OPEN

Subnormothermic Ex Vivo Porcine Kidney Perfusion Improves Energy Metabolism: Analysis Using ^{31}P Magnetic Resonance Spectroscopic Imaging

Thomas Agius, MS,[1] Julien Songeon, MS,[2] Antoine Klauser, PhD,[2,3] Florent Allagnat, PhD,[1] Grégoire Longchamp, MD,[4] Raphael Ruttimann, BS,[4] Arnaud Lyon, MD,[1] Julijana Ivaniesevic, PhD,[5] Raphael Meier, MD, PhD,[6] Sébastien Déglise, MD,[1] James F. Markmann, MD, PhD,[7] Korkut Uygun, PhD,[8] Leo Buhler, MD,[9] Christian Toso, MD, PhD,[4] Jean-Marc Corpataux, MD,[1] Francois Lazeyras, PhD,[2,3] and Alban Longchamp, MD, PhD[1]

Background. The ideal preservation temperature for donation after circulatory death kidney grafts is unknown. We investigated whether subnormothermic (22 °C) ex vivo kidney machine perfusion could improve kidney metabolism and reduce ischemia-reperfusion injury. **Methods.** To mimic donation after circulatory death procurement, kidneys from 45-kg pigs underwent 60 min of warm ischemia. Kidneys were then perfused ex vivo for 4 h with Belzer machine perfusion solution UW at 22 °C or at 4 °C before transplantation. Magnetic resonance spectroscopic imaging coupled with LCModel fitting was used to assess energy metabolites. Kidney perfusion was evaluated with dynamic-contrast enhanced MRI. Renal biopsies were collected at various time points for histopathologic analysis. **Results.** Total adenosine triphosphate content was 4 times higher during ex vivo perfusion at 22 °C than at 4 °C perfusion. At 22 °C, adenosine triphosphate levels increased during the first hours of perfusion but declined afterward. Similarly, phosphomonoesters, containing adenosine monophosphate, were increased at 22 °C and then slowly consumed over time. Compared with 4 °C, ex vivo perfusion at 22 °C improved cortical and medullary perfusion. Finally, kidney perfusion at 22 °C reduced histological lesions after transplantation (injury score: 22 °C: 10.5±3.5; 4 °C: 18±2.25 over 30). **Conclusions.** Ex vivo kidney perfusion at 22°C improved graft metabolism and protected from ischemia-reperfusion injuries upon transplantation. Future clinical studies will need to define the benefits of subnormothermic perfusion in improving kidney graft function and patient's survival.

(*Transplantation Direct* 2022;8: e1354; doi: 10.1097/TXD.0000000000001354).

INTRODUCTION

Transplantation is the preferred treatment for end-stage kidney disease, but it suffers from a severe shortage of available organs. Approximately 100 000 patients are currently waiting for a donor kidney, with only 18 000 kidney transplants performed in the United States each year.[1] This

Received 23 May 2022.

Accepted 24 May 2022.

[1]Department of Vascular Surgery, Centre Hospitalier Universitaire VauDois and University of Lausanne, Lausanne, Switzerland.

[2]Department of Radiology and Medical Informatics, University of Geneva, Geneva, Switzerland.

[3]Center for Biomedical Imaging (CIBM), Geneva, Switzerland.

[4]Visceral and Transplant Surgery, Department of Surgery, Geneva University Hospitals and Medical School, Geneva, Switzerland.

[5]Metabolomics Platform, Faculty of Biology and Medicine, University of Lausanne, Switzerland.

[6]Department of Surgery, University of Maryland School of Medicine, Baltimore, MD, USA.

[7]Department of Surgery, Transplant Center, Massachusetts General Hospital, Harvard Medical School, Boston, USA.

[8]Department of Surgery, Center for Engineering in Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, USA.

[9] Faculty of Science and Medicine, Section of Medicine, University of Fribourg, Fribourg, Switzerland.

T.A., J.S., F.L., and A.L. have contributed equally to this work.

The authors declare no conflicts of interest.

This work was supported by the Swiss National Science Foundation to J.-M.C., F.L. (SNSF 320030_182658), and A.L. (SNSF PZ00P3-185927), as well as the Mendez National Institute of Transplantation and the Leenards Foundation to AL.

A.L., A.K., J.S., T.A., L.B., J.-M.C., and F.L. participated in research design. A.L., A.K., R.M., K.U., J.F.M., and F.L. participated in the writing of the article. A.L., A.K., J.S., T.A., A.N., R.R., Ar.L., J.I., S.M., R.M., S.D., J.-M.C., and F.L. participated in the performance of the research. A.N., R.R., S.M., and F.L. contributed new reagents or analytic tools. A.L., A.K., J.S., T.A., S.M., R.M., L.B., J.-M.C., and F.L. participated in data analysis.

