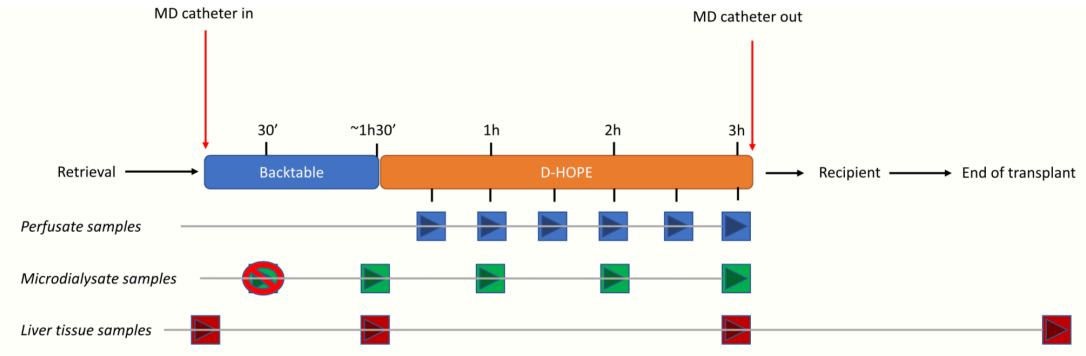

Figure 5. Study design.

The MD catheter was connected to a 107 Microdialysis pump® charged with normal saline with a flow set at 2 μl/min (to reduce the time necessary to fill the system, the pump automatically flushes the catheter at 15 μl/min for 5 minutes upon activation). By this setting, concentration of metabolites in microdialysate represents roughly 40% of extracellular fluid concentration.[52] The first MD vial was connected to the MD catheter after the first drop of microdialysate appeared at the tip of the connection needle and discarded after 30 minutes to allow fluid equilibration, as recommended.[13,53,54] The second vial, which was the first to be analyzed, was collected at the end of backtable preparation, at least one hour after having been positioned, and was therefore representative of liver metabolism during the last part of SCS. Subsequently, the liver was connected to the perfusion device with the MD catheter in place and MD vials were changed hourly during D-HOPE. Thus, at least 3 MD samples were available for every single procedure: the first representing the terminal phase of SCS, and the remaining two samples D-HOPE phase. When D-HOPE time was <120 min, the last MD sample was collected at the end of machine perfusion, thus representing extracellular fluid concentration during the 2nd hour of D-HOPE. During D-HOPE, perfusate samples were collected every 30 minutes.[50] All samples were snap frozen and subsequently analyzed using CMA 600 Microdialysis Analyzer. Concentration of glucose, lactate, pyruvate and

glutamate was measured, and lactate/pyruvate ratio was calculated. Perfusate and microdyalisate flavin mononucleotide (FMN) level was measured with Synergy HTX microplate reader (BioTel Instruments, Winooski, VT, USA) using an excitation wavelength of 460/40 nm and recording the fluorescence with wavelength of 528/20 nm with 100% gain, as previously described.[55,56] FMN levels were expressed as relative fluorescing units (RFU). The MD catheter was removed at the end of machine perfusion, before moving the graft to the recipient for implantation. All perfusate parameters, except FMN, which was expressed as RFU, were normalized to liver weight.

D-HOPE and MD were not used for graft evaluation and all livers in this series were transplanted. A t-tube was routinely positioned during transplant operation and removed at 3 months after a control cholangiogram.

6.1.1 Hepatic biopsies collection

Wedge liver biopsies were collected at the beginning of backtable preparation, before and after D-HOPE, and at the end of transplant. Biopsies were immediately immerged in RNA Later solution (Invitrogen, Thermofisher) and freezed at -20°C for subsequent determination of Interleukin-6 (IL-6), Interleukin-8 (IL-8), Tumor necrosis factor α (TNFα), Toll-like receptor-4 (TLR-4), Intercelular adhesion molecule 1 (ICAM 1) and C-X-C Motif Chemokine Ligand 12 (CXCL12) expression.

6.1.2 RNA extraction and RT-PCR

An equal amount of each biopsy (50 mg) was suspended in 500 ul of TRIzol™ solution (Invitrogen, Thermofisher) together with 0.5 mm size zirconium beads, then homogenized by Bullet Blender (Next Advance, Inc) at a speed of 8 rpm for 3 minutes at 4°C. The homogenized tissue was centifuged at 12,000 g for 15 minutes at 4°C and the collected supernatant was subjected to RNA extraction according to manufacturer's protocol. Briefly, 200 µl of chloroform were added to each sample and after three minutes recovery at room temperature (RT), then samples were centrifuged at 12,000 g for 15 minutes at 4° C. After that, the

chloroform-RNA phase was collected and an appropriate quantity of isopropanol was added for each sample. After 10 minutes recovery at RT, samples were centrifuged at 12,000 g for 10 minutes 4°C. Then, the pelleted RNA was washed with absolute ethanol and centrifuged at 12,000 g for 5 minutes 4°C. After ethanol removal, the isolated RNA was suspended in nuclease free water and quantified spectrophotometrically by NanoDrop 2000 (Thermofisher Scientific). A total amount of 200 ng of RNA per sample was retro-transcribed in cDNA in a 20 µl reaction using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermofisher). Then, a Real Time Polymerase Chain Reaction (RT-PCR) was performed on 5 ng cDNA samples in duplicate, according to the chemistry of Power SYBR® Green PCR Master Mix (Applied Biosystems, Thermofisher), using specific oligonucleotide primers and ran by StepOne Real Time System (Applied Biosystems, Thermofisher). Comparative ΔΔCt method was used to calculate the relative expression of genes of interest normalized to the house-keeping gene expression Actinβ. The hepatic biopsies collected at T1 were used as reference samples for the quantitative analysis. Data were expressed as Relative Quantification (RQ) mean ± SEM. One way or two way Anova were performed as statistical analysis, with Tukey's multiple comparison test. A p-value <0.05 was considered as statistical significant. All analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, California USA, www.graphpad.com).

6.1.3 Hepatic tissue succinate

Succinate level was measured on hepatic biopsies using the Succinate Colorimetric Assay Kit (K649, Biovision Incorporated, Milpitas, CA, USA). Briefly, an equal amount of each biopsy was suspended in ice cold Succinate Assay Buffer (K649-1, Biovision Incorporated, Milpitas, CA, USA) together with 0.5 mm size zirconium beads, then homogenized by a Bullet Blender (Next Advance, Inc), at a speed of 8 rpm for 3 minutes at 4°C. Proteins were collected from the homogenized tissue by centrifugations at 12,000 g for 15 min at 4°C. Then,

sample was analyzed by the Succinate Colorimetric Assay Kit, according to the manufacturer's instructions, and the optical density at 450 nm was read by iMark™ Microplate Absorbance Reader (Bio-Rad, Hercules, CA, USA). Succinate level was normalized towards the T1 biopsy and expressed as Arbitrary Unit (A.U.) mean ± SEM. The Wilcoxon matched-pairs signed rank test was performed between columns with two-sided option. A p-value <0.05 was considered as statistical significant. All analyses were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, California USA, www.graphpad.com).

6.1.4 Histological analysis

Histological ischemia-reperfusion injury and steatosis were determined on liver biopsies obtained at the end of transplant. Tissue samples were fixed and processed to obtain 5-µm-thick sections. Hematoxylin and eosin, periodic acid-Schiff, and periodic acid-Schiff-diastase staining were performed to evaluate steatosis, necrosis, as well as glycogen content and distribution.

6.1.5 Other collected variables

Collected variables included indication for LT, recipient and donor features, as well as times and technical details about retrieval, initial SCS, D-HOPE and transplant operation. Levels and trend of microdialysate and perfusate metabolites were described in the whole cohort and analyzed with regards to their correlation with outcome measures, as outlined below.

