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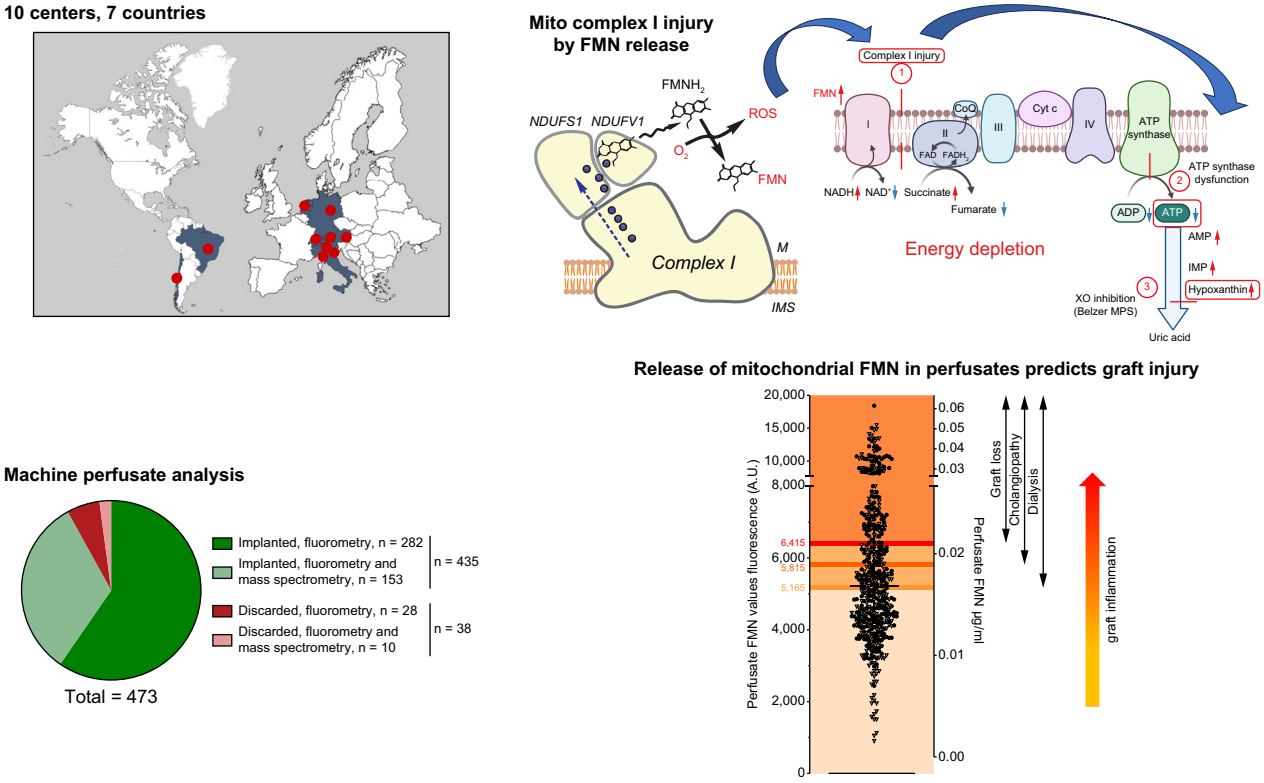
Graphical abstract



Highlights

- Fluorometric perfusate analysis for flavin mononucleotide correlates to mass spectrometric analysis.
- Perfusate flavin mononucleotide values are predictive for graft loss, cholangiopathy, and kidney failure.
- Mitochondrial flavin mononucleotide release relates to the degree of inflammatory activation during hypothermic oxygenated perfusion.

Impact and Implications

Analysis of 473 perfusates, collected from ten international centers during HOPE (hypothermic oxygenated perfusion), revealed that mitochondria-derived flavin mononucleotide values in perfusate are predictive of graft loss, cholangiopathy, and kidney failure after liver transplantation. This result is of high clinical relevance, as recognition of graft quality is urgently needed to improve the safe utilization of marginal livers. *Ex situ* machine perfusion approaches, such as HOPE, are therefore likely to increase the number of useable liver grafts.

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Assessment of liver graft quality during hypothermic oxygenated perfusion: the first international validation study

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Background & Aims: While it is currently assumed that liver assessment is only possible during normothermic machine perfusion, there is uncertainty regarding a reliable and quick prediction of graft injury during *ex situ* hypothermic oxygenated perfusion (HOPE). We therefore intended to test, in an international liver transplant cohort, recently described mitochondrial injury biomarkers measured during HOPE before liver transplantation.

Methods: Perfusate samples of human livers from ten centers in seven countries with HOPE experience were analyzed for released mitochondrial compounds, *i.e.* flavin mononucleotide (FMN), NADH, purine derivatives and inflammatory markers. Livers deemed unsuitable for transplantation served as negative controls.

Results: We collected 473 perfusate samples of human donation after cardiac death (n = 315) and donation after brain death (n = 158) livers. Fluorometric assessment of FMN in perfusate was validated by mass spectrometry (R = 0.7011, p <0.0001). Graft loss due to primary non-function or cholangiopathy was predicted by perfusate FMN values (c-statistic mass spectrometry 0.8418, 95% CI 0.7466-0.9370, p <0.0001; c-statistic fluorometry 0.7733, 95% CI 0.7006-0.8461, p <0.0001). Perfusate FMN values were also significantly correlated with symptomatic non-anastomotic strictures and kidney failure, and superior for the prediction of graft loss than conventional scores derived from donor and recipient parameters, such as the donor risk index and the balance of risk score. Mitochondrial FMN values in liver tissues of non-utilized livers were low, and inversely correlated to high perfusate FMN values and purine metabolite release.

Conclusions: This first international study validates the predictive value of the mitochondrial cofactor FMN, released from complex I during HOPE, and may therefore contribute to a better risk stratification of injured livers before implantation.

Introduction

Ex situ machine perfusion is increasingly recognized as a strategy to optimize the utilization of donor livers for transplantation.[1,2] This approach targets two key applications: first, machine perfusion provides a dynamic platform to mitigate ischemia-reperfusion injury (IRI), reducing post-transplant complications. Secondly, machine perfusion allows for the assessment of organ quality prior to transplantation.[3,4] Machine perfusion has therefore gained interest worldwide, as collection and analysis of circulating perfusate and/or bile during organ perfusion may allow for quick recognition of organ injury, which in turn may aid in the selection of suitable donor liver grafts for subsequent transplantation. However, due to variation in protocols and different machine perfusion approaches by transplant centers, an objective, reliable and universally accepted method for assessment of organ viability is currently lacking.

Two main *ex situ* perfusion concepts have been tested in the last 5 years in randomized clinical studies and are increasingly being adopted into clinical practice. Normothermic machine perfusion (NMP; 37 °C) aims to provide 'near-physiological' conditions to induce and maintain full metabolic activity of the organ.[5] With active metabolism, biochemical parameters of perfusate and bile can be assessed for signs of cellular damage resulting from IRI, providing insight into injury of the biliary tree.[4,6,7] In contrast, (dual) hypothermic oxygenated machine perfusion ([D]HOPE) provides oxygen under cold conditions (8-

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12 °C), using acellular preservation fluids.[3,8] Despite the increasingly demonstrated protective 'HOPE-effect' in human liver transplantation, showing improved graft survival and fewer post-transplant complications,[3,9] the lower metabolic activity of the liver under hypothermic conditions has often been claimed as the main disadvantage for assessment of liver injury and function during (D)HOPE. We have, however, shown in previous work that mitochondrial function through analysis of flavin mononucleotide (FMN), a cofactor of mitochondrial complex I, is detectable during (D)HOPE, and was predictive of graft function after liver transplantation.[10,11] Yet, these results were found in a single center cohort and are in need of external validation by other centers. As the next logical step, we performed an international observational cohort study to analyze the release of FMN during (D)HOPE, validate FMN fluorometry values with mass spectrometry analysis, and investigate the utility of FMN to perform risk stratification as a useful predictor of liver graft quality.