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantationdirect.com).

Correspondence: Alban Longchamp, MD, PhD, Department of Vascular Surgery, Centre Hospitalier Universitaire VauDois and University of Lausanne, Rue du Bugnon 46, 1011 Lausanne, Switzerland. (alban.longchamp@chuv.ch).

ISSN: 2373-8731

DOI: 10.1097/TXD.0000000000001354

scarcity led to the expansion of the donor pool beyond standard-criteria kidney donors, including extended criteria donors and donation after circulatory death (DCD).[1,2] Although organs from these donors allow a higher survival rate than dialysis, their use is complicated by an increased rate of delayed graft function (DGF)[3] and acute rejection.[4]

DCD grafts are particularly vulnerable to ischemia-reperfusion (IR) injury, an issue that is not addressed at all by current standard storage strategies, including static cold storage and nonoxygenated hypothermic machine perfusion (eg, LifePort).[5,6] With this approach, prolonged periods (>24 h) of cold ischemia are associated with tubular necrosis, DGF, and poor graft survival.[4,7,8] Although static cold storage is the most prevalent method for renal allograft preservation, hypothermic machine perfusion without oxygen was shown to reduce DGF and to improve 1- and 3-y graft survival.[9] Hypothermic storage slows but does not entirely suspend cellular metabolism, resulting in a slow but inexorable consumption of cellular energy stores.[10] In a prior preclinical study, we found that, at 4 °C, oxygen supplementation was required to maintain adenosine triphosphate (ATP) levels.[10] In the COMPARE study, oxygen supplementation reduced biopsy-proven acute rejection but did not improve kidney graft survival or glomerular filtration rate at 12 mo.[11]

Although cold anoxic storage aims to arrest cell metabolism, ex vivo perfusion at physiologic normothermic temperature (37 °C) provides a continuous flow of warmed, oxygenated perfusate containing nutritional substrates, thereby maintaining the metabolic activity of the tissue.[12] Normothermic red cell–based-perfusion of porcine kidneys at 37 °C improved early postoperative creatinine and urea clearance in DCD grafts.[13] In addition, normothermic ex vivo perfusion allows graft assessment, reconditioning, and repair.[14,15] Using a red cell–based plasma-free solution, perfusion of marginal kidneys at 37 °C reduced DGF compared with static cold storage.[16] However, perfusion of organ at 37 °C is limited by the availability and cost of a blood perfusion system, complex heating system, tight pH and glucose control, red blood cell hemolysis, and risk of infection and immunization.[17,18] In addition, failure of the perfusion machine would rapidly lead to graft loss.

Subnormothermic (22 °C) ex vivo kidney perfusion was proposed as an alternative to perfusion at 37 °C.[19] Importantly, a previous study demonstrated that, compared with perfusion at 37 °C, kidney perfusion with blood:PlasmaLyte at 22 °C reduced acute tubular necrosis and improved kidney function in a DCD porcine model.[20] In liver grafts, perfusion of a cell-free, oxygenated perfusate at 22 °C promoted mitochondrial respiration and ATP stores before transplantation.[21] Overall, these studies suggest that 22 °C might be the optimal temperature to protect against kidney (IR) injuries, whereas avoiding complex normothermic perfusion machines.

Several tools are used to predict the suitability of kidney grafts. Although MRI is a well-established clinical diagnostic tool for assessing kidney graft function,[22] ^{31}P magnetic resonance spectroscopic imaging (pMRSI) enables the detection of high-energy phosphate metabolites such as ATP.[22] In fact, we previously reported that, in porcine kidneys, warm ischemia reduced energy stores, which correlated with kidney viability.[10]

MATERIAL AND METHODS

Ex Vivo Kidney Perfusion

Kidneys were assigned to the following ex vivo perfusion groups: (i) 4 °C with passive oxygenation of the perfusate (4 °C), (ii) 4 °C with active oxygenation (PO_2 >100kPa) of the perfusate (4 °C + O_2), (iii) 22 °C ex vivo kidney perfusion with active oxygenation (PO_2 >100kPa) of the perfusate (22 °C + O_2, Figure 1A). Passive oxygenation corresponded to ambient air oxygen diffusion.

Immediately after retrieval, kidneys were flushed with Belzer machine perfusion solution (MPS) UW Machine Perfusion Solution and immediately perfused for 4 h (before autotransplantation) or 42 h (time course experiment) using a homemade MRI-compatible pulsatile perfusion machine as published.[10] Belzer MPS UW solution can be stored between 2 °C and 25 °C and has a pH of 7.4 at 22 °C. Active oxygenation was achieved using a 0.15 m^2 membrane oxygenator (Biochrom Ltd, Cambridge, United Kingdom), maintaining the PO_2 levels at 100 kPa for the whole preservation time. The PO_2 levels during passive oxygenation were set at 20 kPa. The perfusion module was kept in an isolating box that passively kept the kidney at the desired temperature. Systolic and diastolic pressure were set at 40 and 20 mmHg, respectively.