6.2 Outcome measures

Primary outcome measures were early allograft dysfunction (EAD)[57] and L-GrAFT score.[58] Secondary outcome measures were duration of hospital stay, onset and grade of acute kidney injury (AKI)[59], and comprehensive complication index (CCI)[60] calculated at hospital discharge and at 6-month follow-up. Graft

survival and occurrence of biliary complications were also evaluated. Minimum follow-up was 6 months.

6.3 Statistical analysis

Variables were expressed as number (%) or median (interquartile range) and compared using standard parametric and non-parametric tests. All perfusate values were normalized to liver weight. For repeated measures Anova, normal distribution of variables was verified by Shapiro-Wilk test and by visual inspection using quantile-quantile plots. P-values of pairwise t-tests between different timepoints were adjusted using the Bonferroni multiple testing correction method. Correlation between variables was evaluated using Pearson correlation coefficient. Optimal cutoff points in ROC analyses were calculated using Youden method. Considering the exploratory nature of this study, no sample size calculation was made. All analyses were performed using R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/).

7. RESULTS

7.1 D-HOPE procedure and clinical outcome

Recipient and donor features are summarized in Table 1.

TABLE 1 Baseline characteristics of recipients and donors

Recipient features							Donor features				
n	S	Age (years)	BMI	Indication	HCC	MELD	Age (years)	Type	BMI	M (%)	μ (%)
1	M	68	27	ETOH+NASH	No	15	75	DBD	45	20	70
2	M	60	27	PBC	Yes	11	60	DCD.3	26	1	5
3[a]	M	62	33	ETOH+NASH	yEs	8	48	DBD	37	25	30
4[b]	M	53	33	HCV+NASH	Yes	20	63	DBD	29	40	30
5	M	66	22	ETOH	No	13	58	DBD	41	5	35
6[a]	F	64	21	ETOH	No	13	83	DBD	23	10	60
7	F	63	26	HBV+NASH	Yes	9	83	DBD	24	3	40
8	F	61	18	HBV+HDV	Yes	18	80	DBD	27	10	70
9	M	67	27	NASH	Yes	9	53	DBD	34	1	2
10	F	66	28	NASH	Yes	9	83	DBD	20	0	5
Median		63	23			12	69		28	7	32
25th %		61	27			9	59		24	1	11
75th %		66	28			14	82		36	17	55

Abbreviations: BMI, body mass index; Crea, creatinine; DBD, donation after brain death; DCD, Maastricht category 3 donation after circulatory death; ETOH, alcoholic cirrhosis; GGT, gamma glutamyl transferase; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDV, hepatitis D virus; M, macrovesicular steatosis; Na, sodium; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; S, sex; μ, microvesicular steatosis.

[a]Developed early allograft dysfunction.

[b]Developed graft failure.

Machine perfused grafts were preferentially allocated to low MELD (12.1[9.1, 14.5]) patients, of whom 7 (70%) had hepatocellular carcinoma. The indication for D-HOPE was based on advanced donor age (cases 6, 7, 8, and 10), high BMI and steatotic graft appearance (cases 3, 4, 5, and 9), advanced donor age in association with graft steatosis (case 1) and donation after circulatory death (case 2). Table 2 summarizes times and graft weight before and after machine perfusion. Cold ischemia and D-HOPE time were 344.5 (295.2, 366.8) and 116 (103, 143) minutes. In 3 grafts weight increased ≥ 5% during D-HOPE. Total preservation time never exceeded 10 h 15 min.

TABLE 2 Operational characteristics of liver grafts

n	Retr. (min)	CIT[a] (min)	BT (min)	D-HOPE (min)	Weight pre (g)	Weight post (g)	Delta weight (%)	rWIT[b] (min)	Tot (min)
1	47	341	95	106	1550	1480	−4	30	491
2	37	360	94	147	1370	1370	0	23	550
3	57	369	130	103	2290	2410	+5	25	508
4	80	443	120	131	2400	2800	+17	37	614
5	67	407	135	103	1980	2090	+6	21	530
6	48	291	72	151	1500	1480	−1	24	497
7	41	296	115	125	1180	1120	−5	21	453
8	35	295	105	190	1000	1010	+1	24	524
9	43	348	170	91	1750	1760	+1	21	474
10	50	268	110	101	940	960	+2	22	404
Median	47	344	112	116	1525	1480	0.8	23	502
25th %	41	295	97	103	1227	1182	−1.0	21	478
75th %	55	367	127	143	1922	2007	4.4	25	528

Abbreviations: BT, back table preparation; CIT, cold ischemia time; D-HOPE, dual hypothermic oxygenated machine perfusion; Retr, donor hepatectomy time; rWIT, recipient warm ischemia time; Tot, total preservation time.

[a]Cold ischemia time from cold perfusion in the donor to D-HOPE start.

[b]Time from start of vascular anastomoses to graft reperfusion into recipient. The liver was weighed before and after machine perfusion and the weight variation was expressed as a percentage of the initial weight.

Median MD time, from insertion of the first microvial to removal of the last one, was 228 (210, 245) minutes. No major adverse events occurred and occasional mild bleeding from MD catheter entry site was easily controlled by diathermy. No patient developed catheter-related bleeding or intra-or extra-hepatic hematoma, as assessed by ultrasound scan performed after LT.

Study variables and outcome measures are summarized in Table 3. Three patients developed EAD after LT and, of those, one later developed delayed non-function and required retransplantation. All three patients had a transaminase peak ≥2000 IU, whereas only the patient who subsequently suffered from graft failure had bilirubin level ≥10 mg/dl on day 7th after LT. Patients who developed EAD had higher transaminase peak, as well as higher day 7th bilirubin, INR, and alkaline phosphatases levels. L-GrAFT score was higher in the EAD group, with a difference approaching statistical significance (estimated risk of graft loss 22.1% vs. 7.9%, p = 0.09). Concerning baseline variables the only significant difference between EAD and non-EAD cases was a higher percentage of macrovesicular steatosis (25% versus 3%, p = 0.04) in EAD group. Degree of macrovesicular steatosis was 25% and 10% in patients who recovered from EAD, whereas it was 40% in the patient who developed graft failure.

TABLE 3 Study variables in the whole series and according to the onset of early allograft dysfunction