Patients and methods

Study design

In this international, observational cohort study we included donor livers from ten international liver transplant centers between 2019 and 2022. End-of-life care and donor management were carried out according to national legislation and in accordance with ongoing, standard clinical practice for each individual center. Both donation after brain death (DBD) and donation after circulatory death (DCD) donor livers were included, respecting the considerable variability in donor management, particularly with DCD donors, between countries.[1] Both HOPE (perfusion via portal vein only) and (D)HOPE (dual perfusion via portal vein and hepatic artery) were used for a minimum of 2 h prior to transplantation, using either the commercially available, pressure-regulated perfusion device Vitasmart® (Bridge to life Ltd., Chicago, IL, United States) or the LiverAssist device (XVIVO B.V. Groningen, the Netherlands). Oxygenated (pO_2 ≥80 kPa) University of Wisconsin machine perfusion solution was recirculated at temperatures between 8-12 °C, with a maximum perfusion pressure of 3-5 mmHg. Perfusate samples were collected at 60 min after the start of (D)HOPE, stored in -80 °C, and shipped per batch to University Hospital Zurich, Switzerland, for further analysis. All DCD livers in Zurich underwent routine perfusate analysis for FMN since 2018, and the decision regarding whether to utilize DCD livers or not was exclusively dependent on perfusate analysis with varying cut-offs.

An additional cohort of 38 perfused livers from the University Hospital Zurich which underwent HOPE were included, but were not transplanted after FMN assessment (further referred to as 'non-utilized'). The non-utilized donor livers underwent a further estimation of graft quality by additional *ex situ* evaluation of liver injury and function using a normothermic perfusion system, as previously reported.[11] These non-utilized livers were considered as high-risk liver grafts not suitable for transplantation, and were therefore used as a reference group for poor liver graft quality.

The study protocol relates to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the following regulatory bodies in the individual centers: Zurich University Hospital, Switzerland: 2019-01000; Bologna, Italy: RF-2016-02364732: Turin, Italy: 739/2019; Milan Policlinico, Italy: 1792/22; Berlin/Aachen, Germany: EA2/270/20; Erasmus MC Rotterdam, the Netherlands: METC 2014-060; University Medical Center Groningen, the Netherlands: METc 2014/077; Santiago, Chile: 000021/Code: D/UTH/520; São Paulo O: 4.740.772/C: 45630421.0.0000.0071, Vienna, Austria: 2209/2018. When writing the manuscript, STROBE guidelines were adhered to.[12]

Perfusate fluorometric spectroscopy analysis

Perfusate fluorometric spectroscopy was performed with a Cytation 3 imaging microplate reader (BioTek Cytation Hybrid Multimode Reader, Agilent Crosslab, Santa Clara, USA), as established previously.[8,10,11,13] Briefly, FMN values were measured on a 96-well microplate (350 µl, Merck, Switzerland) using the following settings, set at excitation at 485 nm; emission at 528 nm and gain of 130. NADH values were measured, set at excitation at 360 nm; emission at 460 nm and gain of 100. Fluorometric measurements were calibrated using FMN standard concentrations (Sigma-Aldrich F2253, Saint Louis, USA).

Metabolite extraction and profiling by targeted liquid chromatography-mass spectrometry

Fifty µl of perfusate per sample was used for metabolite profiling. Proteins were precipitated by the addition of two volumes of acetonitrile and an internal standard mixture, containing chloramphenicol and C13-labeled L-glutamine, L-arginine, L-proline, L-valine, and uracil, added to each supernatant (10 mM final concentration), followed by vortexing for 5 min, and centrifugation (16,000 x g) at 4 °C for 10 min. Pure FMN (Sigma-Aldrich F2253, Saint Louis, USA) was used for liquid chromatography-mass spectrometry. The protein pellet gained after centrifugation was used in a bicinchoninic acid-based protein assay for normalization among samples. Metabolites in the supernatants were lyophilized, dissolved in 15 µl of water, sonicated in an ice water bath for 5 min, and centrifuged again (16,000 x g). Five microliters of the supernatant were mixed with 45 µl of water for metabolite analysis on a reversed-phase column (Reprosil-PUR C18-AQ (1.9 µm, 120 Å, 150 x 2 mm ID; Dr. Maisch; Ammerbuch, Germany) or with 45 µl of acetonitrile for metabolites separated on a zicHILIC column (3.5 µm, 100 Å, 150 x 2.1 mm ID; di2chrom; Marl, Germany). Subsequent separation of the metabolites was performed on an LC instrument (1290 series UHPLC; Agilent, Santa Clara, CA, USA), online coupled to a triple quadrupole hybrid ion trap mass spectrometer QTrap 6500 (Sciex, Foster City, CA, USA), based on the multiple reaction monitoring method, as reported previously.[14] Three transitions per selected metabolite were measured. Relative quantification was performed using MultiQuantTM software v.2.1.1 (Sciex, Foster City, CA, USA). Peak integrations were reviewed manually, and peak intensities were normalized, first against the internal standards, and subsequently against protein abundances obtained from the bicinchoninic acid assay. The first transition of each metabolite was used for relative quantitation between samples. Metabolomics data have been deposited in the publicly available repository PeptideAtlas with the identifier PASS05861 and can be downloaded via http://www.peptideatlas.org/PASS/PASS05861.

