Screening for mitochondrial function before use-routine liver assessment during hypothermic oxygenated perfusion impacts liver utilization

Janina Eden,[a] Eva Breuer,[a] Dominique Birrer,[a] Matteo Müller,[a] Matthias Pfister,[a] Hemma Mayr,[a] Keyue Sun,[a] Jeannette Widmer,[a] Florian Huwyler,[b] Udo Ungethüm,[a] Bostjan Humar,[a] Anurag Gupta,[a] Stefanie Schiess,[a] Martin Wendt,[a] Franz Immer,[c] Andreas Elmer,[c] David Meierhofer,[d] Andrea Schlegel,[e,f] and Philipp Dutkowski[a,f,*]

[a]Department of Surgery and Transplantation, Swiss HPB Centre, University Hospital Zurich, Switzerland
[b]Department of Mechanical and Process Engineering, ETH Zurich, Zurich, Switzerland
[c]Swisstransplant, The Swiss National Foundation for Organ Donation and Transplantation, Effingerstrasse 1, Bern 3011, Switzerland
[d]Max Planck Institute for Molecular Genetics, Mass Spectrometry Facility, Berlin 14195, Germany
[e]Transplantation Center, Digestive Disease and Surgery Institute and Department of Immunology, Lerner Research Institute, Cleveland Clinic, Cleveland, OH, USA

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Summary

Background To report on a concept of liver assessment during *ex situ* hypothermic oxygenated perfusion (HOPE) and its significant impact on liver utilization.

Methods An analysis of prospectively collected data on donation after circulatory death (DCD) livers, treated by HOPE at our institution, during a 11-year period between January 2012 and December 2022.

Findings Four hundred and fifteen DCD Maastricht III livers were offered during the study period in Switzerland, resulting in 249 liver transplants. Of those, we performed 158 DCD III liver transplants at our institution, with 1-year patient survival and death censored graft survival (death with functioning graft) of 87 and 89%, respectively, thus comparable to benchmark graft survivals of ideal DBD and DCD liver transplants (89% and 86%). Correspondingly, graft loss for primary non-function or cholangiopathy was overall low, i.e., 7/158 (4.4%) and 11/158 (6.9%), despite more than 82% of DCD liver grafts ranked high (6–10 points) or futile risk (>10 points) according to the UK-DCD score. Consistently, death censored graft survival was not different between low-, high-risk or futile DCD III livers. The key behind these achievements was the careful development and implementation of a routine perfusate assessment of mitochondrial biomarkers for injury and function, i.e., release of flavin mononucleotide from complex I, perfusate NADH, and mitochondrial CO_2 production during HOPE, allowing a more objective interpretation of liver quality on a subcellular level, compared to donor derived data.

Interpretation HOPE after cold storage is a highly suitable and easy to perform perfusion approach, which allows reliable liver graft assessment, enabling surgeons to make a fact based decision on whether or not to implant the organ. HOPE-treatment should be combined with viability assessment particularly when used for high-risk organs, including DCD livers or organs with relevant steatosis.

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Abbreviations: ATP, Adenosine triphosphate; BAR, Balance of risk score; BMI, body mass index; AST, Aspartate amino transferase; ALT, Alanine amino transferase; EAD, Early allograft dysfunction; FMN, Flavin mononucleotid; DCD, Donation after circulatory death; DBD, Donation after brain death; DWIT, Donor warm ischemia time; fDWIT, functional donor warm ischemia time; aDWIT, asystolic donor warm ischemia time; HAT, Hepatic artery thrombosis; HCC, Hepatocellular carcinoma; HOPE, Hypothermic oxygenated perfusion; IGL, Institute George Lopez; INR, international normalized ratio; MELD, Model for end stage liver disease; NADH, Nicotinamid adeninedinucleotide; NMP, Normothermic machine perfusion; NRP, Normothermic regional perfusion; PNF, Primary non function; PSC, Primary slerosing cholangitis; 8-OHdG, 8-Hydroxydeoxyguanosine

*Corresponding author. Department of Surgery & Transplantation, University Hospital Zurich, Switzerland.
E-mail address: philipp.dutkowski@usz.ch (P. Dutkowski).
[f]These authors contribute equally.

Keywords: Donation after circulatory death; Hypothermic oxygenated perfusion; Flavin mononucleotide; Liver utilization

Research in context

Evidence before this study

Machine liver perfusion allows better preservation compared to conventional cold storage based on several randomized controlled trials for normothermic and for hypothermic oxygenated perfusion. An additional significant advantage would be the identification of reliable perfusate biomarkers for recognizing liver function in real-time before implantation.

Added value of this study

This study shows that assessment of liver quality is possible during hypothermic oxygenated perfusion of livers donated after circulatory death, based on detection of mitochondrial complex I injury and function. Notably, this novel approach was superior compared to donor derived data.

Implications of all the available evidence

Testing subcellular liver injury before use is the main advantage of dynamic preservation strategies and opens the door for a safe increase of liver utilization worldwide.

Introduction

In Switzerland, a controlled donation after circulatory death (DCD) liver transplant program was introduced in 2012, with the aim to increase the donor pool in the presence of long liver waiting times, often exceeding 10 months.[1] The DCD pathway included in all cases the withdraw of treatment of DCD III candidates in the operating theatre, with a mandatory 10 min stand-off period after cardiac arrest, verified by echocardiography, and subsequently additional brain death diagnostic.[2] This resulted in long donor warm ischemia times (DWIT), frequently exceeding 30 min for functional donor warm ischemia (fDWIT).[3] Due to an expected high risk for graft loss, and based on our experimental research, we decided from the beginning to treat all DCD III livers with an endischemic *ex-situ* hypothermic oxygenated perfusion (HOPE), applied with a minimal perfusion time of 1 h, mostly during recipient hepatectomy.[4] In the majority of cases, perfusion was continued until graft implantation. The primary policy for DCDIII liver utilization was to use this additional graft resource for predominantly low risk recipients, e.g., candidates with a low lab model of end-stage liver disease (MELD) and with hepatocellular carcinoma (HCC). Between 2012 and 2017, DCD Maastricht III livers were exclusively transplanted at our own institution (University Hospital Zurich). In 2018, the 10 min no touch period was reduced to 5 min, DWIT remained however relatively high, compared to other countries.[5,6] The DCD III program was at the same time expanded to the other two Swiss liver transplant centers, e.g., Bern and Geneva. Both centers implemented their own preservation strategies for DCD III liver transplants, e.g., normothermic regional perfusion (NRP) in Geneva, and HOPE on demand in Bern.

The aim of this study is to report our 11-year experience with all DCD liver transplantations at our institution with emphasis on evolved liver utilization rates, implementation of routine graft assessment during machine perfusion, and consecutive outcomes.

Methods

Data collection

DCD III liver offers were reported by Swiss Transplant between 01/01/2012 and 31/12/2022, including the number of discarded DCD III livers during the same period, as well as donor risk factors, including donor age, donor BMI, and donor warm ischemia times.

We documented all DCD III liver transplants between 01/1/2012 and 31/12/2022 in our institution with a follow-up until May 31st 2023. All available donor and recipient data were recorded to enable calculation of several prediction scores, e.g., UK DCD risk score, balance of risk (BAR) score, and lab model for end stage liver disease (MELD) score. The study endpoints of this analysis included primary non-function (PNF), early allograft dysfunction (EAD) according to Olthoff criteria,[7] biliary complications, i.e., bile leak, anastomotic and non-anastomotic strictures, postoperative renal replacement therapy (RRT), total length of intensive care unit (ICU) and hospital stay; hepatic artery thrombosis (HAT), and patient, as well as death-censored graft survival, i.e., death with functioning graft.