	Overall	EAD	No EAD	
	10	3	7	*p*
Recipient age (years)	63.5 [61.0, 66.3]	62.1 [57.7, 63.0]	65.8 [61.8, 66.6]	0.21
Recipient BMI	26.8 [23.1, 28.1]	33.1 [27.1, 33.1]	26.7 [24.1, 27.1]	0.30
Donor age (years)	69.1 [58.6, 82.2]	63.5 [55.9, 73.4]	74.8 [59.1, 81.5]	0.73
Donor BMI	28.0 [24.4, 36.1]	29.3 [26.2, 33.0]	26.8 [25.0, 37.6]	0.91
Donor ICU stay (days)	4.0 [3.2, 5.5]	4.0 [3.0, 5.0]	4.0 [3.5, 5.5]	0.72
Donor GGT (IU/L)	25 [18, 46]	32.00 [25, 41]	23 [15, 93]	0.65
Macrosteatosis (%)	7.5 [1.5, 17.5]	25.0 [17.5, 32.5]	3.0 [1.0, 7.5]	0.04
Microsteatosis (%)	32.5 [11.2, 55.0]	30.0 [30.0, 45.0]	35.0 [5.0, 55.0]	0.91
Donor hepatectomy (min)	47 [41, 55]	57 [52, 68]	43 [39, 48]	0.09
Cold ischemia time (min)	344 [29, 366]	369 [330, 406]	341 [295, 354]	0.42
Backtable time (min)	112 [97, 127]	120 [96, 125]	110 [100, 125]	0.91
D-HOPE (min)	113 [103, 123]	120 [111, 120]	106 [98, 136]	1.00
Graft weight pre (g)	1525 [1227, 1922]	2,290 [1895, 2345]	1,370 [1090, 1650]	0.09
Graft weight post (g)	1,480 [1182, 2007]	2,410 [1945, 2605]	1,370 [1065, 1620]	0.07
Delta weight (%)	0.80 [−0.98, 4.43]	5.20 [1.95, 10.95]	0.60 [−2.25, 1.55]	0.30
Total preservation (min)	502 [478, 528]	508 [502, 561]	491 [463, 527]	0.30
Surgery time (min)	365 [287, 455]	395 [354, 455]	335 [278, 448]	0.42
PRBC units	4.0 [4.0, 7.5]	4.0 [4.0, 4.0]	6.0 [2.0, 10.5]	0.48
End lactate (mmol/L)	2.15 [1.48, 2.30]	2.10 [1.60, 2.15]	2.30 [1.65, 2.30]	0.42
Induction (basiliximab)	8 (80.0%)	3 (100.0%)	5 (71.4%)	0.86
Day of IS start				0.24
0	3 (30.0%)	1 (33.3%)	2 (28.6%)	
1	6 (60.0%)	1 (33.3%)	5 (71.4%)	
4	1 (10.0%)	1 (33.3%)	0 (0.0%)	
AST peak (IU/L)	1,020 [676, 1850]	3,375 [2695, 7725]	721 [616, 1020]	0.02
ALT peak (IU/L)	837 [463, 1003]	1,000 [937, 1048]	510 [419, 902]	0.21
Bilirubin day 7th (mg/dl)	2.0 [1.4, 4.8]	5.5 [3.1, 9.0]	2.0 [1.5, 2.4]	0.57
INR day 7th	1.2 [1.2, 1.3]	1.3 [1.3, 1.4]	1.2 [1.2, 1.3]	0.64
ALP day 7th (IU/L)	192.0 [143.8, 231.5]	237.0 [169.0, 340.5]	170.0 [149.5, 214.5]	0.49
L-GrAFT (risk %)	8.7 [7.7, 19.2]	22.1 [15.6, 51.3]	7.9 [6.3, 9.4]	0.09
AKI stage				0.28
No AKI	2 (20.0%)	1 (33.3%)	1 (14.3%)	
1	5 (50.0%)	1 (33.3%)	4 (57.1%)	
2	2 (20.0%)	0 (0.0%)	2 (28.6%)	
3	1 (10.0%)	1 (33.3%)	0 (0.0%)	
Clavien-Dindo ≥3 complications	2 (20.0%)	2 (66.7%)	0 (0.0%)	0.12
Hospital CCI	21.7 [11.7, 27.8]	33.5 [16.7, 51.5]	20.9 [14.8, 22.6]	0.42
6-month CCI	30.6 [22.6, 38.7]	47.4 [40.5, 73.7]	22.6 [21.7, 31.9]	0.05

Note: Data are presented as median [IQR] or number (%), as appropriate.

Abbreviations: AKI, acute kidney injury; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; CCI, comprehensive complication index; D-HOPE, dual hypothermic oxygenated machine perfusion; EAD, early allograft dysfunction; GGT, gamma glutamyl transferase; ICU, intensive care unit; INR, international normalized ratio; IS, immunosuppression; L-GrAFT. Liver graft assessment following transplantation; PVT, portal vein thrombosis; TIPS, transjugular intrahepatic portosystemic shunt.

With regards to clinical outcome, patients in the EAD group had in general poorer outcome and suffered from a higher rate of surgical complication postoperatively and at 6-month follow-up, as demonstrated by a higher CCI (47.4 vs. 22.6, p = 0.05). Only patient 4 developed Clavien-Dindo ≥3b complications, represented by coagulopathy-related bleeding requiring relaparotomy and

temporary packing on postoperative day 1, followed by renal failure requiring renal replacement therapy and graft failure leading to retransplantation on postoperative day 31st.

Four (40%) patients developed biliary complications (anastomotic, n = 3; ischemic-type, n = 1). Patients 3, 8, and 10 presented at 3-month cholangiogram with sludge and anastomotic stricture, which were successfully managed endoscopically. Patient 6 developed an ischemic-type stricture of the biliary confluence, which was successfully managed by percutaneous balloon bilioplasty and no evidence of recurrence thereafter.

Median follow-up was 10.9 (9.8, 11.6) months. Patient and graft survival were 90%. Patient 4, after making a good recovery after re-LT, died 6 months after LT due to complications of HHV8 infection.

7.2 Microdialysate and perfusate metabolites

Figure 6 depicts values of MD metabolites during SCS and D-HOPE. Only 3 patients had D-HOPE lasting > 2 hours and had samples representative of this time frame. We observed a significant raise of MD glucose level upon initiation of D-HOPE, which increased from 49 (42, 68) mg/dl to 133 (118, 146) and 152 (119, 216) mg/dl at 1^{st} and 2^{nd} hour of D-HOPE, respectively (p = 0.01). A similar trend, albeit non-significant, was observed for lactate, which increased from 1.9 (1.4, 2.3) mmol/L to 2.8 (2.2, 3.8) mmol/L at 1^{st} hour and 2.9 (2.2, 4.4) mmol/L at 2^{nd} hour (p = 0.08). Levels of glutamate in MD fluid persisted high throughout the procedure (200 [189, 206], 192 [187, 194] and 196 [190, 199] μmol/L during SCS, D-HOPE 1^{st} and 2^{nd} hour, respectively) and were unaffected by D-HOPE, whereas pyruvate levels were persistently low (4 [2, 4], 4 [2, 6] and 6 [4, 8] μmol/L at three timepoints, respectively). Lactate/pyruvate ratio trend closely mimicked that of lactate, as an effect of low pyruvate levels.