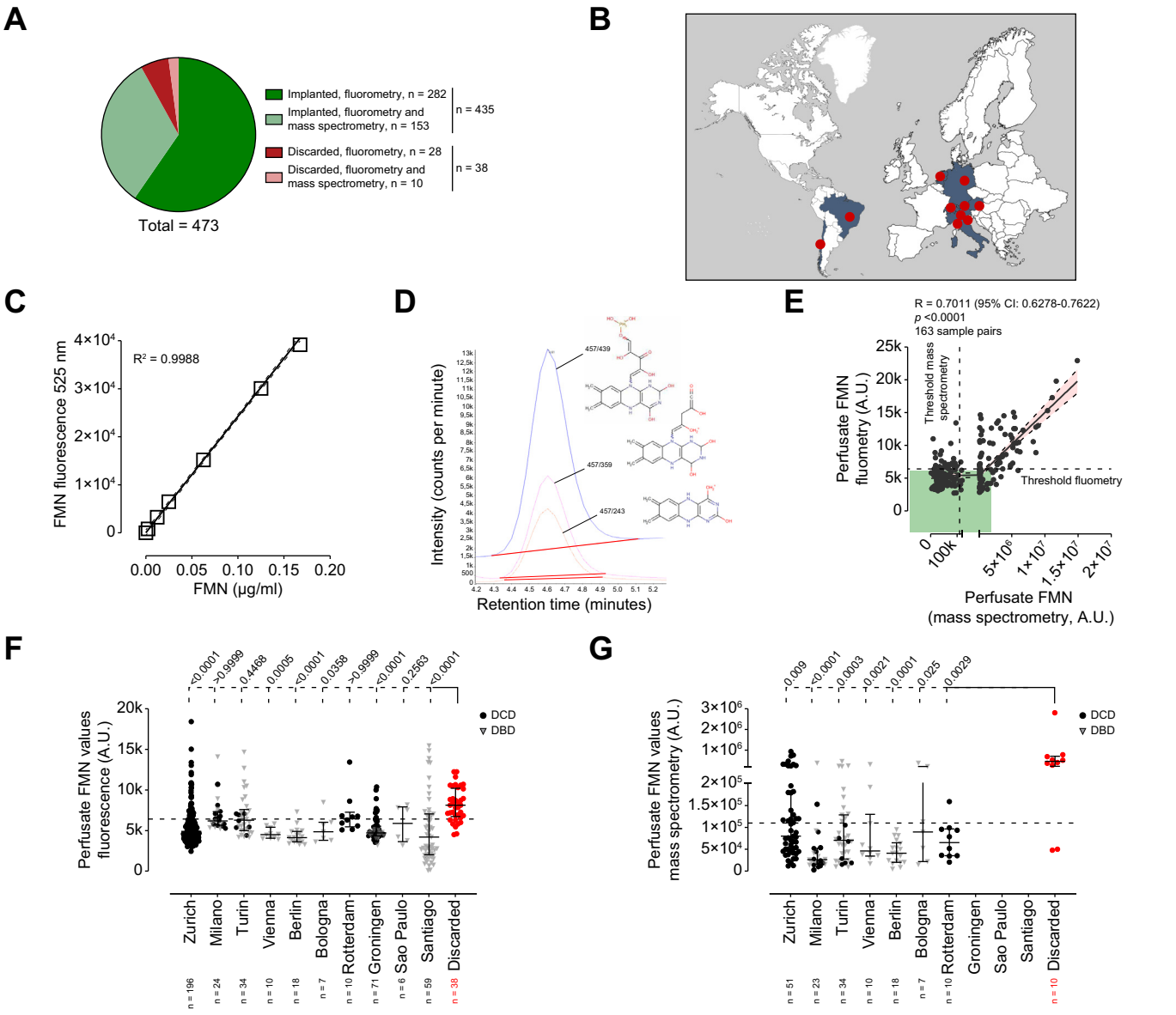

Fig. 1. Study design, correlation of fluorescence and mass spectrometry. The study refers to 473 machine liver perfusates, 435 livers were implanted, and 38 livers were discarded. All perfusates were analyzed fluorometrically for FMN, 163 perfusates were additionally analyzed by mass spectrometry. (A). Ten centers from seven countries participated in this study (B). Calibration of fluorometric measurements allowed calculation of µg FMN/ml perfusate (C). Next, MRM transitions for FMN are shown with overlay of three chromatograms for FMN specific ion transitions in positive ionization mode: m/z 457→ (439, 359, 243) (D). The corresponding fragment structures are shown on the right. The areas above the red lines were used for peak integrations (Gaussian smoothing width: 2 points). Mass spectrometry for FMN correlated with fluorometric values (E) (Pearson's parametric correlation). The single values of all centers are visualized with medians and IQR in scatter plots for fluorescence and mass spectrometry (F, G, respectively). *p* values are two-tailed and refer to discarded livers (Mann-Whiney *U* test, levels of significance *p* <0.01). FMN, flavin mononucleotide; MRM, multiple reaction monitoring; m/z, mass over charge.

Study endpoints

The main outcome parameters for this study were (1) graft loss due to either primary non-function (PNF, defined as organ failure resulting in death or retransplantation within 7 days) or (2) incidence of symptomatic (defined as need for either antibiotic treatment, radiologic, endoscopic, or surgical intervention, retransplantation or patient death) non-anastomotic strictures (NAS, defined as cholangiopathy outside the biliary anastomosis region, and in the presence of a patent hepatic artery), and (3) kidney injury (defined as the need for new onset renal replacement therapy [RRT] after transplantation). Secondary outcome parameters include liver lab values during the first week after transplantation, as a surrogate marker for IRI. The median follow-up for all transplanted patients was 2.3 years (1.1-6.9 years).

Tissue mitochondrial analysis

To further investigate mechanistic details and the link between perfusate FMN values and tissue mitochondrial FMN content, a subset of paired perfusate and tissue samples from human donor livers that underwent HOPE treatment at the University Hospital Zurich were analyzed. Perfusates, liver tissues and

Table 1. Donor and recipient parameters of implanted livers from ten international centers (n = 435).

	DBD, n = 158	DCD, n = 277
Donor age (yr)	61 (51-75)	58 (49-65.3)
Donor BMI (kg/m^2)	27 (24-31)	25 (23-28)
Cold ischemia time (min)	360 (290-459)	294 (233-344)
Functional warm ischemia time (min)	–	29 (26-33)
HOPE perfusion time (min)	183 (116-330)	154 (120-218)
Donor risk index	1.94 (1.56-2.30)	2.48 (2.16-2.85)
Recipient age (years)	59.5 (51-64.3)	60 (51-65.3)
Recipient MELD	15 (9-25)	14 (10-19)
Primary transplant (n/%)	3/158 (1.9)	6/277 (2.2)
Peak AST (U/L)	865 (500-1,501)	2,177 (1,006-4,310)
Peak ALT (U/L)	586 (308-1,077)	1,176 (610-2,414)
Peak creatinine (μmol/L)	123 (75-186)	182 (91-276)
Intensive care unit stay (days)	3.5 (2-6)	2 (1-4)
Hospital stay (day)	15 (10-21.8)	15 (12-24)
PNF (n/%)	3/158 (1.9)	8/277 (2.9)
NAS (n/%)	8/158 (5.1)	28/277 (10.1)
Graft loss due to PNF, NAS (n/%)	10/158 (6.3)	31/277 (11.2)
Kidney failure post-LT (RRT) (n/%)	15/158 (9.5)	72/277 (25.9)
5-yr graft survival (%)	95.8 ± 2.1	79.8 ± 4.6

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBD, donation after brain death; DCD, donation after circulatory death; FMN, flavin mononucleotide; HOPE, hypothermic oxygenated perfusion; LT, liver transplantation; MELD, model for end-stage liver disease; NAS, non-anastomotic strictures; PNF, primary non-function; RRT, renal replacement therapy.

isolated mitochondria of both implanted and non-utilized livers were further analyzed for mitochondrial injury and function, and were tested in correlation with perfusate and mitochondrial FMN content. Isolation of mitochondria was done according to previous studies (11).

Perfusate injury marker analysis

Perfusate samples obtained 60 min after the start of (D)HOPE were further quantified for their content of mitochondrial DNA and cytoplasmic DNA, as well as proinflammatory molecules, including damage-associated molecular patterns (DAMPs), such as high-mobility-group-box-1 (Hmgb1, IBL International GmbH; ST51011)), Toll-like receptor 4 and 9, inflammasome (NLRP-3), interleukins (IL-1β, IL-18) and alanine and aspartate aminotransferase (ALT and AST).

Staining

A novel staining procedure for the NDUFS1 (FMN docking site at complex I) subunit of human mitochondrial complex I (Complex I-75 kD (CI-75 kD) also known as the NADH-ubiquinone oxidoreductase 75 kDa subunit) was developed according to the staining protocol provided by Akoya biosciences for multiplex staining. Paraffin-embedded tissue sections (5 μm) were used. Heat-mediated antigen retrieval was performed with citrate buffer pH6, blocked and incubated with primary antibody overnight at 4 °C (ABN302) in antibody diluent and blocking buffer. The specimen was incubated with the secondary antibody (Akoya, OPAL polymer HRP Ms+Rb) and fluorophore (Akoya, OPAL 520) at room temperature for 10 min (each). Additional staining for NF-κB, CD68 (macrosialin; MCA341R, RRID:AB_2291300) and von Willebrand factor was performed. Quantification was performed in a blinded fashion.

Statistical analysis

Data are presented as median (IQR) for continuous variables and n (%) for dichotomous variables. The non-parametric Mann-Whitney *U* test was used to test for differences between two groups. One-way ANOVA (Kruskal-Wallis) was used for testing of multiple non-parametric data. A p value of <0.05 was considered statistically significant. Pearson's parametric correlation was used to assess the relationship between two metric variables. ROC curves were used to determine the threshold of highest sensitivity and specificity (Youden index) in human perfusate FMN samples for liver graft loss, symptomatic NAS, and kidney injury requiring RRT. Metabolite activities were measured in duplicates or quadruplicates at 25 °C. All activities are shown in mmol substrate/min/mg protein. All data were analyzed using SPSS (IBM v.24.0; Armonk, NY: IBM Corp.) and GraphPad Prism, version 10.0 (San Diego, CA, USA).