Definitions and surgical technique

No interventions, including heparinization before withdraw, or preemptive cannulation, were permitted before donor death declaration in Switzerland. All livers were procured by super rapid retrieval until 2018 using IGL-1 preservation solution for aortic perfusion with concomitant topical cooling by slushed ice. Following the national implementation of the DCD program, some livers underwent since 2018 NRP before procurement, when the donor procedure was started in Geneva.

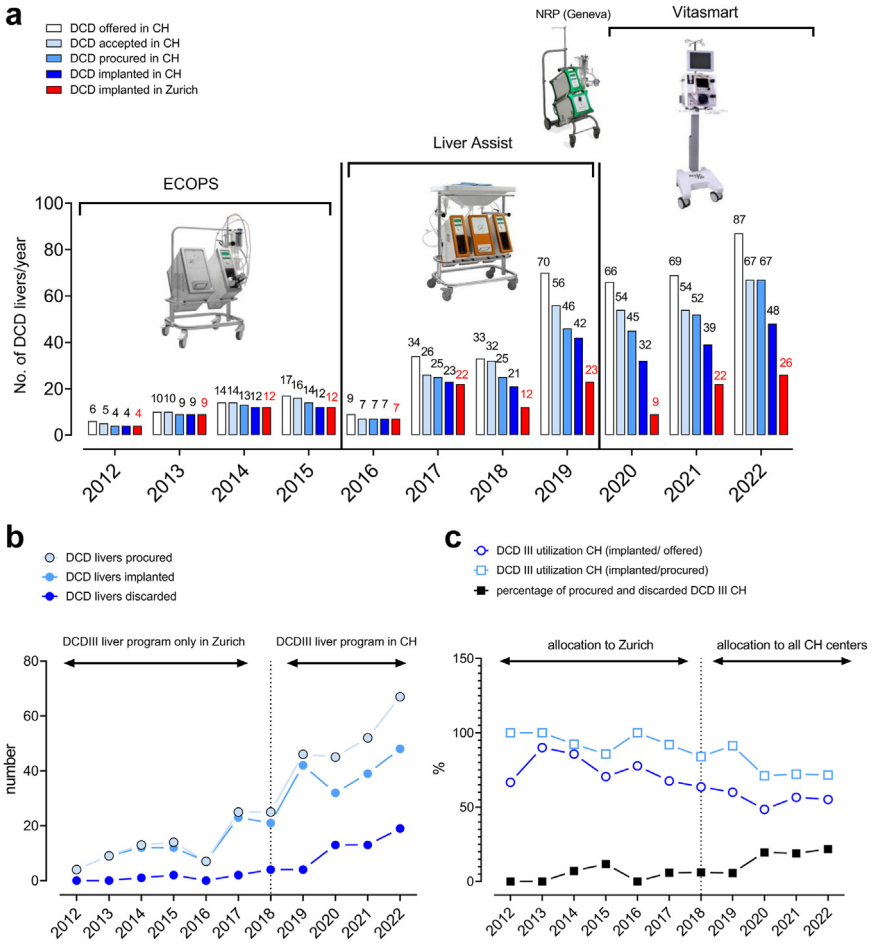

Fig. 1: Number of DCD III liver offers and number of implanted or discarded DCD III livers in Switzerland 2012–2022. The number of offered DCD III livers in Switzerland increased continuously in the study period (a). While until 2017, all liver were offered only to one center (Zurich), all three centers participate in the DCD III liver transplant program since 2018 (b). The DCD III utilization decreased during the study period due to introduction of *ex situ* assessment before transplantation (c).

The gallbladder was opened in all cases, and the bile duct was divided and flushed with IGL-1 after vascular perfusion. Total donor warm ischemia time (DWIT) was defined as the time between donor withdraw of life support and cold *in-situ* flush. Functional donor warm ischemia time (fDWIT) was defined as the time interval between systolic blood pressure < 50 mmHg and start of cold *in-situ* flush. Asystolic donor warm ischemia time (aDWIT) was defined as the time between cardiac arrest and cold *in-situ* flush. Cold ischemia time (CIT) was defined as the time interval between cold in-situ aortic flush with Institute George Lopez solution (IGL-1) and start of HOPE-treatment.

The standard liver implantation technique used at our institution is classical liver transplantation with cava replacement. All livers were reperfused through the portal vein first with previous blood flush. The biliary anastomosis was routinely duct to duct, with Roux-en-Y hepaticojejunostomy for recipients with primary sclerosing cholangitis (PSC). EAD was defined using the criteria by Olthoff et al.[7] PNF was defined as graft loss or death within 7 days post-transplant excluding technical issues or vascular complications. HAT was defined as thrombosis of the hepatic artery diagnosed on imaging. The immunosuppression protocol at our center included intraoperative steroids and basiliximab, with tacrolimus starting at postoperative day 2.

Hypothermic oxygenated perfusion (HOPE)

After initial cold storage, all livers were *ex-situ* perfused through the portal vein only with cold (8–12 °C) oxygenated (80–100 kPa) recirculating Belzer machine perfusion solution (MPS) (3 L). The perfusion flow was between 150 and 300 ml/min (median 180 ml/min) at a perfusion pressure of 3 mm Hg. Machine perfusion was continued for a minimum of 1 h until liver