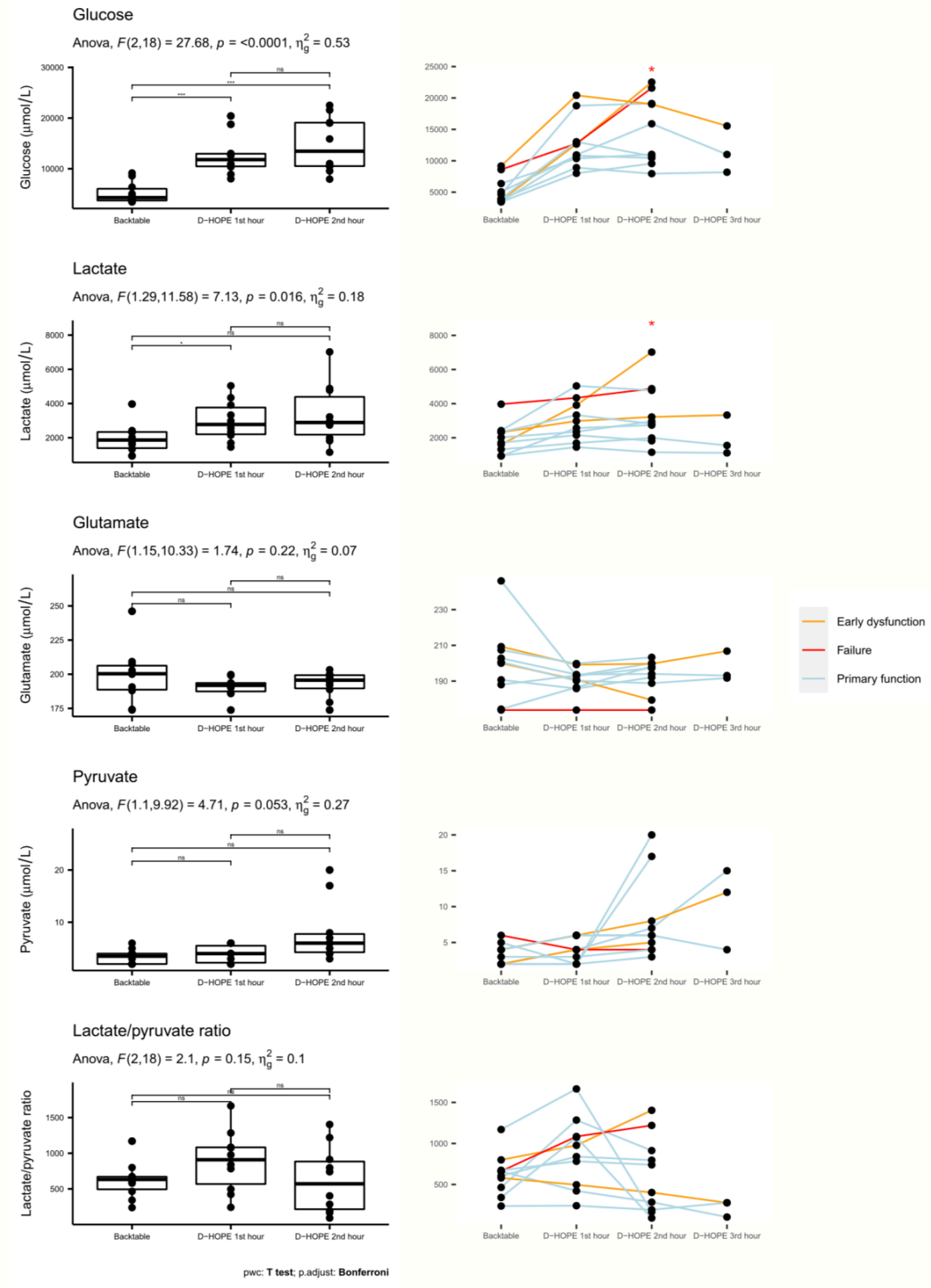

Figure 6. Glucose, lactate, glutamate, pyruvate levels and lactate/pyruvate ratio in microdialysate during backtable preparation and subsequent machine perfusion. In the left column, levels across different timepoints are compared using ANOVA for repeated measures. Degrees of freedom, F-statistic (F), p-value, and generalized effect size squared (η2) are provided for each biomarker.

Levels of significance of pairwise t-test across different time points is indicated as non-significant (ns), <0.01 (**) or <0.001 (***). Y axis scale changes across different plots to improve data visualization. As only 3 grafts had a D-HOPE time exceeding 2 h, the 3-h timepoint is not visualized. In the right column, line plots depicting the trend of study metabolites in each patient are provided. Line colors identify patients who had primary graft function (light blue), early allograft dysfunction (orange), or required retransplantation (red). 2nd hour samples were collected 2 h after the beginning of D-HOPE or at the end of machine perfusion when D-HOPE time was <120 min. Asterisks indicate that glucose and lactate levels during the 2nd hour of D-HOPE were significantly higher in patients developing early allograft dysfunction. pwc, pairwise comparison

Kinetics of MD lactate and glucose was different in grafts that developed EAD (Figure 6). In particular, levels of glucose and lactate were significantly higher during 2nd hour of D-HOPE (244 vs. 121 mg/dl, p = 0.03 and 4.9 vs. 2.7 mmol/L, p = 0.03) (Table 4). In contrast with glucose and lactate levels on perfusate, levels of MD metabolites clearly diverged, being significantly higher in dysfunctioning grafts at two hours of machine perfusion (Figure 7, Table 4, Table 5).

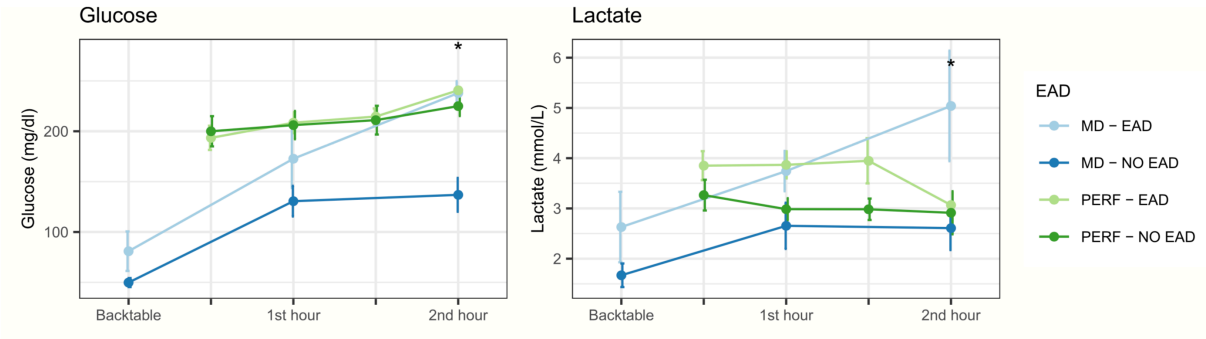

Figure 7. Line plots depicting trend of glucose and lactate in perfusate (green and light green) and microdialysate (blue and light blue), according to subsequent development of early allograft dysfunction. In cases in which D-HOPE time was <120 min, 2nd hour samples were collected at the end of machine perfusion. Values are represented as mean ± standard error (vertical error bars).

For 2nd-hour MD glucose, the area under the receiver operating characteristic curve evaluating its association with EAD was 0.952. With a cut-off value of 215 mg/dl, 2nd hour MD glucose had 100% sensitivity, 86% specificity, 75% positive predictive value, and 100% negative predictive value for the development of EAD.

Table 4. Microdialysate values stratified by early allograft dysfunction

Value	Overall	No EAD	EAD	p
	10	7	3	
Glucose (μmol/L)				
Backtable	4300 [3740, 6050]	3960 [3680, 4850]	8600 [6150, 8885]	0.21
1st hour	11800 [10475, 12958]	10820 [9635, 11985]	12740 [12700, 16580]	0.14
2nd hour	13460 [10520, 19092]	10730 [10005, 13460]	21580 [20295, 22050]	0.03
3rd hour	11010 [9595, 13280]	9595 [8888, 10302]	15550 [15550, 15550]	0.22
Lactate (μmol/L)				
Backtable	1865 [1390, 2335]	1710 [1135, 2180]	2320 [1960, 3145]	0.31
1st hour	2775 [2200, 3765]	2350 [1920, 2950]	3910 [3445, 4125]	0.14
2nd hour	2890 [2178, 4390]	2740 [1905, 2890]	4880 [4050, 5950]	0.03
3rd hour	1550 [1330, 2440]	1330 [1220, 1440]	3330 [3330, 3330]	0.22
Glutamate (μmol/L)				
Backtable	200 [189, 206]	201 [189, 205]	200 [187, 205]	0.73
1st hour	192 [187, 194]	193 [188, 193]	191 [182, 195]	0.73
2nd hour	196 [190, 199]	197 [193, 199]	179 [177, 190]	0.21
3rd hour	193 [192, 200]	192 [192, 193]	207 [207, 207]	0.22
Pyruvate (μmol/L)				
Backtable	4 [2, 4]	3 [2, 4]	4 [3, 5]	0.47
1st hour	4 [2, 6]	3 [2, 5]	4 [4, 5]	0.29
2nd hour	6 [4, 8]	6 [5, 12]	5 [4, 6]	0.65
3rd hour	12 [8, 14]	10 [7, 12]	12 [12, 12]	1.00
L/P ratio				
Backtable	632 [494, 670]	605 [404, 667]	662 [621, 731]	0.43
1st hour	909 [568, 1082]	840 [603, 1180]	978 [737, 1031]	0.91
2nd hour	571 [215, 884]	284 [179, 768]	1220 [811, 1312]	0.09
3rd hour	278 [190, 278]	190 [147, 234]	278 [278, 278]	0.48

Data are expressed as median [interquartile range] and compared using non-parametric Mann-Whitney test. Abbreviations: EAD, early allograft dysfunction; L/P, lactate/pyruvate

Table 5. Normalized perfusate values stratified by early allograft dysfunction.