Results

International comparison of perfusate FMN by fluorometry and mass spectrometry

We included a total of 473 perfusate samples collected during (D)HOPE treatment of 315 DCD and 158 extended criteria DBD livers (Fig. 1A) performed at ten centers from seven countries (Fig. 1B). Of these, 435 livers were transplanted (91.7%), whereas 38 livers were considered not suitable for transplantation (non-utilized) after FMN assessment. Twenty DCD livers (13 in Milano, and 7 in Turin) underwent mandatory NRP prior to HOPE (20/435, 4.6%). Relevant donor and recipient information of implanted livers is provided in Table 1.

First, we aimed to identify the presence of FMN in (D)HOPE perfusates among different international centers. Fluorometric absorbance measurements were strongly correlated with FMN standard concentrations within the linear range (R^2 = 0.99) (Fig. 1C). Secondly, we identified FMN abundance in the perfusate samples through mass spectrometry analyses (Fig. 1D). In a subset of 163 perfusate samples, fluorometric measurements of perfusate FMN values correlated with FMN abundance measured through mass spectrometry (R = 0.70, p <0.0001) (Fig. 1E). Next, we compared FMN perfusate values of implanted livers from all involved centers with non-utilized livers from Zurich, separated for DBD and DCD livers. FMN

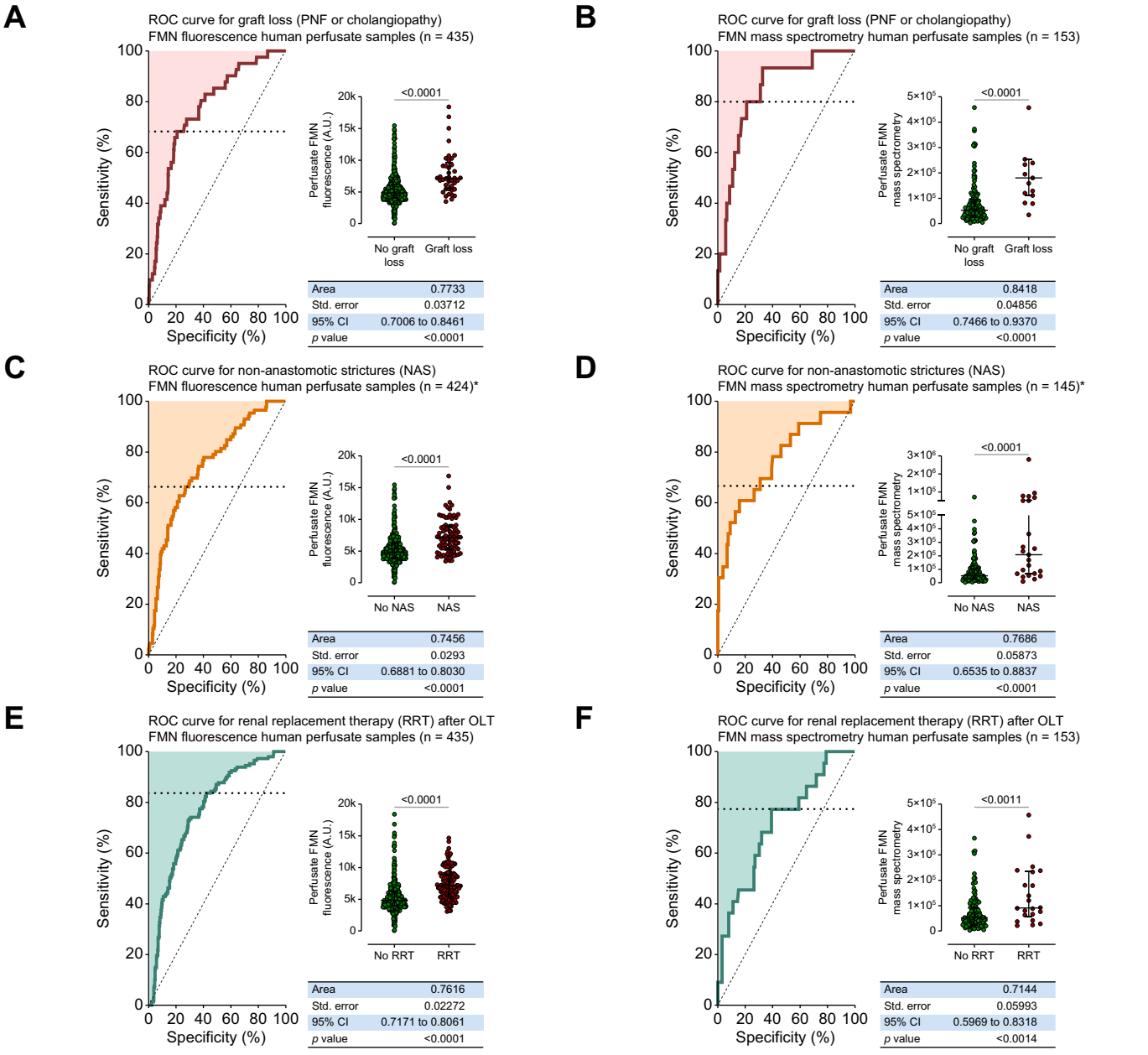

Fig. 2. Predictive capacity of perfusate FMN by fluorescence and mass spectrometry. ROC curve-based thresholds for clinical decisions on perfused liver grafts are illustrated for fluorometric perfusate FMN measurements or mass spectrometry for graft loss (A and B), non-anastomotic strictures (C and D) and renal replacement therapy (E and F). The AUC represents the c-statistic, quantifying the overall ability of the test to discriminate between positive and negative values. The reported *p* values test the null hypothesis that the AUC equals 0.5, according to Berrar D *et al.* (supplementary reference 1). Confidence intervals are calculated by Prism using the hybrid Wilson/Brown method (supplementary reference 2-3). FMN, flavin mononucleotide.

fluorometric measurements were significantly different in implanted livers compared to non-utilized livers, with the exception of Milan, Turin, Rotterdam, and São Paulo (Kruskal-Wallis; p >0.99, p = 0.45, p >0.99, and p = 0.26, respectively) (Fig. 1F), possibly related to the presence of free hemoglobin in samples from livers after NRP pretreatment (Millan, Turin), or to a sample size effect (Rotterdam, São Paulo). In the subset of 163 perfusate samples of which FMN values were measured by mass spectrometry, all centers showed significantly lower FMN values for transplanted livers when compared to non-utilized livers (Kruskal-Wallis; all p <0.05; Fig. 1G).

Perfusate FMN predicts graft loss, cholangiopathy and kidney failure

Next, for all livers that were subsequently transplanted, we sought to estimate the predictive value of FMN levels for clinically relevant outcomes, related to any injury the graft may have suffered due to the donation, preservation, or the ischemia and reperfusion process upon transplantation. We analyzed the predictive value of perfusate FMN values for graft loss due to either primary non-function (PNF) or cholangiopathy. We found a c-statistic of 0.77 (95% CI 0.70-0.85,