	Total (n = 158)	Low risk (n = 28)	High risk (n = 77)	Futile (n = 53)	p-value[a]
Donor parameter:					
Age, years	60 (49–71)	50.5 (30–59)	59 (49–73)	64 (57–72)	**<0.001**
Sex male/female	103/55	21/7	39/38	43/10	**0.016**
Height, cm	173 (165–178)	173 (168–181)	172 (165–178)	175 (167–180)	ns
BMI, kg/m^2	26 (23–27)	23 (21–25)	25 (23–28)	27 (26–29)	**<0.001**
DWIT, min	32 (28–37.5)	30 (23–34)	31 (28–36)	37 (33–41)	**<0.001**
fDWIT,min	28.5 (24.75–34)	21 (17–27)	26 (22–30)	33 (29–36)	**<0.001**
aDWIT, min	18 (14–20)	14 (11–18)	16 (13–20)	19 (17–21)	**<0.001**
UK DCD risk score (points)	8 (6–11)	3 (3–5)	8 (7–9)	12 (11–13)	**<0.001**
Benchmark DCD cases, n (%)	28/158 (17.7%)	9/28 (32.1%)	17/77 (22.1%)	2/53 (3.8%)	**<0.001**
Preservation parameter:					
Cold storage, min	248 (195–314)	328 (216–475)	264 (209–399)	276 (199–407)	ns
Hope duration, min	142 (107–188)	122 (82–194)	140 (110–189)	144 (110–196)	ns
Perfusion flow, ml/min	180 (180–250)	215 (180–250)	180 (180–250)	180 (180–250)	ns
Recipient parameter:					
Age, years	59.5 (53–66)	56 (43–59)	58 (51–64)	62 (56–67)	**<0.001**
Sex male/female	122/36	19/9	59/18	44/9	**0.018**
MELD, points	12 (9–17)	14 (9–19)	12 (8–17).	12 (10–19)	ns
BAR score, points	4 (2–7)	2.5 (1.8–7.5)	4 (2–7)	4 (4–8)	ns
HCC, n (%)	100/158 (63.3%)	12/28 (42.8%)	56/77 (72.7%)	32/53 (60.3%)	ns
Tumorsize, cm	2.4 (1.7–3.1)	2 (1.5–3.0)	2.2 (1.7–3)	2.9 (1.5–3.7)	ns
Tumor number	2 (1–3)	2 (1–3)	2.5 (1–3)	2 (1–3)	ns
AFP, ng/ml	8 (5–26)	8 (4–26)	6 (5–97)	8 (5–11)	ns
Operation time, hrs	4 (3.2–5)	3.9 (3.2–5)	4.2 (3.6–5.2)	4.1 (3.5–5.2)	ns
Intraoperative transfusions (U)					
RBC	1 (0–3)	1 (0–2)	1 (0–2)	1 (0–3)	ns
FFP	0	0	0	0	ns
Number of patients without transfusions during OLT	78/158 (49.4%)	19/28 (67.8%)	34/77 (44.1%)	25/53 (47.2%)	**0.015**
Bloodloss, ml	300 (200–500)	280 (150–490)	250 (210–510)	310 (280–550)	ns
AST day 3, U/L	2333 (1410–4960)	1840 (123–248)	2560 (174–558)	2640 (117–494)	ns
AST day 10, U/L	47 (34–77)	43 (20–81)	51 (34–85)	46 (35–72)	ns
ALT day 3, U/L	600 (383–1058)	468 (326–927)	543 (383–954)	641 (394–1163)	ns
ALT day 10, U/L	139 (94–200)	104 (65–251)	137 (86–200)	143 (117–199)	ns
Bilirubin day 3	1.5 (0.8–3)	1.6 (0.7–3.3)	1.5 (0.8–3.5)	1.3 (0.8–3.0)	ns
Bilirubin day 10	1.1 (0.5–2.3)	1.1 (0.4–3.2)	1.5 (0.5–3.1)	0.9 (0.5–1.9)	ns
INR day 3	1.1 (1.0–1.2)	1.1 (1–1.1)	1.1 (1–1.1)	1.1 (1.0–1.3)	ns
INR day 10	1.1 (1.0–1.1)	1.1 (1.0–1.1)	1.1 (1.0–1.1)	1.0 (1.0–1.1)	ns
Graft loss due to:					
PNF	7/158 (4.4%)	1/28 (3.6%)	4/77 (5.2%)	2/53 (3.8%)	ns
Cholangiopathy	11/158 (6.9%)	2/28 (7.1%)	5/77 (6.6%)	4/53 (7.5%)	ns
Biliary anastomotic strictures	53/158 (33.5%)	6/28 (21.4%)	28/77 (36.4%)	19/53 (35.8%)	ns
Biliary leakage	9/158 (5.7%)	1/28 (3.6%)	6/77 (7.8%)	2/53 (3.8%)	ns
Renal replacement therapy	39/158 (24.7%)	3/28 (10.7%)	19/77 (24.7%)	17/53 (32.0%)	**0.008**
Hepatic artery thrombosis (HAT)	4/158 (2.5%)	1/28 (3.6%)	1/77 (1.3%)	2/53 (3.8%)	ns
ICU stay, hours	72 (48–120)	72 (42–72)	72 (48–144)	72 (48–96)	ns
Hospital stay, days	17 (12–23)	14 (11–19)	18 (13–28)	15 (11–19)	ns
1 y patient survival	87%	88%	84%	85%	ns
5 y patient survival	72%	77%	71%	77%	ns
1 y death censored graft survival	89%	84%	93%	88%	ns
5 y death censored graft survival	86%	89%	85%	82%	ns

Statistically significant values are indicated in bold ($p < 0.05$). [a]Kruskal Wallis test for ordinal or metric scaling comparing low risk/high risk/futile groups, log rank test for survival.

Table 1: **DCD III liver transplants in Zurich between 2012 and 2022.**

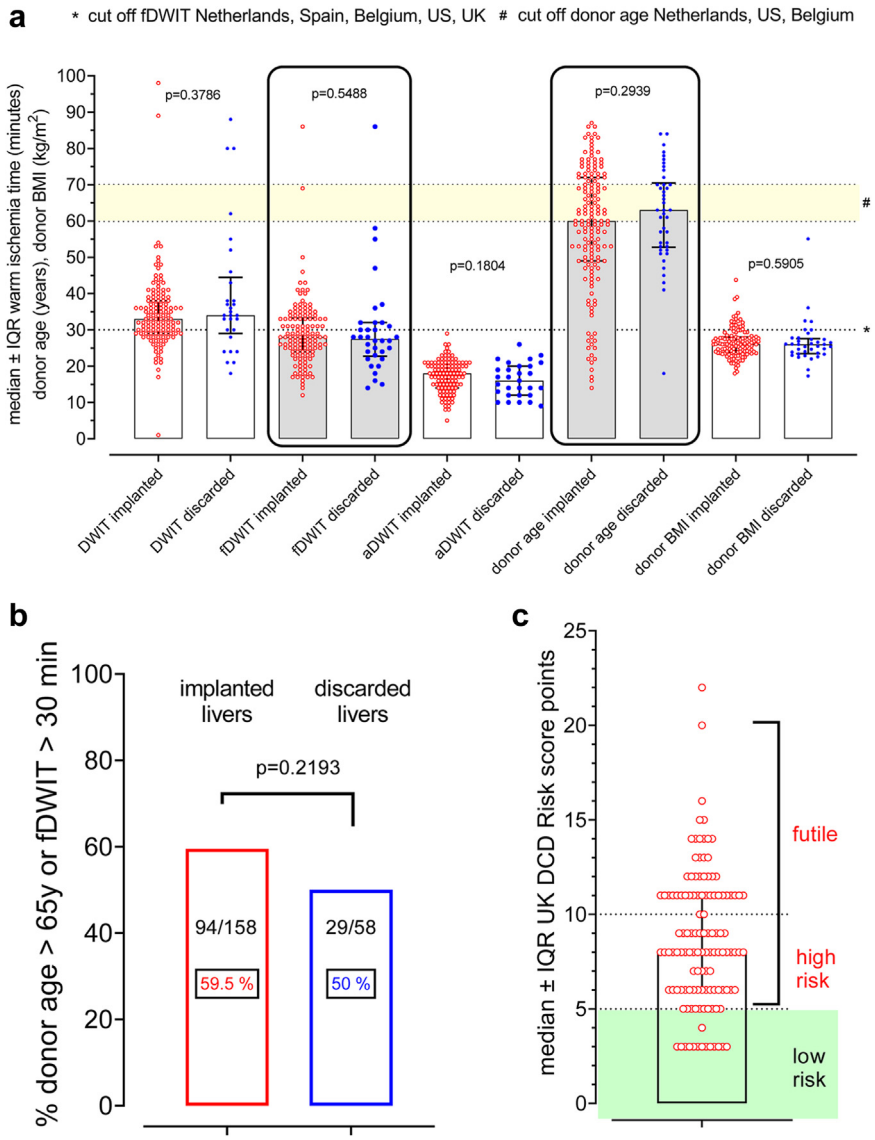

Fig. 2: Risk factors in implanted and discarded DCD III livers in Switzerland. Donor age, donor BMI, and donor warm ischemia times were not different between implanted and discarded DCD III livers (a). The percentage of implanted livers outside clinical cutoffs was high (94/158, 59.5%), and not different from the percentage of discarded livers within clinical cutoffs (29/59, 50%) (b). Eighty percent of implanted DCD III livers qualified as high risk or futile according to the UK DCD risk score (c). P values refer to the Mann–Whitney U test.

implantation. The median perfusion time was 142 min (107–188 min).