Value	Overall	No EAD	EAD	p
	10	7	3	
Glucose (mg/dl)				
30’	193 [174, 212]	194 [171, 224]	191.2 [182, 203]	0.91
60’	198 [189, 223]	194 [180, 229]	198.8 [198, 214]	0.57
90’	204 [189, 225]	194 [185, 232]	208.3 [206, 219]	0.31
120’	226 [210, 244]	210 [209, 233]	240.7 [240, 240]	0.65
Lactate (mmol/L)				
30’	3.4 [3.0, 3.9]	3.4 [2.8, 3.7]	3.9 [3.6, 4.1]	0.43
60’	3.3 [3.0, 3.7]	3.1 [2.6, 3.3]	4.1 [3.7, 4.1]	0.03
90’	3.3 [2.9, 3.5]	3.3 [2.6, 3.4]	4.2 [3.7, 4.4]	0.14
120’	2.8 [2.4, 3.3]	2.5 [2.3, 3.3]	3.1 [3.1, 3.1]	0.65
Glutamate (μmol/L)*				
30’	214 [181, 261]	233.1 [191, 296]	202.6 [139, 211]	0.21
60’	222 [202, 275]	241.3 [211, 305]	199.5 [150, 214]	0.14
90’	224 [203, 267]	249.6 [210, 317]	196.2 [146, 214]	0.09
120’	224 [221, 275]	275.3 [249, 324]	163.1 [134, 191]	0.08
Pyruvate (μmol/L)*				
30’	9 [6, 16]	12.8 [8, 18]	5 [3, 6]	0.09
60’	12 [10, 20]	12.0 [10, 21]	10 [7, 16]	0.57
90’	18 [8, 23]	22.3 [17, 24]	4 [2, 7]	0.03
120’	15 [15, 19]	19.0 [17, 24]	8 [5, 11]	0.08
L/P ratio*				
30’	450 [241, 488]	269.3 [188, 450]	625 [559, 1612]	0.02
60’	241 [159, 365]	240 [154, 243]	404 [277, 661]	0.43
90’	156 [134, 408]	146 [127, 155]	881 [594, 3165]	0.03
120’	170 [116, 226]	131 [100, 203]	209 [209, 209]	0.65

*Perfusate values of glutamate and pyruvate needs to be interpreted with caution as they were constantly close to the low (pyruvate) or high (glutamate) limit of determination of the analyzer. Data are expressed as median [interquartile range] and compared using non-parametric Mann-Whitney test. Abbreviations: EAD, early allograft dysfunction; L/P, lactate/pyruvate

2nd-hour MD glucose and lactate level were positively correlated with L-GrAFT score, 6-month CCI, graft weight variation during D-HOPE, cold ischemia time, and macrosteatosis (Figure 8).

Levels of MD metabolites were not associated with the development of biliary complications. However, both surviving grafts that initially developed EAD subsequently developed biliary complications, including one case of ischemic cholangiopathy.

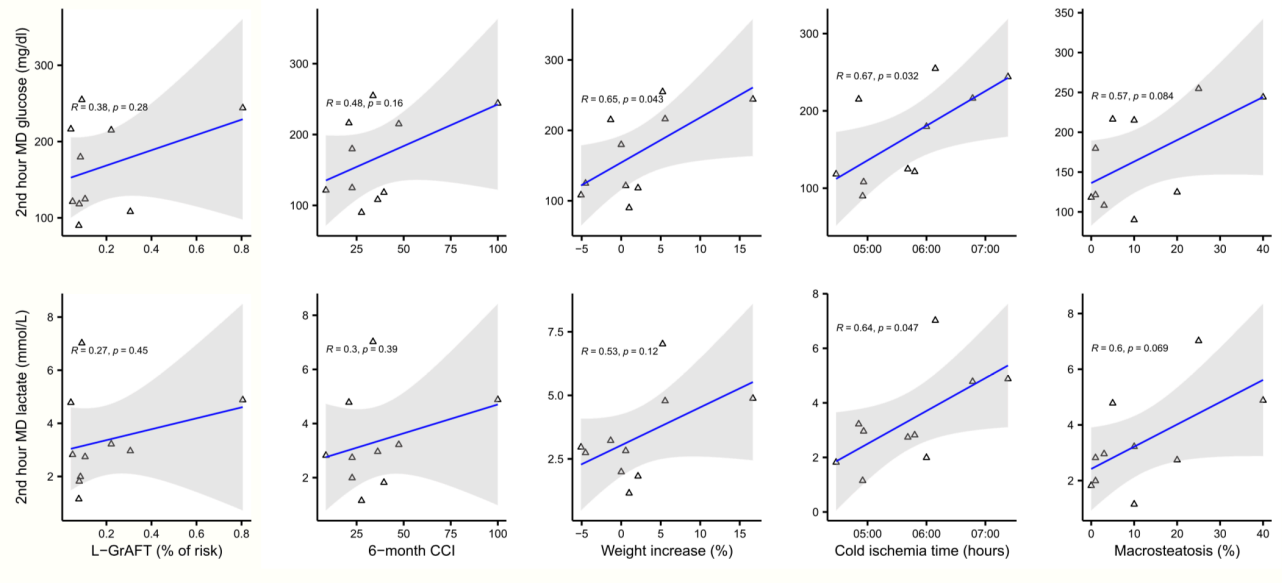

Figure 8. Correlation of 2nd hour MD metabolites with L-GrAFT score, 6-month CCI and graft characteristics.

7.3 Microdialysate and perfusate FMN

Figure 9 depicts FMN levels in perfusate and microdialysate. As opposed to glucose and lactate, FMN levels in microdialysate significantly dropped after initiation of D-HOPE, independently of graft function. In contrast, adjusted and non-adjusted perfusate FMN levels were higher in grafts who developed early dysfunction, although this difference did not achieve statistical significance (Table 6). The only graft that developed delayed non-function and required re-LT was characterized by the highest perfusate FMN levels, which progressively increased throughout D-HOPE. Perfusate FMN levels were positively correlated with L-GrAFT score, 2nd-hour MD glucose and lactate, and with clinical outcome measures (Figure 10).