MRI Imaging

Measurements were performed on a 3 Tesla multinuclear Prisma-fit 3T whole-body MRI scanner (Siemens Healthineers, Erlangen, Germany). ^{1}H imaging was performed with the body coil using a T2-weighted sequence (turbo SE, TR 6 530 ms, TE 110 ms, 2 mm slices) for kidney localization and structural imaging. Dynamic-contrast enhanced MRI with gadolinium (Gd-MRI) was used to determine the perfusion distribution between the cortex and the medulla and as an estimate of glomerular filtration rate as previously described.[7,10] Data were collected using a dynamic 2D saturation-prepared turbo flash sequence with the scanner body coil. This sequence has an inversion time of 255 ms, a flip angle of 12°, 1.3 mm × 1.3 mm resolution, and 6 slices of 4 mm (1 mm gap), TR 500 ms, and a TE of 1.4 ms. The perfusion-descending cortical slope was determined using the angle of the linear regression between the maximum signal value and the lowest intensity point after the initial peak.[21]

^{31}P Magnetic Resonance Imaging Spectroscopy

pMRSI was performed as described previously.[7] Briefly, a single loop ^{31}P-tuned coil fixed at the bottom of the perfusion tank allows the measurement of the signal. Scanner embedded body coil was used for ^{1}H imaging and for shimming to ensure field homogeneity. pMRSI consisted of 3D spatial encoding, with a field of view 250 × 250 × 160 mm^3, matrix size 16 × 16 × 8, nominal spatial resolution 15.6 × 15.6 × 20 mm^3, TR 1.0 s, flip angle of 35°, echo delay 0.6 ms, bandwidth 4000 Hz, and 2k sampling points. Elliptical encoding with 18 weighted averages resulted in an acquisition time of 45 min. The resonance of the inorganic phosphate (Pi, 5.2 ppm), which is uniformly present in the container and the kidney, was used as a reference for quantification of the pMRSI signal. Excitation pulse bandwidth has been adjusted to the ATP frequency range (Pi resonance—500 Hz). An exponential time filter with 20 Hz frequency width and zeroth and first order phase corrections were used to process the spectra. The metabolites (ATP, phosphomonoesters [PME], Pi, phosphocreatine/PCr) were fitted