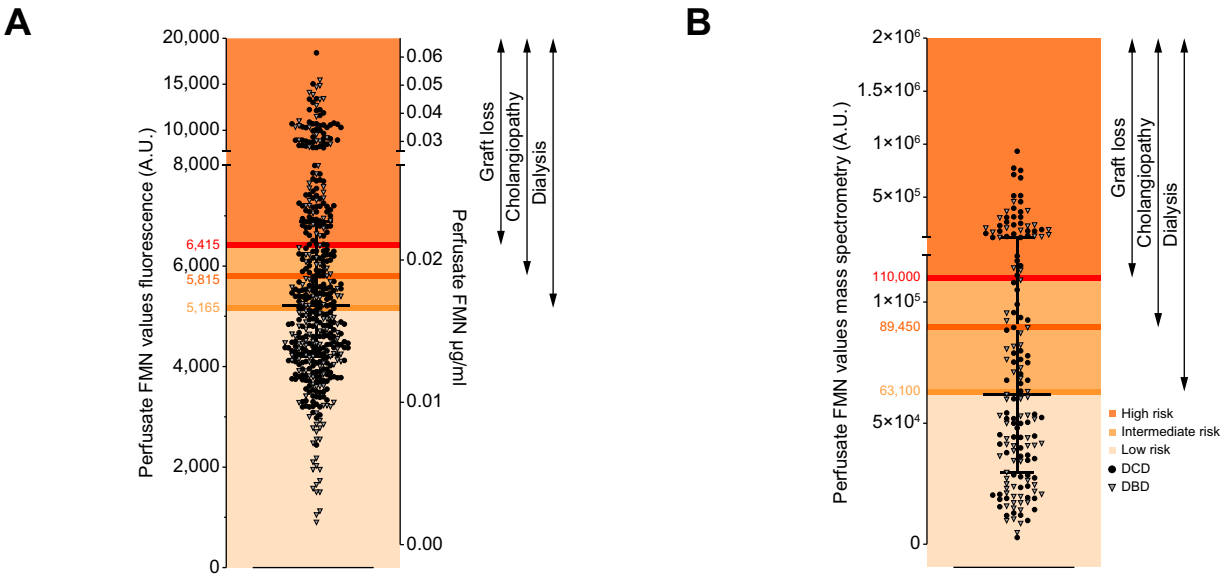

Fig. 3. Determination of thresholds for accepting or discarding liver grafts during HOPE. Proposed acceptance, risk and discard thresholds determined by the maximal Youden index of the ROC curves by fluorescence (A) and mass spectrometry (B). FMN, flavin mononucleotide.

p <0.0001) for perfusate FMN by fluorometry (Fig. 2A) and a c-statistic of 0.84 (95% CI 0.75-0.94, *p* <0.0001) for perfusate FMN analysis by mass spectrometry (Fig. 2B). Likewise, perfusate FMN analysis was also predictive for the occurrence of symptomatic NAS with a c-statistic of 0.75 (95% CI 0.69-0.80, *p* <0.0001) by fluorescence (Fig. 2C), and with a c-statistic of 0.77 (95% CI 0.65-0.88, *p* <0.0001) by mass spectrometry (Fig. 2D). In addition, perfusate FMN values had a strong predictive value for post-transplant kidney failure requiring RRT, with a c-statistic of 0.76 (95% CI 0.71-0.81, *p* <0.0001) by fluorometry (Fig. 2E), and with a c-statistic of 0.71 (95% CI 0.59-0.83, *p* <0.0014) by mass spectrometry (Fig. 2F). Detailed results of the ROC analysis are available in Table S1.

Accordingly, we calculated the maximal Youden index of the ROC curves to define safety thresholds for accepting donor livers by fluorometry (Fig. 3A) as well as mass spectrometry (Fig. 3B). Of note, perfusate FMN values of DBD and DCD livers were relatively homogenously distributed (Fig. 3A,B). Low-risk livers were defined as those that did not result in graft loss, cholangiopathy or kidney injury post-transplantation. Intermediate-risk livers were defined as those that did result in cholangiopathy and/or kidney injury, but not in graft loss post-transplantation. High-risk livers were defined as those that resulted in graft loss, and/or cholangiopathy, and/or kidney injury. Perfusate FMN threshold values, as measured by fluorometry for low-to medium-risk livers, was set at 5,165 A.U. (0.017 µg/ml), whereas for medium-to high-risk livers this was set at 6,415 A.U. (0.021 µg/ml). For FMN values measured by mass spectrometry, the cut-off values were 63.100 A.U. and 110.000 A.U. for low-to medium-risk livers and for medium-to high-risk livers, respectively. These results show that perfusate FMN values, detected during (D)HOPE by either fluorometry or mass spectrometry analysis, are related to key outcome parameters after transplantation.

Perfusate FMN correlates with first week lab values

We proceeded in assessing the association of perfusate values of FMN with injury markers and liver function after transplantation. For example, international normalized ratio levels at day 1, 2 and 7 correlated significantly with perfusate FMN values, as well as ALT and AST (Fig. S1A-F, I), while serum bilirubin and peak creatinine showed no correlation (Fig. S1G and H) with perfusate FMN values. These results indicate that the degree of mitochondrial injury occurring during procurement and preservation is associated with impaired liver graft function after implantation.

Perfusate FMN correlated with mitochondrial function and purine metabolites

In another step, we analyzed machine liver perfusates, collected in the first hour of (D)HOPE, to assess the effect on other mitochondrial processes, including direct complex I FMN content, citric acid and purine metabolism. Initially, we observed that high perfusate FMN values inversely correlated with the FMN content of mitochondrial complex I in several exemplary livers (Fig. 4A). The key role of complex I in proton pumping, and thus ATP synthesis, was further supported by the strong correlation of mitochondrial FMN values with mitochondrial ATP (R = 0.93, *p* <0.0001, Fig. 4B). Consistently, complex I bound mitochondrial FMN was a prerequisite for complex I function (R = 0.68, *p* <0.0001, Fig. 4C), and NADH presence was associated with FMN levels in perfusate (R = 0.29, *p* <0.0001, Fig. 4D) pointing to impaired oxidation of NADH with increased FMN release, *i.e.* complex I injury. These results suggest that perfusate FMN is released as a result of mitochondrial injury during HOPE. With regard to other metabolites, we observed a significant increase of succinate compared to fumarate, and a significant increase of adenylate breakdown products AMP, IMP, inosine, and