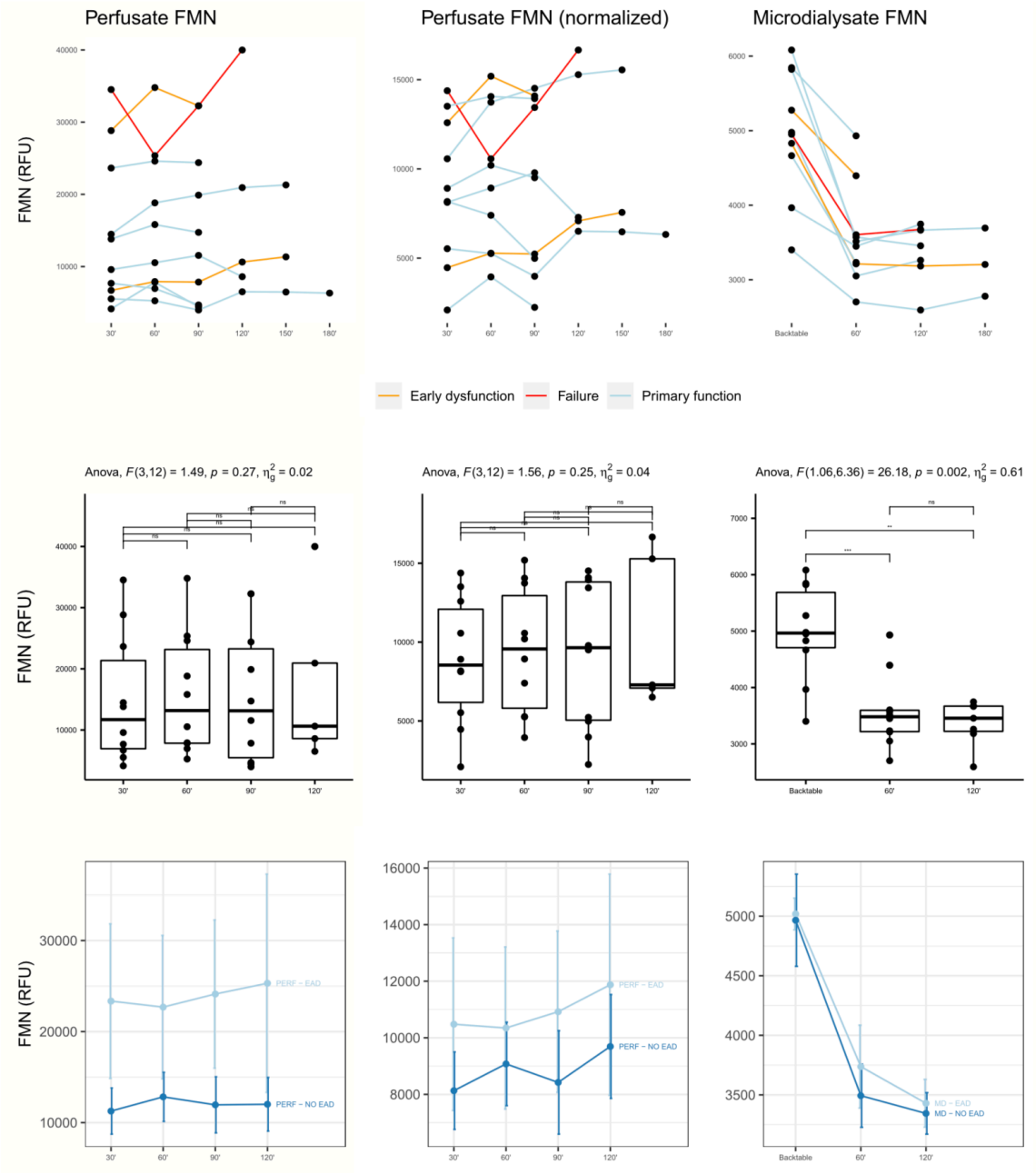

Figure 9. Non-adjusted (left column) and adjusted (middle column) flavinmononucleotide level in perfusate and in microdialysate (right column). Individual trends for each patient are presented in the first row, with colors differentiating cases according to early graft function. Levels across different timepoints are compared with ANOVA for repeated measures in the second row. In the third row, levels (mean ± standard error) are presented according to the development of early allograft dysfunction.

Table 6. Flavin mononucleotide values (RFU) stratified by EAD.

Value	Overall	No EAD	EAD	p
	10	7	3	
Perfusate				
30’	11701 [6945, 21356]	9588 [6606, 14146]	28830 [17764, 31675]	0.21
60’	13175 [7843, 23158]	10537 [7388, 17318]	25362 [16638, 30078]	0.14
90’	13140 [5477, 23268]	11548 [4562, 17313]	32265 [20056, 32267]	0.14
120’	10629 [8600, 20939]	8600 [7553, 14769]	25311 [17970, 32652]	0.25
150’	11343 [8908, 16324]	13889 [10181, 17597]	11343 [11343, 11343]	1.00
180’	6326 [6326, 6326]	6326 [6326, 6326]	NA [NA, NA]	NA
Perfusate (normalized to liver weight)				
30’	8543 [6177, 12084]	8174 [6827, 9740]	12590 [8528, 13486]	0.43
60’	9566 [5808, 12946]	8929 [6329, 11971]	10567 [7922, 12881]	0.43
90’	9646 [5048, 13815]	9505 [4484, 11863]	13444 [9338, 13767]	0.43
120’	7288 [7086, 15284]	7288 [6897, 11286]	11875 [9481, 14269]	0.56
150’	7562 [7018, 11556]	11012 [8742, 13281]	7562 [7562, 7562]	1.00
180’	6326 [6326, 6326]	6326 [6326, 6326]	NA [NA, NA]	NA
Microdialysate				
Backtable	4964 [4706, 5686]	4978 [4316, 5834]	4951 [4890, 5113]	0.91
60’	3482 [3218, 3596]	3449 [3142, 3543]	3604 [3408, 4000]	0.43
120’	3456 [3222, 3670]	3456 [3261, 3665]	3430 [3307, 3552]	1.00
180’	3205 [2992, 3450]	3236 [3008, 3465]	3205 [3205, 3205]	1.00

Data are expressed as median [interquartile range] and compared using non-parametric Mann-Whitney test. Abbreviations: RFU, relative fluorescing units; EAD, early allograft dysfunction;

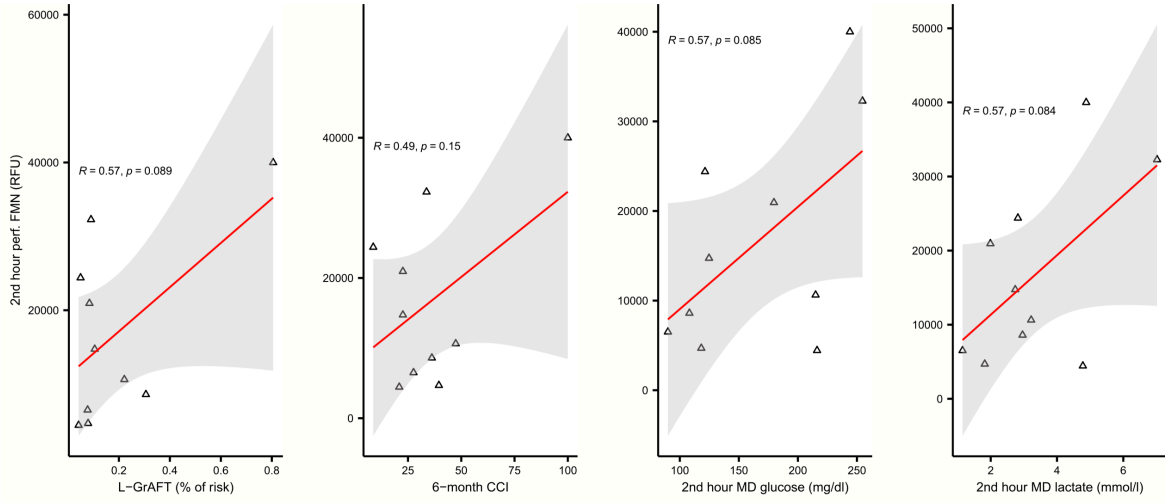

Figure 10. Correlation of 2nd hour perfusate FMN with L-GrAFT score, 6month CCI and 2nd hour MD metabolites.

7.4 Histology and expression of inflammatory cytokines

Histological injury (Figure 11 — Panel A) correlated with EAD onset and was more severe in the graft that required retransplantation. Interestingly, reduced glycogen content was observed in grafts that subsequently developed EAD. Upon reperfusion, a significant increase of inflammatory cytokines (IL-6, IL-8, and TNFα) and adhesion molecules (ICAM1) expression was observed, with no significant differences according to EAD development (Figure 11 — Panel B). There was no correlation between MD and perfusate parameters and the expression of inflammatory cytokines or adhesion molecules. Succinate tissue content exhibited a downward trend from cold preservation to reperfusion into recipient, with no significant differences between study groups (Figure 12).