Perfusate assessment

The perfusate underwent fluoroscopic analysis assessed for released FMN and NADH, as previously reported[2,8] (Fig. 5a).

Measurement of produced CO_2 during HOPE

The production of $C^{13}O_2$ was monitored during HOPE with Laser spectrometry (^{13}CORlab, ArgosMED GmbH), captured at the outlet of the oxygenator (Fig. 8e).

Mitochondrial isolation and mass spectrometry

Mitochondrial isolation and mass spectrometry were done as previously reported.[9]

Normothermic reperfusion of discarded livers

After the decision to discard livers due to exceeding FMN and NADH thresholds during HOPE, livers were completely cannulated and *ex situ* reperfused up to 3

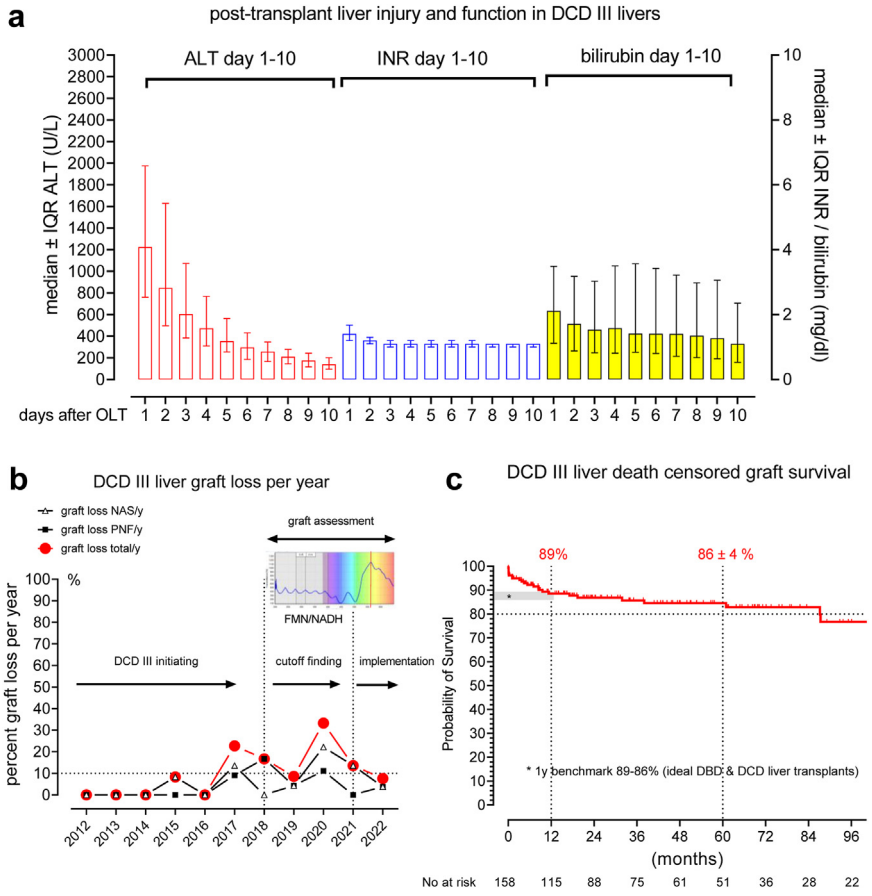

Fig. 3: Outcome in DCD III livers implanted in Zurich 2012–2022. DCD III livers showed overall low liver enzyme release and excellent liver graft function (a). Graft loss per year was low during the first 5 years and increased from 2017 to 2020, due to use of DCDIII livers also in sick and retransplant recipients (b). Overall death censored graft survival was 89 and 85% at 1 and 5 years, respectively (c).

days, as reported earlier, with detection of hepatocellular and cholangiocyte injury.[8]

Ethics statement

The data collection and analysis were approved by local ethics (Switzerland: KEK No. 2019-01000). Organ retrieval and transplantation was approved by the Swiss Medical Government (BT2007-nTx0080-N2V02/BSC), all recipients signed informed consent when included on the waiting list for liver transplantation.

Statistics

All data were analyzed using descriptive statistics, e.g., reported as indicated in legends, i.e., median and interquartile range (IQR), mean and standard deviation, or numbers and percentages. Correlations and comparisons were calculated using non-parametric tests (Spearman r, Mann Whitney U-test, Kruskal Wallis test). Survival was compared using the logrank test. The Software packages used were Graphpad Prism 9.5.1 and IBM SPSS Statistics 25. Utilization rates were calculated according to two main definitions: first, transplanted livers divided by livers offered, and second, transplanted livers divided by livers procured.[1]

Role of funders

This study is independent from funding through the Swiss National Foundation in terms of study design, data collection, interpretation, or writing report.

Results

Between January 2012 and December 2022, a total of 415 DCD III liver grafts were offered in Switzerland, which resulted in 249 nationwide liver implants (Fig. 1). The majority of these livers were implanted at our institution (158/249, 63.5%), with routine end-ischemic HOPE treatment, using three perfusion devices throughout this time period, i.e., ECOPS®, Liver Assist® and VitaSmart® (Fig. 1a). Thirteen livers of this cohort were pre-treated with NRP due to initiation of the DCD process in Geneva (13/158, 8.2%).