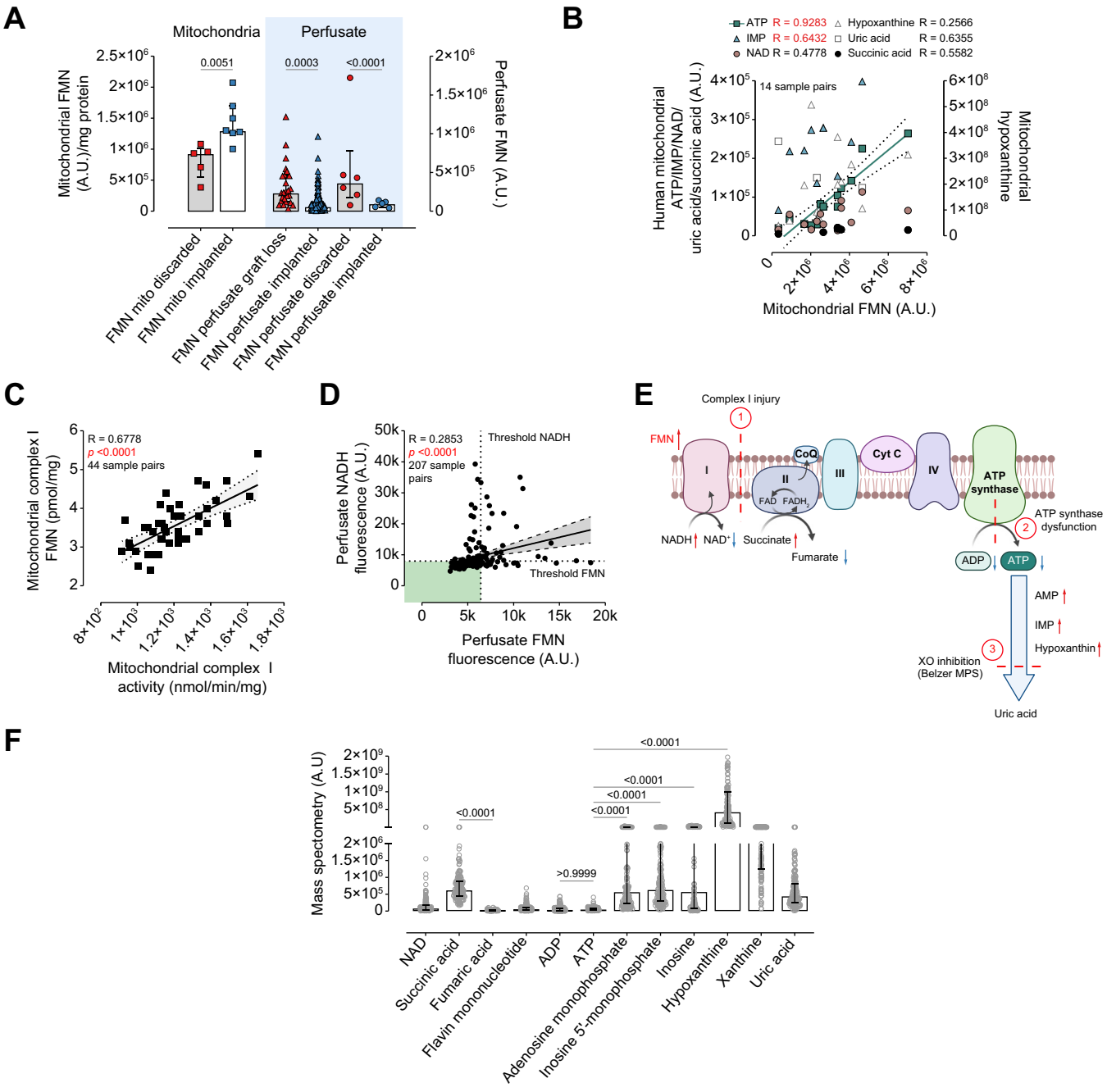

Fig. 4. Correlation of mitochondrial FMN and perfusate FMN, correlation of perfusate FMN and complex I function. Perfusate FMN inversely correlated with mitochondrial FMN (A), and mitochondrial FMN correlated with mitochondrial content of ATP and IMP (B). Consistently, complex I bound mitochondrial FMN correlated with complex I activity (C), and perfusate FMN was associated with perfusate NADH (D). Perfusates collected within the first hour of HOPE showed an increasing level of purine metabolites, underlining mitochondrial activity under hypothermic conditions (E, F). *p* values are two-tailed (Mann-Whiney *U* test, levels of significance $p < 0.01$ (A,F). Pearson's parametric correlation was used in B, C, D, levels of significance $p < 0.01$, two-tailed *p* values. FMN, flavin mononucleotide; HOPE, hypothermic oxygenated perfusion.

hypoxanthine, compared to ADP and ATP (Fig. 4E,F), which was significantly more pronounced in DCD livers (Fig. S2). Accordingly, perfusate FMN values also showed moderate correlation with perfusate NAD^+, succinate, ADP and IMP (Fig. 5A–D), as well as with other purine and citric acid metabolites (Fig. S3A–D). Of note, xanthine and uric acid values were less increased, possibly due to inhibition of xanthine oxidase through the presence of allopurinol in the machine perfusion solution. These findings point to increased hypoxic conditions before or during organ procurement, and consequently to a high need for ATP during the first hour of *ex situ* perfusion. Perfusate FMN values did not correlate with hepatocyte enzyme release (AST, ALT), underlining its mitochondrial origin (Fig. S4).

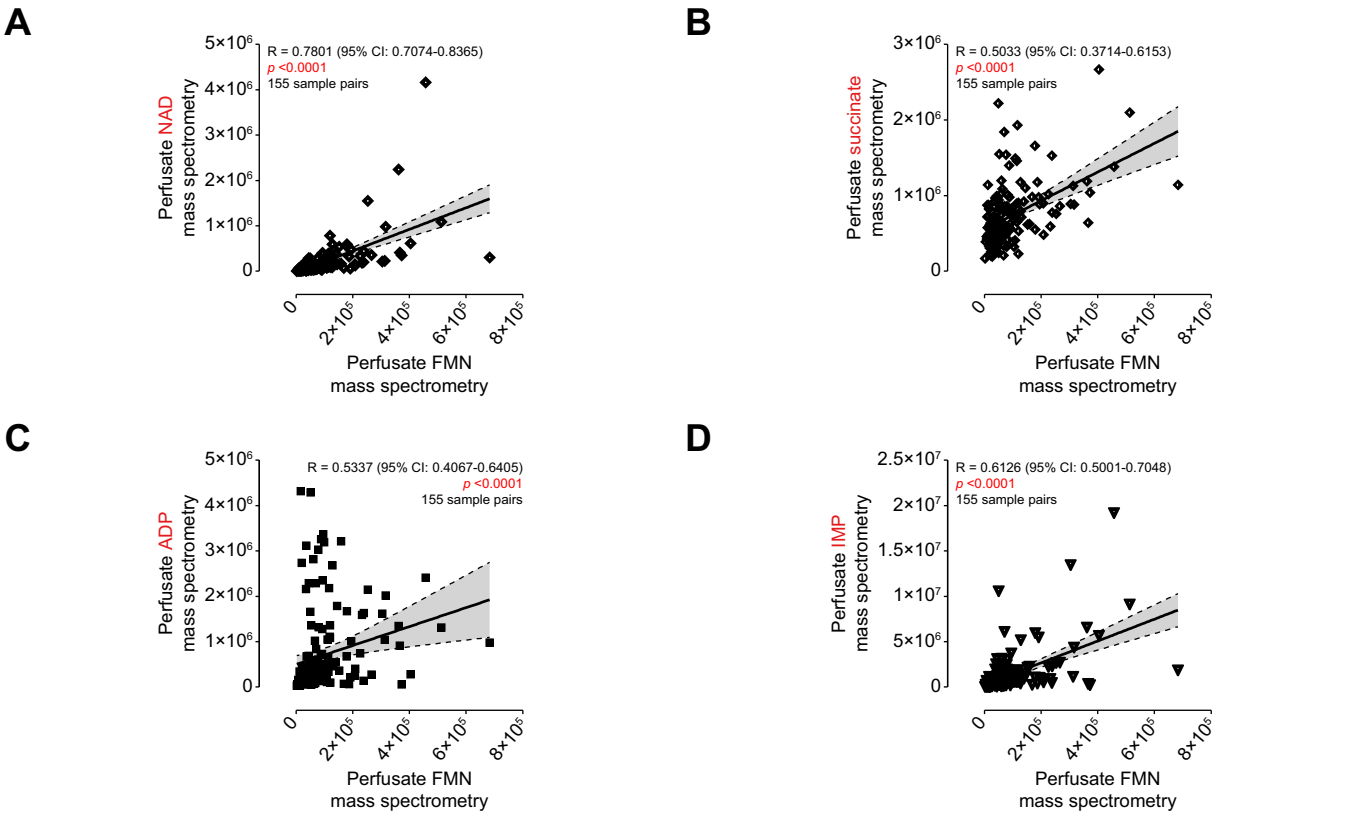

Fig. 5. Perfusate FMN and purine metabolites. Perfusate FMN measure by mass spectrometry shows positive correlations with perfusate (A) NAD, (B) succinate, (C) ADP and (D) IMP. Pearson's parametric correlation was used in A-D, levels of significance p <0.01, two-tailed p values. FMN, flavin mononucleotide.

Mitochondrial injury and downstream inflammation in discarded livers

As a proof of concept, in a subset of perfused livers we compared paired liver perfusates and tissues of implanted livers with samples of non-utilized livers. For this purpose, we investigated mitochondrial injury and downstream inflammation, *i.e.* mitochondrial DNA release, Toll-like receptor activation, inflammasome activation and cytokine release. These parameters were correlated with staining for the NDUFS-1, release of NF-κB, Kupffer cell activation (CD68), and endothelial cell activation (vWF). All tested parameters showed significant differences between discarded and implanted livers (Figs 6 and 7). These findings confirmed that non-utilized livers displayed high levels of mitochondrial injury and inflammation.