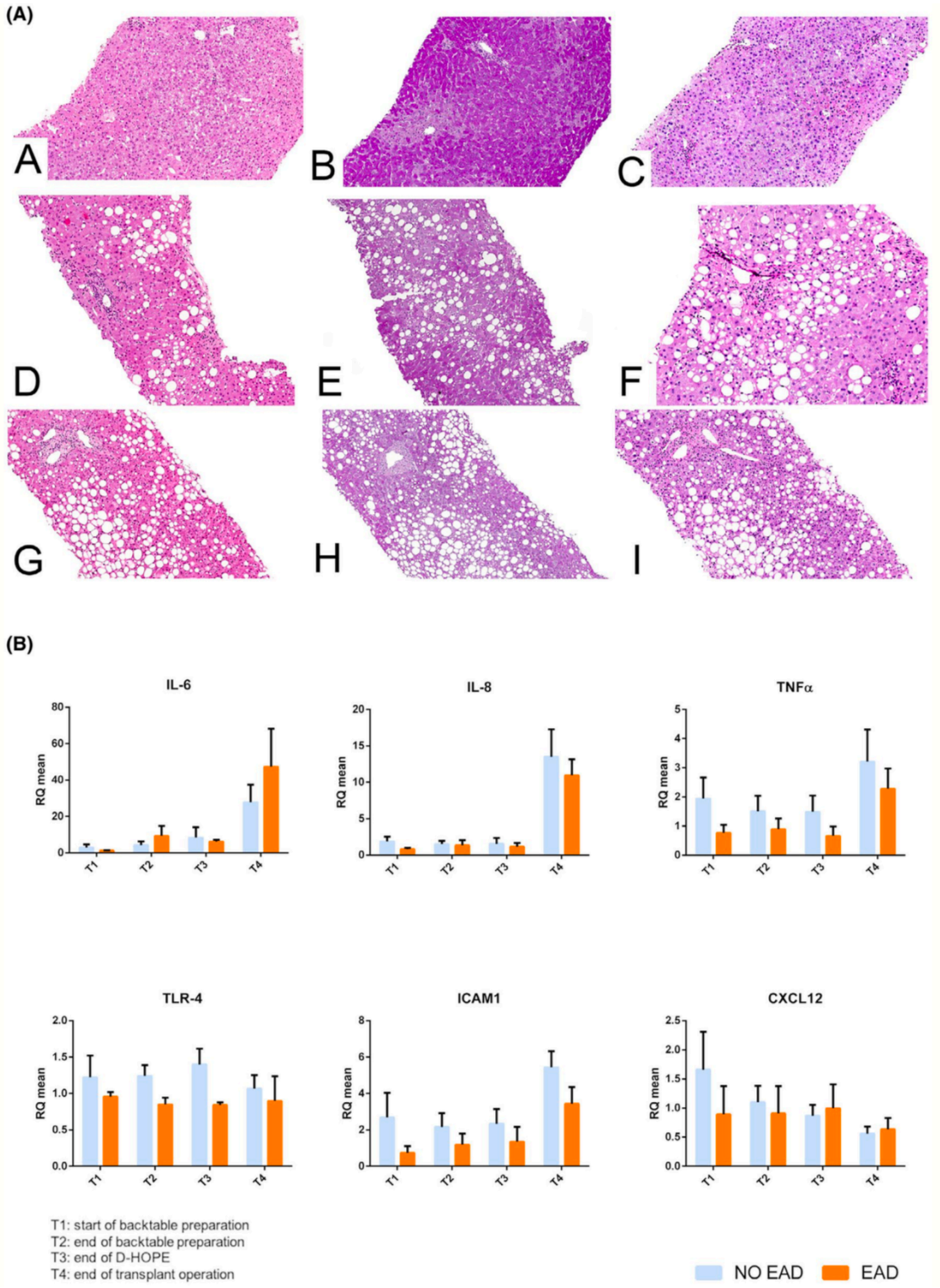

Figure 11. Panel A. Representative histological images of liver grafts at the end of transplant (100× original magnification). Non-EAD case (case 10; images A–C) showed mild signs of steatosis and reperfusion injury (A). PAS staining (B) enhanced cytoplasmatic hepatocytes' glycogen deposits, confirmed with PAS-D staining (C). Glycogen was diffusely distributed with a zone-1-to-zone-3 gradient pattern. EAD case (case 3; images D–F) was characterized by mild steatosis and focal parcellar necrosis (D). Glycogen was present in few periportal hepatocytes (E,F). Graft failure case (case 4; images G–I) showed severe steatosis (G) and minimal signs of glycogen

deposits (H,I). Microscope liver histologic slides were scanned with the NanoZoomer S210 Digital slide scanner (Hamamatsu Photonics K.K.) using an objective lens with a numerical aperture of 0.75. Slides were focused at 400× original magnification (scanning resolution: 0.23 µm/ pixel), and images were acquired with the NDP.scan image acquisition software (Hamamatsu Photonics K.K.). Then, contrast and brightness corrections were performed to the whole image and data exported with the NDP.view2 viewing software (Hamamatsu Photonics K.K.).
Panel B. Cytokines levels at the start of backtable preparation (T1), before (T2) and after (T3) machine perfusion, and at the end of transplant operation (T4). No significant differences were observed between EAD and non-EAD patients at any timepoint

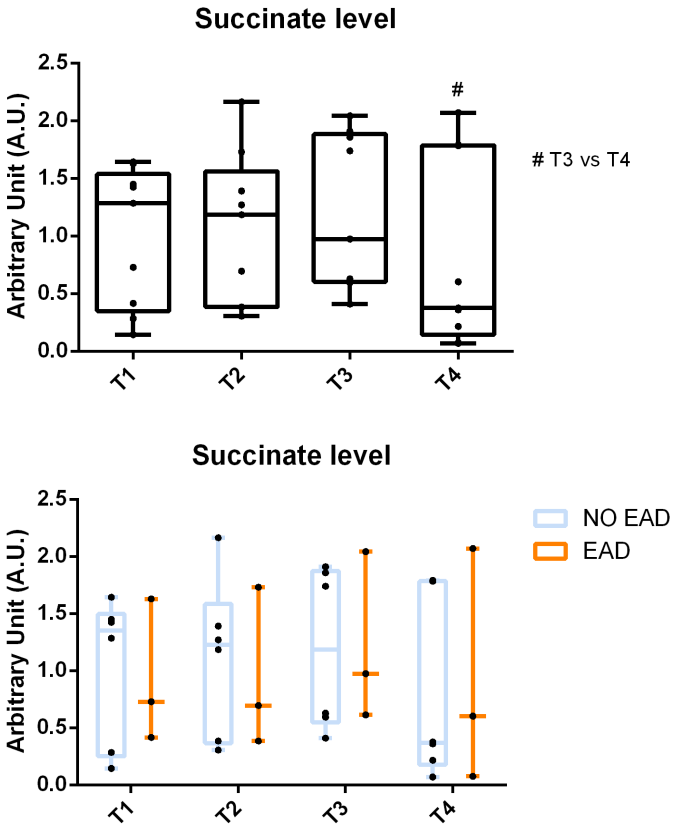

Figure 12. Succinate level of liver biopsies.