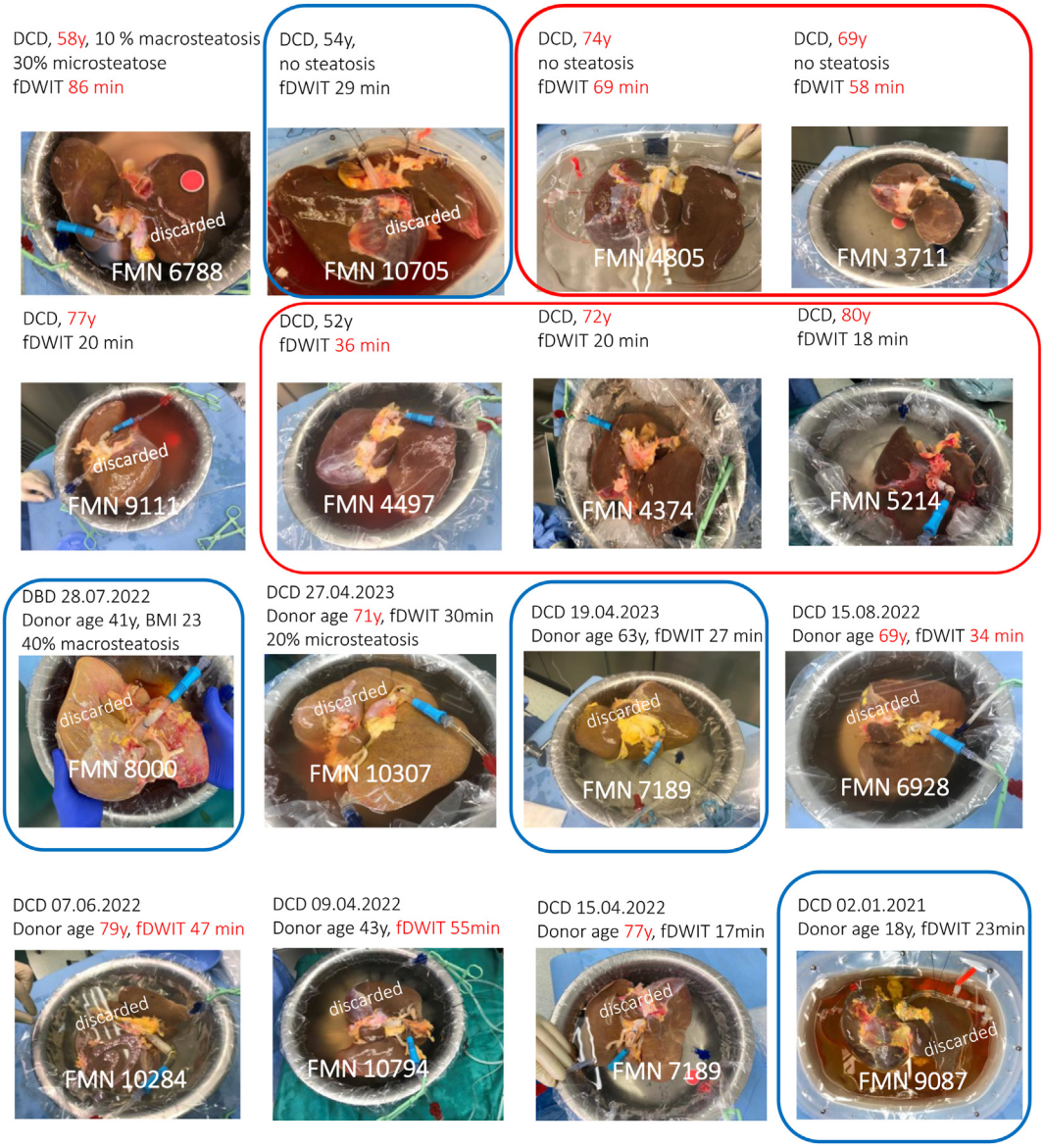

Fig. 4: Examples of DCD III livers assessed during HOPE. Sixteen examples of assessed livers during HOPE are shown, with twelve livers, exceeding clinical thresholds for donor age or fDWIT (>65 y, >30 min). Five of these livers (42%) were implanted based on low mitochondrial injury within the first hour of HOPE (red encircled, **FMN ≤ 6000 A.U., i.e., 0.04 µg/g liver**). In contrast, four livers demonstrated no clinical signs for risk, but the perfusate assessment resulted in unexpected high values for mitochondrial injury (**FMN > 6000 A.U.**), leading to discard of these livers (blue encircled).

The liver utilization rate, defined by the ratio of livers offered per livers implanted,[1] ranged between 70 and 80% in the first 5 years, with only one active center in Switzerland (Zurich) (Fig. 1b and c). Since 2018, the other two Swiss centers (Geneva and Bern) also participate in the DCD liver transplant program (Fig. 1b and c). The number of discarded DCD III livers significantly increased since 2020 with the implementation of *ex-situ* viability assessment before use (Fig. 1c). Correspondingly, the national Swiss liver utilization rate decreased and was 55% (48 livers implanted/87 livers offered) and 72% (48 livers implanted/67 livers procured) in 2022 (Fig. 1b and c).

Donor characteristics in Switzerland and our institution

The median donor age of implanted DCD III livers at our institution was high, i.e., 60 years (49–72), compared to other DCD populations,[1,10] with a median body mass index (BMI) of 26 kg/m^2 (23–27). The

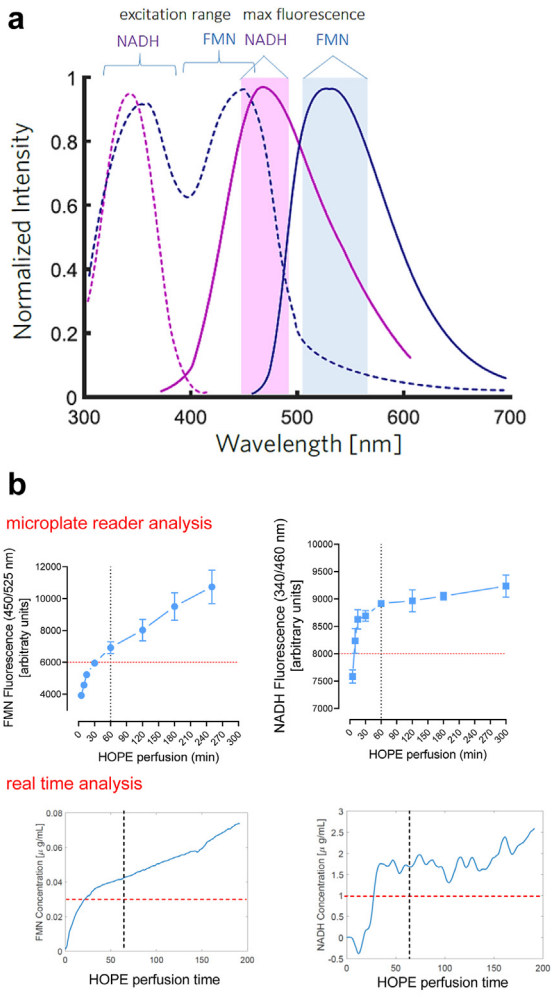

Fig. 5: Excitation and emittance wavelengths of NADH and FMN, as well as perfusate fluorescence during HOPE. The excitation wavelengths for NADH were between 310 and 390 and for FMN between 400 and 470 nm. The emitted light was detected at 450–490 for NADH and at 505–570 nm for FMN[8] (a). Perfusate fluorescence was detected by plate reader analysis or real time during HOPE (b).

median fDWIT was 28 min (24–33), and the total DWIT was 33 min (29–38), both outside benchmark criteria.[5] This resulted in a high proportion, i.e., 130/158, 82%, of high risk or futile ranked DCD III livers, based on a UK DCD Risk score between 6 and 10 points, or above 10 points, respectively (Table 1, Fig. 2c).

Importantly, a comparison of donor age, donor BMI, and also donor warm ischemia times showed no major differences between implanted and discarded DCD III livers (Fig. 2a), likely because the final decision to use DCD III livers was mostly based on perfusate assessment rather than donor data or macroscopic liver assessment. Consistently, the percentage of implanted DCD III livers outside of clinical cutoffs for donor age (>65 y) or fDWIT (>30 min) was high (59.5%, 94/158), and did not significantly differ from the proportion of discarded livers within these clinical criteria (50%, 29/58 (Fig. 2b)).

Recipient characteristics at our institution

The median recipient age was 59.5 years (53–66), and the median lab MELD score was 12 points (9–17), with many recipients listed for liver transplantation due to HCC (Table 1). Patients with HCC (100/152) had a median tumor size of 2.4 cm (1.7–3.1) and a median tumor number of 2 (1–3) as well as a median αfetoprotein (AFP) of 8 ng/ml (5–26). Due to the low lab MELD and mostly primary transplants, the median BAR score was also low in the whole DCD III population, e.g., 4 points (2–7).

Perfusion and preservation parameters at our institution

The median cold storage remained short due to short distances in Switzerland, e.g., 248 min (195–314), and IGL-1 was used as universal static preservation solution. The median HOPE duration was 142 min (107–188), the median perfusion flow at the end of perfusion was 180 ml/min (180–250). The perfusate was in all cases 3 l of recirculating oxygenated Belzer MPS at a temperature of 8–12 °C.

Outcome at our institution

The median duration of liver transplant surgery was 4 h, with low transfusion requirements (median 1 RBC, 0 FFP). Recipients stayed after transplant 72 h on the ICU (48–120), and 17 days in hospital (12–23). The median AST on day 3 was 2333 U/L (141–496), and decreased quickly thereafter to 47 (34–77) on day 10, respectively (Table 1). The median ALT on day 3 was 600 U/L (383–1058) and 139 U/L (94–200) on day 10, respectively. The median bilirubin on day 3 was 1.5 mg/dl (0.8–3) and 1.1 mg/dl (0.5–2.3) on day 10, respectively. The INR of most recipients recovered very fast, and remained in the normal range over a 10 day period (Fig. 3a).