Donor and recipient parameters are inferior compared to perfusate analysis

Finally, we investigated the predictive value of conventional donor and recipient scores in the same cohort. We used the DRI (donor risk index) and the BAR (balance of risk) score, which both showed only weak prediction of graft loss (c-statistics 0.5997 and 0.5488, respectively), compared to perfusate FMN values (Fig. S5A and B). These results underline that donor and recipient parameters are not useful for evaluation of liver graft quality.

Discussion

In this observational cohort study involving ten international centers, we demonstrate that perfusate FMN values, measured during (D)HOPE, enable stratification of liver grafts into different risk categories correlating with patient outcomes. This offers a new and highly needed tool to select individual grafts for transplantation, where the final decision remains in the hands of the medical team (considering the risk they are willing to take).

Mitochondria are regarded as the primary trigger of IRI and play an important role in organ transplantation.[15] Machine perfusion before transplantation modifies mitochondrial metabolism via delivery of both oxygen and metabolites. At the same time, machine preservation perfusates are an easily obtainable and highly informative source of organ cellular and subcellular injury, due to analysis of perfusate compounds, *i.e.* biomarkers. While such assessment of liver mitochondrial injury by FMN release during (D)HOPE has previously been reported,[10,11] it remains to be demonstrated whether these results could also be replicated in other, independent, international transplant centers.

Our results show that perfusate FMN values, measured during the first hour of (D)HOPE of human liver grafts, correlated with graft loss, with symptomatic NAS, and with kidney failure requiring RRT after liver transplantation. In addition, perfusate FMN values inversely correlated with mitochondrial FMN levels, underlining its mitochondrial origin. Third,

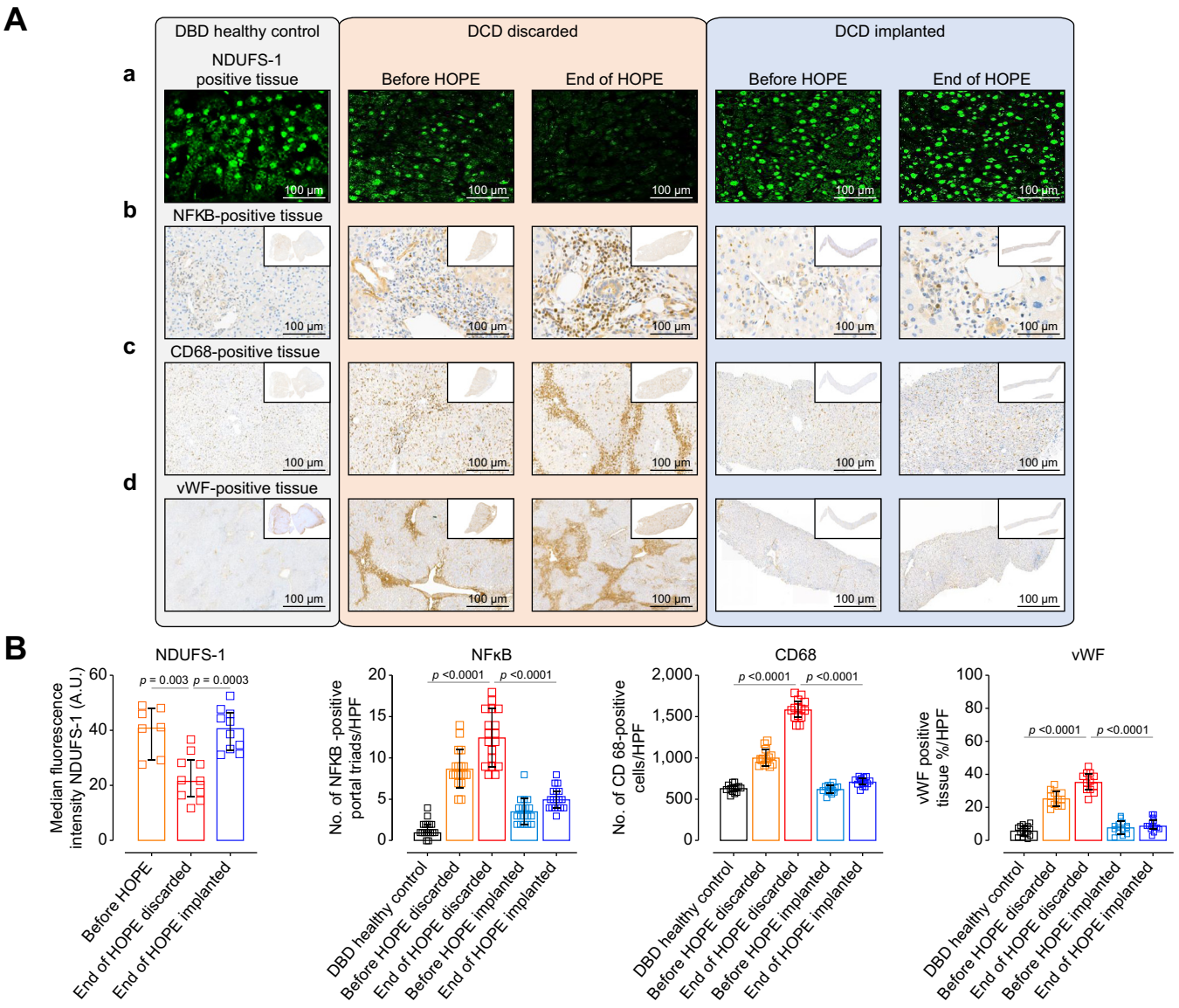

Fig. 6. Mitochondrial injury and inflammation in implanted and discarded livers (liver tissue samples). Liver tissue of discarded and implanted livers were compared for changes at the NDUFS1 region of complex I, for NF-κB, CD68, and for vWF (A) with respective quantifications (B). *p* values are two-tailed (Mann-Whiney *U* test, levels of significance $p < 0.01$ (B).

increased perfusate FMN values were associated with markers of downstream inflammation, including mitochondrial DNA, DAMPs, and cytokines. Fourth, and importantly, perfusate FMN values had higher predictive performance than conventional risk scores based on donor or recipient factors, and have also been shown to be superior than other conventional perfusate markers, *e.g.* AST, ALT or lactate.[10,11] Consistently, we found in this study that DCD livers can have low perfusate FMN signals, while DBD livers potentially range at a very high level (Fig. 1F,G). This underlines the strength of an objective perfusate assessment apart from the conventional donor information, including graft type.

Whether FMN analysis in liver machine perfusate can also predict later occurring biliary injury, alongside PNF and kidney injury, is currently debated. For example, several researchers rely only on testing biliary viability under normothermic and therefore more physiologic conditions, as livers produce minimal-to-no bile in the cold.[16] However, mitochondrial aerobic energy production plays an important role in the development of biliary injury, as it generates ATP necessary for bile salt transporters.[17] Accordingly, mitochondrial injury leads to ATP depletion, and secondly triggers an inflammatory state in cholangiocytes via DAMP signaling.[18] Third, mitochondrial ATP also controls the CFTR (cystic fibrosis transmembrane conductance regulator) protein on cholangiocytes, which in turn regulates bicarbonate secretion.[19] Consecutively, defective CFTR causes changes in bile composition and mucinous obstruction.[20] For all these reasons, mitochondrial injury is likely to have many consequences, not only for hepatocytes but also for cholangiocytes, which explains why monitoring mitochondrial injury during (D)HOPE is also informative for assessment of biliary injury. This has important clinical