8. DISCUSSION

In the setting of clinical LT, MD has been mainly applied as a tool for monitoring and early detection of ischemic complications and acute rejection[23–29]. However, one fascinating feature of this technology is the possibility of evaluating liver metabolism during different phases of organ retrieval, static cold storage, implantation and early postoperative course[18,19,21,22]. In particular, the studies by Nowak et al.[19] and Silva et al.[21], have clearly shown that both glucose and lactate slowly build up during SCS, whereas their levels increase steeply during implantation and organ reperfusion, to return progressively to baseline levels within 3 hours following reperfusion. Perera et al.[61] have shown that end-ischemic interstitial lactate level and lactate/pyruvate ratio are higher in grafts from DCD donors, this finding being associated with more severe glycogen depletion. Similarly, glutamate also appears to accumulate during SCS, reaching end-ischemic levels of approximately 300 μmol/L, which progressively normalize in the hours following graft reperfusion.[20] In contrast, pyruvate quickly becomes undetectable during SCS, (reflecting lack of metabolic activity during this phase), rises above pre-SCS values upon reperfusion (suggesting a phase of hypermetabolism) to return to baseline in the following hours.[19]

The release of glucose in the extracellular space represents a hepatic-specific response to ischemia and it has been interpreted as a result of glycogen breakdown.[19] In other tissues, ischemic events are associated with very low interstitial glucose. Interestingly, the rate of glucose and lactate accumulation increases during implantation[19] and back table preparation,[21] suggesting a potential role for temperature during these phases. Previous studies have suggested a prognostic value of MD metabolites, as patients developing initial poor function had higher MD lactate level during back table preparation and delayed clearance after graft reperfusion.[21,61]

In this study, we sought to use MD to deepen our understanding of liver metabolism during SCS and D-HOPE. Our main finding is that glucose and lactate are released in the extracellular space upon initiation of D-HOPE and their levels correlate with other known predictors of graft function (cold ischemia time and macrosteatosis), FMN perfusate level, graft dysfunction, and worse clinical outcome.

In our study, D-HOPE did not alter pyruvate and glutamate levels, as compared to the expected kinetics during SCS. In particular, pyruvate levels were almost undetectable and glutamate levels were constantly high, in keeping with previous findings.[20] The lower concentration of glutamate observed in our study (~ 200 μmol/L) can be explained by the relatively short ischemia time before D-HOPE (5 h 44 min) in our series. Interestingly, the only graft that failed in our series had constantly very low levels of MD glutamate during back table and D-HOPE.

Why D-HOPE was associated with a significant increase of glucose and lactate into microdialysate is open to several interpretations. Temperature may have played a role, as the device employed for machine perfusion operates at ~ 10°C, which is warmer than conventional temperatures observed during SCS. During rewarming, it is possible that increased metabolism enhanced glycogenolysis, which in turn resulted in increased glucose release. The composition of the perfusion fluid used for D-HOPE, containing 180 mg/ml of dextrose, may also influence MD glucose level. However, it should be noted that glucose levels in microdialysate were lower than those in perfusate and their kinetics was different (Figure 7). In addition, perfusion fluid composition was unlikely to affect lactate levels and progressive accumulation of lactate in the renal cortex has also been observed in a study using MD in hypothermically perfused kidneys.[33]

Whatever the mechanism, glucose and lactate released in the extracellular space during D-HOPE can be interpreted as markers of graft injury. The rise of MD glucose and lactate during the first 2 h of D-HOPE was more

important in grafts that developed early dysfunction, whereas it was mild or absent in those exhibiting primary function (Figure 7). However, in the few cases that had a 3-h MD sample available, glucose and lactate levels started to decrease also in EAD group (Table 4). Consequently, it seems that restoration of aerobic metabolism during D-HOPE could take longer in more severely damaged grafts.

Importantly, there was a significant discrepancy between perfusate and MD kinetics of glucose and lactate (Figure 7). Perfusate analysis, as a measure of larger and global compartment, did not seem to entirely capture metabolic changes happening at a cellular level, a finding that was more evident for glucose and which is in keeping with our previous study on ex-vivo lung perfusion.[30] Perfusate glucose level increased during D-HOPE independently of subsequent development of EAD, whereas MD levels of glucose were higher in EAD patients, with a significant difference during the 2nd hour of machine perfusion. As lactate is produced during anaerobic glycolysis and glucose is released during ischemia due to hepatocytes glycogenolysis, we might hypothesize that the grafts that developed EAD did not fully recover after SCS and continued to experience a certain degree of hypoxia during machine perfusion, as demonstrated by their metabolic profile. In line with this observation, glycogen content was reduced in EAD livers, likely due to glycogen depletion during cold preservation, an observation which is in keeping with findings from Perera et al.[61]

The next logical step was comparing these findings with perfusate and MD levels of FMN, a marker of mitochondrial injury that recently emerged as a promising tool for graft viability assessment.[55,56] As opposed to glucose and lactate, D-HOPE start determined a steep decrease of MD FMN levels, suggesting its rapid washout from the extra- cellular space and confirming protection of mitochondria during D-HOPE. In contrast, FMN appeared to accumulate in perfusate, with an incremental trend in grafts who developed EAD and with the highest levels observed in the only failing graft of this series (Figure 9). This finding, which is in keeping with those from Mueller et al.,[55] also suggests a

mechanistic interpretation to the association between microdialysate metabolites and graft dysfunction. Taken as a whole, our data seem to point to the same direction: mitochondrial injury sustained during SCS, which is proportional to cold ischemia time and macrosteatosis, is reflected by the accumulation of FMN in perfusate and release of glucose and lactate in the extracellular space during D-HOPE, highlighting their potential as viability markers. Noteworthy, these metabolites can be measured point-of-care using MD equipment during D-HOPE.

This study has numerous implications. First, it confirms that precious information on graft function and injury can be gathered also during hypothermic perfusion, challenging the concept that normothermic perfusion is a prerequisite for graft viability assessment.[62,63] Second, while perfusate analysis appears handier, this study and previous experiences[23,24,27,28] suggest that MD is feasible without major modifications of routine practice. In our opinion, microdialysate and perfusate analysis should be seen as complementary rather than alternative techniques, as metabolites kinetics appears to be different in these two compartments. Real-time assessment of graft function could drive not only graft acceptance but also influence the duration of machine perfusion, as more severely damaged grafts may require longer perfusion time to recover from ischemic injury.

Third, this pilot study represents a baseline based on which other potential applications of MD during machine perfusion could be explored. In a recent study on perfusate analysis during D-HOPE,[50] we identified alanine aminotransferase as the most reliable predictor of EAD. Unfortunately, MD ALT could not be evaluated due to its molecular weight (100 kDa). Further studies on the subject should ideally employ available 100 kDa MD catheters to allow measurement of other molecules, including inflammatory cytokines, as also suggested by a recent study.[64] Finally, MD could be used to assess liver metabolism and explore new viability criteria during normothermic machine

perfusion, a setting mimicking normal physiology and characterized by longer perfusion times.[49]

Limitations of our study include the small sample size of 10 grafts with rather heterogeneous characteristics, the lack of a power analysis, and the choice of EAD as an end-point, which has been discouraged in machine perfusion trials as it strongly relies on transaminase level after transplant and could be influenced by the washout phenomenon during machine perfusion.[65]

In this study, microdialysis time was limited to less than 4 hours by design, allowing complete measurement of MD metabolites at only 3 time points. As previous studies have shown a quick recovery of aerobic metabolism after liver reperfusion[21,61] and due to concerns about sterility breaches, we did not consider leaving the MD in place during this phase, possibly missing relevant metabolic changes.[31] Rapid sampling MD[33] could improve the yield of MD as applied to procedures of limited duration, whereas the use of an MD catheter with higher molecular weight cutoff membrane would allow measuring MD concentration of other potentially important molecules.

9. CONCLUSIONS

In conclusion, this study expands previous knowledge on liver metabolism during D-HOPE and confirms that liver graft injury and function can be assessed during hypothermic machine perfusion. These preliminary findings require validation in larger studies allowing correlation of MD parameters with clinically relevant endpoints and possibly exploring further applications of MD in machine perfusion.

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