The median follow-up of all patient was 31.6 months (10.9–69.3). The overall graft losses due to PNF and cholangiopathy were 7/158 (4.4%) and 11/158 (6.9%), respectively, resulting in an overall patient survival of 87% at one year and 72% at 5 years (Table 1). The death censored 1 year graft survival, e.g., censoring death with functioning graft, was 89%, the 5 year death censored graft survival was 86% (Fig. 3c). There was no difference in survival rates between low risk, high risk or futile ranked patients (Table 1).

Evolution of *ex situ* assessment during machine liver perfusion

Clinical implication of routine liver assessment

Fig. 4 shows exemplary sixteen livers (15 DCD, 1 DBD), which we accepted upfront for HOPE treatment and

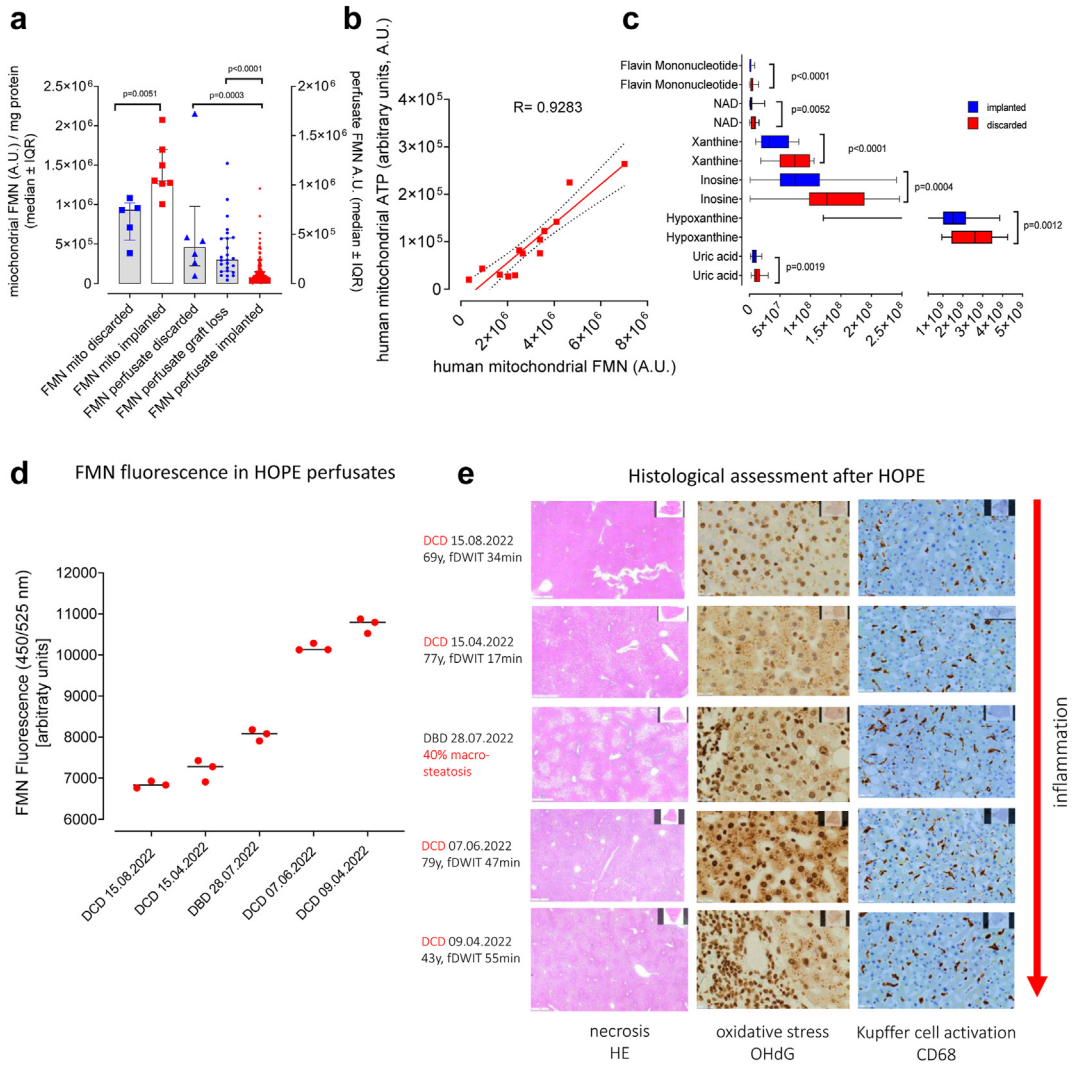

Fig. 6: Mitochondrial and perfusate metabolites, correlation of perfusate FMN and liver histology. Mitochondrial FMN content was significantly lower in discarded (n = 5) compared to implanted livers (n = 7), and inversely correlated with high (n = 7) and low perfusate FMN (n = 110) (a). Mitochondrial FMN highly correlated with mitochondrial ATP (b) (Pearson correlation coefficents). Perfusate FMN and purine metabolites were significantly different between implanted and discarded livers (n = 33 each) (c). With increasing perfusate FMN, the degree of inflammation increased, as quantified by necrosis (HE staining), oxidative DNA injury (OHdG staining), and Kupffer cell activation (CD68 staining) (d,e). Presented p values refer to the Mann–Whitney U test.

assessment despite high donor risk in twelve of them, with exceeding cutoffs for donor age or fDWIT, e.g., >65 y, >30 min, respectively. Instead of upfront rejection, as practiced by several centers around the world, we decided to further assess their potential, which is possible by inducing and measuring reperfusion injury during oxygenated *ex-situ* machine perfusion before implantation.[9,11] As this injury is known to start in mitochondria[12–14] we have implemented a fluometric real time detection of released mitochondrial compounds, e.g., flavin-mononucleotide (FMN) and NADH (Fig. 5a)[2,9] which serve as perfusate biomarkers for liver graft quality. Accordingly, since 2018 we analyze in all DCD III and in some extended criteria DBD livers the amount of perfusate FMN and NADH during HOPE at specific time points, e.g., 5 min, 10 min, 15 min, 30 min, 60 min. At 30 min of HOPE, the decision whether to implant or discard the organ was made, based on predefined cut offs developed over the past five years (Fig. 5b). Five of the twelve example livers, classified as high risk according to clinical cutoffs (donor age > 65 years or functional DWIT > 30 min), in fact appeared suitable for implantation and resulted in successful transplantations with excellent recipient outcomes. In contrast, four livers, without any clinical criteria of elevated donor risk, showed high FMN values and were

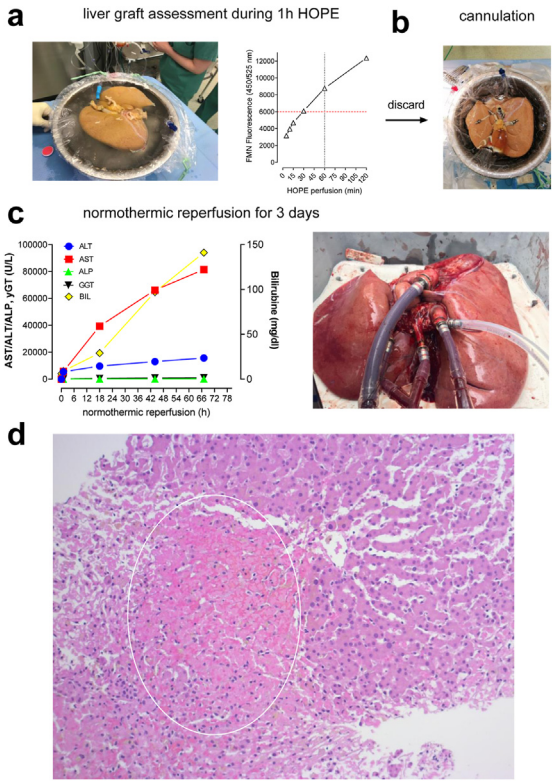

Fig. 7: Example of a discarded human liver based on perfusate fluorescence and subsequent *ex situ* normothermic reperfusion. Example of a discarded human DCD liver exceeding the FMN threshold (a), and subsequent cannulation (b) and normothermic *ex situ* reperfusion for 3 days, with high liver enzyme release and bilirubin increase (c) and large amounts of liver necrosis (circle) (d).

subsequently discarded. Among these, one liver was from a brain dead donor (DBD) (Fig. 4).