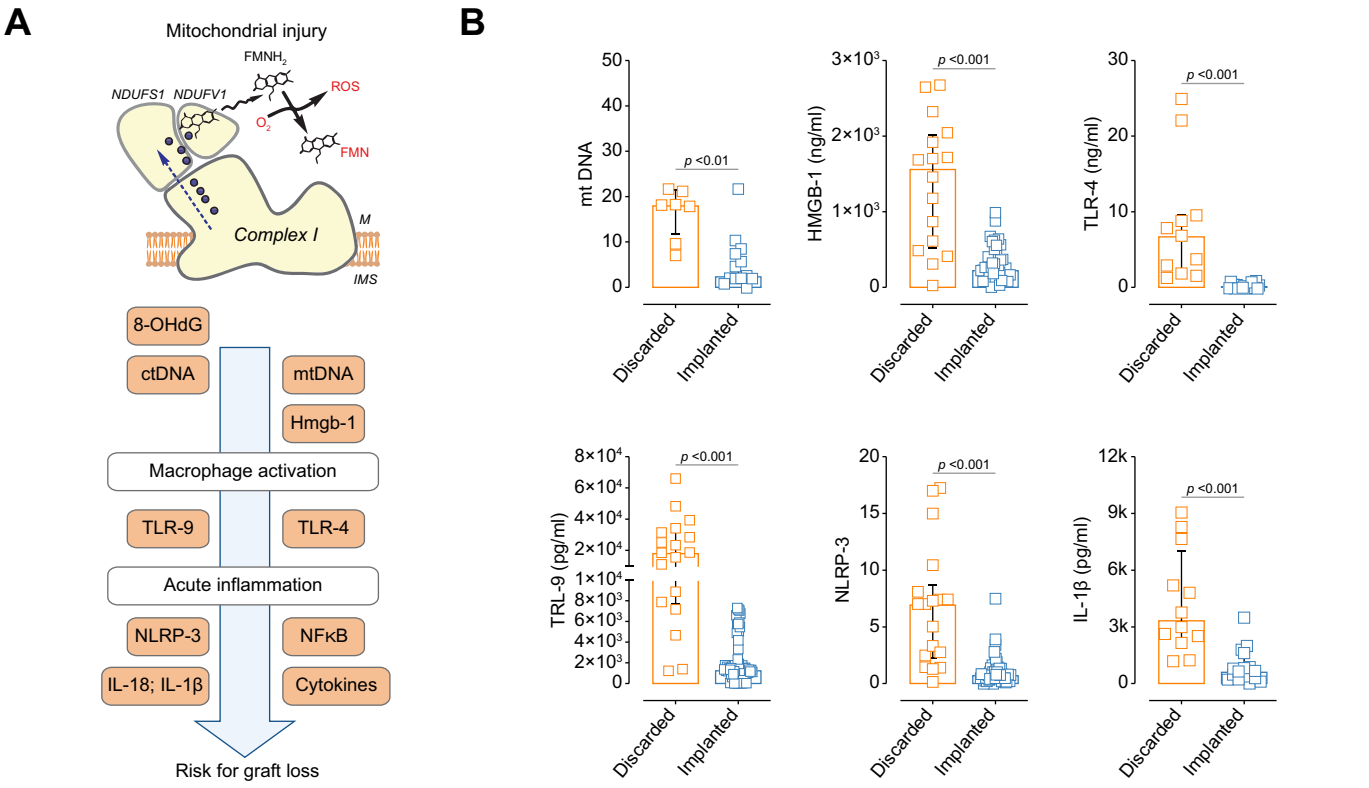

Fig. 7. Mitochondrial injury and inflammation in implanted and discarded livers (perfusate analysis). Schematic of mitochondrial-related inflammatory cascades (A). Inflammatory markers differed significantly between perfusates of discarded and implanted livers (B). *p* values are two-tailed (Mann-Whiney *U* test, levels of significance $p < 0.01$ (B).

implications, as many centers currently perform HOPE only for treatment, but not for assessment of livers. We show here, in contrast, that assessment of livers during HOPE is possible by a relatively simple and rapid fluorometric measurement of perfusate, which is even feasible by real-time monitoring, as previously reported.[21] Consistent with our analysis, several recent reports have been published on the key role of mitochondrial function for evaluation of organ quality in livers and kidneys before implantation. This includes for example high-resolution respirometry,[22] real-time confocal microscopy,[23] and circulating cell-free mitochondrial DNA in perfusates or flush solutions.[24] We propose therefore further evaluation of perfusate FMN together with additional mitochondrial biomarkers, such as oxidative phosphorylation function, microRNA, proteomics, or lipidomics.

This study has limitations. We focused on one biomarker only, due to good prediction of graft loss (c-statistic 0.8418 and 0.7733). Yet, while sensitivity and specificity values are useful and widely accepted measures of general accuracy, methods for optimal cut-off findings are debatable.[25] We relied here on the maximum Youden index for threshold determination, but discrimination against other thresholds was relatively weak. These values are therefore not absolute but should be interpreted more as a guideline threshold rather than as a sharp cut-off. In addition, while the predictive value of FMN outperforms conventional risk scores used for organ acceptance at the time of donor offer, no perfusate measurement accounts for subsequent intraoperative and recipient risk factors that may also result in NAS, kidney injury requiring RRT, and PNF. We recognize that every liver assessment to date, including ours, is a snapshot of a dynamic process. As a methodological limitation, perfusate fluorometry can result in higher values in the presence of free hemoglobin despite probe centrifugation, which we observed in some of the HOPE perfusate samples of DCD livers pretreated by NRP (Milan and Turin). Mass spectrometry analysis appears more accurate in this respect, resulting also in better ROC curves (Fig. 2). Another limitation is the lack of proof of clinical non-function in discarded livers. Non-utilized livers in Zurich were therefore either tested on normothermic *ex situ* perfusion systems for IRI, or underwent biochemical analysis for mitochondrial injury (Figs. 6 and 7), with solid confirmation of the predicted injury. Finally, all perfusates were collected and shipped from perfusion centers with dry ice to either Zurich (fluorometric and ELISA measurements) or Berlin (mass spectrometry). We regard this strategy, however, as an important step for centrally testing a new method before establishing individual center-derived perfusate assessment. In the future, real-time prospective assessment of machine perfusates for released FMN will be possible with specific sensors, providing centers calibrated fluorescence values in perfused livers. Such a trial is planned and will be registered.

We envision that FMN can be used as a biomarker to accept a liver graft when the perfusate FMN values are in the low-risk area. We suggest furthermore, that intermediate- or high-risk perfusate FMN values may be a reason to add further graft evaluation by, for example, controlled oxygenated rewarming

and NMP after HOPE/(D)HOPE, with functional assessment of biliary injury by bile analysis.[4,7] Our study also underlines that machine perfusion omitting assessment, for example of FMN release, does not ensure a good outcome.

In conclusion, this first international study validates the predictive value of the mitochondrial cofactor FMN, released from complex I during (D)HOPE. It may therefore contribute to a better risk stratification of injured livers before implantation.

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Abbreviations

ALT, alanine aminotransferase; AST, aspartate aminotransferase; DAMPs, damage-associated molecular patterns; DBD, donation after brain death; DCD, donation after circulatory death; (D)HOPE, (dual) hypothermic oxygenated perfusion; FMN, flavin mononucleotide; IRI, ischemia-reperfusion injury; NAS, non-anastomotic strictures; NMP, normothermic machine perfusion; PNF, primary non-function; RRT, renal replacement therapy.

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Conflict of interest

JVG: has had funding from and is a consultant with Organ Recovery Systems, Itasca, IL. The other authors declare no conflict of interest.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study design: AS, PD. Funding Acquisition: AS, PM, AG, DM and PD. Data collection: all co-authors. Data analysis: JE, AS, FA, AG, DM, ED, PD, AMT. Manuscript writing: JE, AMT, DW, AS, PD, VM. Structured Discussion: JE, AS, PD, VM. Manuscript revision: all authors revised and approved the manuscript.

Data availability statement

Metabolomics data have been deposited in the publicly available repository PeptideAtlas with the identifier PASS05861 and can be downloaded via http://www.peptideatlas.org/PASS/PASS05861.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2024.08.030.

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Author names in bold designate shared co-first authorship

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