As a proof of concept, we isolated mitochondria of discarded human livers and analyzed their FMN and ATP content by mass spectrometry. These data confirmed significantly lower mitochondrial FMN in livers with high perfusate FMN, while livers with high mitochondrial FMN displayed lower perfusate FMN and metabolites (Fig. 6a and c). Mitochondrial FMN correlated also well with mitochondrial ATP content, pointing to the key role of an intact complex I and overall respiratory chain for successful energy production during HOPE (Fig. 6b). Histological assessment of discarded livers after HOPE treatment demonstrated a proportional increase of inflammation, e.g., oxidative DNA injury (8-OHdG), Kupffer cell activation and necrosis, with increasing perfusate FMN (Fig. 6d and e). Finally, we perfused several discarded livers under normothermic conditions, to simulate reperfusion injury during implantation, confirming immediate graft failure due to excessive liver necrosis (AST > 80,000 U/L, ALT > 10000 U/I, Fig. 7a–d).[8]

Underlying mechanisms

During any sort of ischemia, cellular respiration switches from aerobic to anaerobic due to the lack of oxygen. Consequently, the oxidation of NADH to NAD (nicotinamide adenine dinucleotide) in mitochondria is on hold, as well as the proton transport across the inner mitochondrial membrane. This results in interrupted electron flow throughout the respiratory chain. Simultaneously, the electron donors NADH and succinate, together with other precursors of the TCA-cycle further accumulate in mitochondria, paralleled by a fast ATP-breakdown.[15] When oxygen is re-introduced after ischemia, the respiratory chain aims for a rapid re-establishment of the electron flow, with subsequent ATP production to fuel the augmenting metabolic demand.[15] The accumulated succinate is therefore immediately oxidized by complex-II (SDH), which can lead, however, to electron overflow at complex I and reverse electron transfer (RET) instead of forward electron transport.[16,17] Major electron overflow at complex I triggers under these conditions the release of a small compound from complex I, reduced flavin mononucleotide,[18] which is non-covalently bound. The release of $FMNH_2$ leads in the presence of oxygen to massive production of ROS and oxidized FMN, which in turn is released to the extracellular environment (Fig. 8a). This is underlined by a gradual decrease in complex I function with increasing FMN loss (Fig. 9a), further leading to increasing, i.e., unoxidized NADH levels in perfusates (Fig. 9b). Mitochondria generate thus large amounts of ROS during reperfusion (after ischemia), which trigger a cascade of inflammation and danger signals, e.g., the release of mitochondrial DNA (mtDNA), release of HMGB-1, activation of toll-like-receptors and NLRP-3 inflammasome. Notably, we found a clear correlation between perfusate FMN and the activity of the citric acid cycle in the first hour of HOPE, measured by the amount of produced CO_2 (Fig. 8)e. These results support the view that down regulation of mitochondrial activity is key to prevent mitochondrial oxidative injury (Fig. 8b–e).

Discussion

We report here the largest single center series of liver transplantation with HOPE-treated DCD livers undergoing routine pre-transplant assessment of liver quality. We show, that donor derived parameters are rather inadequate for estimation of graft quality and prediction of post-transplant outcomes. Secondly, we demonstrate that machine perfusion alone is not always sufficient to avoid severe complications. Therefore, a careful perfusate assessment strategy is needed in order to safely increase the use of DCD livers with any donor risk.

During the last ten years, a significant evolution and change in liver preservation strategies has occurred, and

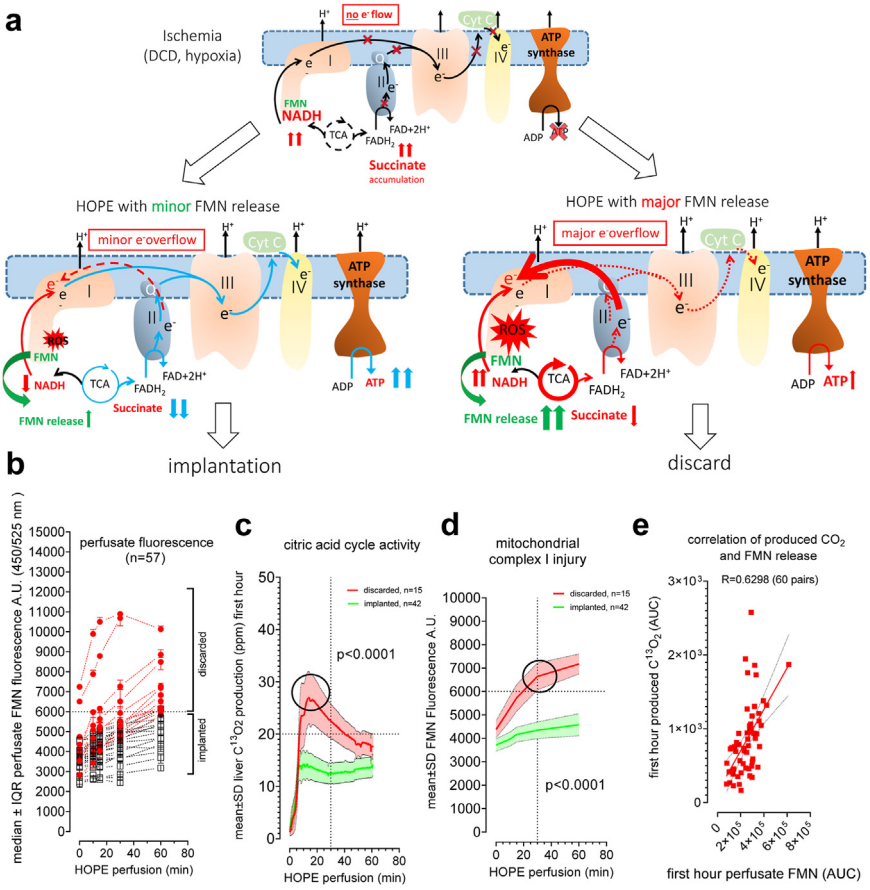

Fig. 8: Mechanism of mitochondrial injury and assessment of mitochondrial function during HOPE. Mechanism of mitochondrial injury during ischemia reperfusion induced by HOPE. Accumulated succinate and NADH during oxygenated reperfusion induce electron overflow at complex I and release of FMN at complex I (a). The level of complex I derived FMN or NADH can be detected by means of perfusate fluorescence (a). Detection is possible because of the natural fluorescence properties of FMN and NADH with excitation wavelengths between 310–390 nm and 400–470 nm, and emission wavelengths at 450–490 nm and 505–570 nm, respectively[8] (b). Perfusate measurements of HOPE treated DCD III livers (n = 37) are shown with levels below (open square) or above (red circle) the arbitrary cutoff of 6000 A.U. (arbitrary units). An increased CO_2 activity in the first 30 min (c) was detected in cases with increased perfusate FMN (d), with correlation of produced CO_2 and perfusate FMN (e); CO_2 produced during HOPE was detected at the oxygenator outlet with laser spectrometry (C^{13}ORlab, Argos GmbH). Presented p values refer to the Mann Withney U test.

three main dynamic liver perfusion techniques, e.g., normothermic machine perfusion (NMP), NRP and HOPE, have been implemented in clinical practice.[19,20] Despite a continuous shift from static cold storage to liver pumps for improved viability of marginal organs, however, assessment of liver graft quality remains not widely adopted in most programs. This is caused by an inherent difficulty in interpreting liver function during *ex situ* perfusion, leading to the report of several so-called liver biomarkers, measured in the circulating perfusate or in bile.[21–25] These include perfusate transaminases, pH, lactate clearance, bile flow, bile pH, bile glucose, coagulation based values of perfusate INR and factor V, or methacetin metabolism. While most of these parameters are used during NMP, their discrimination between good or bad livers remains limited.[26–28] This is based on the fact, that all of these markers are rather down-stream consequences of impaired liver function, but not cause related. On the subcellular level, however, clear evidence points to mitochondria as the source of ischemia reperfusion injury not only in hepatocytes, but likewise in cholangiocytes and endothelial cells. Mitochondrial transition pore opening, together with mtDNA and danger signal release, are therefore more upfront signals of liver injury and also representative for an impaired liver function. Measurement of mitochondrial injury during machine perfusion has therefore gained attendance. Based on our research in this field, we have focused on fluorometric perfusate analysis for surrogate markers of

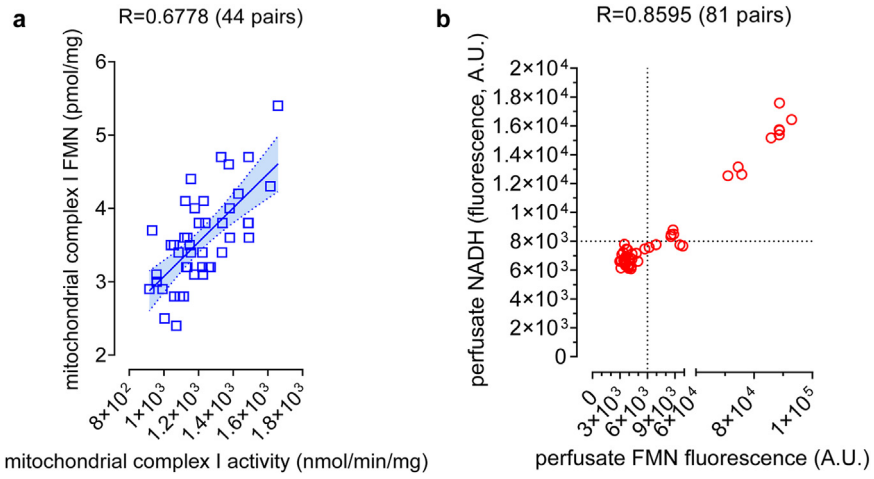

Fig. 9: Correlation of complex I function and loss of FMN, correlation of perfusate FMN with perfusate NADH. Complex I function decreased with increasing FMN loss (a). High perfusate FMN correlated with high perfusate NADH (b) (Pearson correlation coefficients).

mitochondrial injury and function.[2,9] Consistently, FMN release during ischemia reperfusion has been reported in the brain,[29] in kidneys[30] and in hearts.[31]

Of note, the course of the first 50 patients receiving a HOPE treated DCD liver was rather uneventful with no PNF,[32] despite accepting nearly every offer and no standardized perfusate assessment. With the increasing tendency to use DCD livers also for rescue options in sick recipients, e.g., high MELD or retransplants, we observed however an increase in PNF and also in cholangiopathies in 2017 and 2018 (Fig. 3b). We implemented subsequently a perfusate assessment, based on released FMN and NADH.[2] The establishment of reliable thresholds for these perfusate biomarkers needed another two year period for developing cutoffs to accept or reject DCD livers. Our threshold is thus the result of clinical transplant practice with HOPE-treatment of human DCD livers and needs to be validated in other centers.

In the last two years, we have implanted 48 DCD III livers with a standardized perfusate assessment in our department avoiding graft loss due to PNF or ischemic cholangiopathy. This is based on a higher detection and selection of pre-injured liver grafts with perfusate assessment, and consequently an increase in overall discard rates from 5% to 20% within the least 3 years. Liver utilization rates (livers implanted/livers offered and livers implanted/livers procured) are therefore lower, e.g., approximately 60% and 71%, compared to the utilization rates at the start of the DCD program (80% and 100%, Fig. 1c). This figure illustrates a feeling of false security and lack of experience in the first years of program implementation, specifically by using a perfusion technique without graft assessment. We therefore conclude that any liver perfusion technology without assessment is not advisable, similarly as are high utilization rates at the expense of serious complications. These insights evolved throughout our years of graft assessment illustrating the need for advanced assessment strategies, considering the fact that organ shortage is one of the greatest obstacles in liver transplantation in many European countries, including Switzerland.[1]

This study has limitations due to the retrospective design and due to the difficulty to proof the damage of discarded livers. For this purpose, we examined histological and cellular injury from tissues of discarded graft and simulated also reperfusion injury during *ex situ* reperfusion of discarded livers. Notably, there are many down-stream markers of injury, which can be determined in machine liver perfusates, as for example HMGB-1, mitochondrial DNA, toll-like receptor 4 and 9, NLRP 3, etc. All of these markers need however more time to be released into perfusates during perfusion, and several hours for reliable assessment. The advantage of measuring perfusate FMN, NADH, and produced CO_2, is the quick and real time assessment by perfusate fluorimetry and gas analysis at the oxygenator outflow.

Whether the combinations of different perfusion strategies, for example NRP plus HOPE or NRP pus NMP yields even better results is unclear. In a recent international comparison of utilization rates, we demonstrated higher DCDIII liver utilization rates in countries with routine use of machine technology, regardless of their technique, i.e., HOPE; NMP, or NRP.[1] Graft assessment before use is therefore likely to counteract organ shortages throughout Europe and worldwide.

Contributors

All authors read and approved the final version of the manuscript.

J.E.: Conceptualization, methodology, validation, formal analysis, investigation, data curation, writing original draft, data verification.

E.B.: Investigation, Editing.

D.B.: Investigatin, Editing.

M.M.: Investigation, Editing.

M.P.: Investigation, Editing.

H.M.: Investigation, Editing.

K.S.: Investigation, Editing.

J.W.: Investigation, Editing.

F.H.: Validation, methodology, formal analysis, data curation, investigation, editing.

U.U.: Resources.

B.H.: Investigation, Editing.

A.G.: Investigation, Editing.

S.S.: Resources, data curation.

M.W.: Resources, data curation.

F.I.: Resources, data curation.

A.E.: Resources, data curation.

D.M.: Formal analysis, validation, editing.

A.S.: Formal analysis, validation, investigation, editing, supervision, data verification.

P.D.: Formal analysis, investigation, methodology, validation, editing, supervision, project administration, funding acquisition, data verification.

Data sharing statement

Data are available from the corresponding author upon reasonable request.

Future Data on perfusate measurements will be shared in a machine perfusion registry initiated by ESOT.

Declaration of interests

A.S. received consultant fees for presentations from Bridge to life LTD, all other authors declare no competing interests.

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