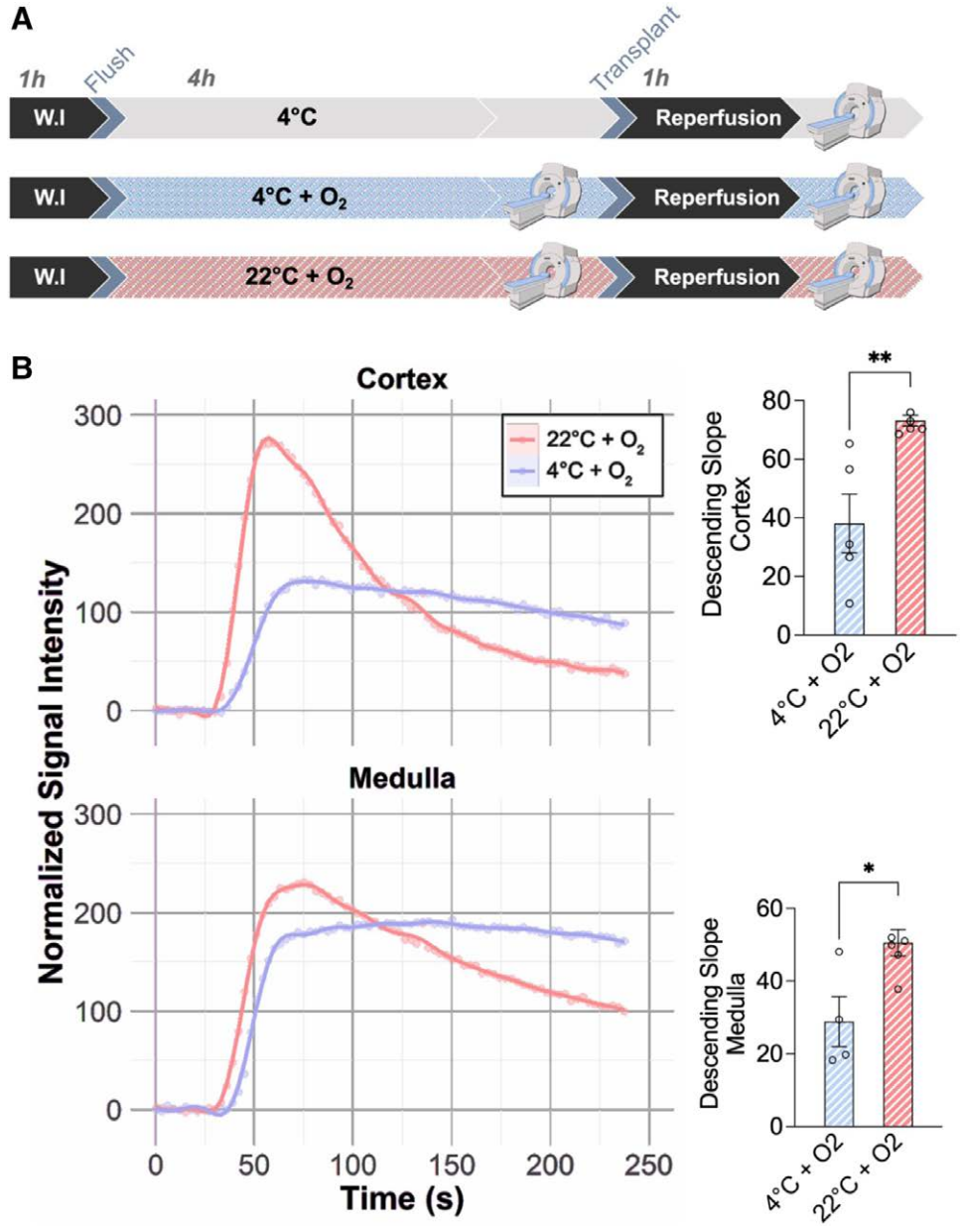

FIGURE 1. Ex vivo subnormothermic perfusion improves kidney perfusion. A, Experimental groups and design. Kidneys were retrieved after 60 min warm ischemia and placed into a hypothermic + passive oxygenation (4 °C), hypothermic + active oxygenation (4 °C + O_2), or subnormothermic + active oxygenation (22 °C + O_2) perfusion machine with or without oxygen for 4 h. Kidney were autografted into the same pig. Dynamic-contrast enhanced MRI analysis was performed prior (4 °C + O_2, 22 °C + O_2) and after transplantation (4 °C, 4 °C + O_2, 22 °C + O_2). B, Pretransplant representative Gd uptake in cortex (top) and medulla (bottom) of kidneys during hypothermic (blue line) or subnormothermic (red) perfusion and Gd perfusion-DS quantification (right, n = 5/group). Bars indicate mean ± SEM, and asterisks indicate the significance of the difference between perfusions methods by Student's *t* test or 1-way ANOVA and Tukey test. **P* < 0.05, ***P* < 0.01. n = 4 to 6 per group. DS, descending slope.

with Gaussian peaks using the syngo.via software (SIEMENS, Erlangen, Germany) and were estimated over the whole kidneys by averaging pMRSI voxels containing graft tissue, resulting in a single spectrum. Quantification results provided by 3D ^{31}P-MRSI at 3 T were analyzed with LCModel for magnetic resonance spectroscopy fitting as previously described.[23] The 3 ATP peaks were quantified separately to prevent methodological bias because of excitation profile imperfection. In each condition, pMRSI allowed the detection of α-, β-, and γ-ATP and PME composed by phosphocholine, phosphoethanolamine (PE), and adenosine monophosphate (AMP). ATP and PME concentration (mM) were quantified from the fitting and using the concentration of the inorganic-phosphate buffer (Pi, 25 mmol/L) as reference.[7] As single ATP concentration was calculated by average of the α-, β-, and γ-ATP values, ATP maps were generated using the spectroscopy software (Syngo MR Spectroscopy Evaluation, Siemens Healthineers, Erlangen, Germany). The colors represent the metabolite concentration normalized to the Pi for each voxel.

Animals and Surgery

The study was approved by the University of Geneva's animal ethics committee (protocol number: GE83/33556). Female pigs of 5 mo old were obtained from the animal facility

of Arare, Switzerland (n = 16). All pigs were maintained under standard conditions. Water and food were provided ad libitum. Animals were premedicated and anesthetized as previously described.[24] Animals were kept intubated and ventilated during the procedure. An arterial line was inserted in the internal carotid artery. Monitoring included heart rate, systemic blood pressure, pulse oximetry, and end-tidal CO_2.

Kidneys were explanted and transplanted back into the same animal (autotransplantation).[24] To mimic circulatory arrest during DCD procurement, renal arteries were crossed clamped for 60 min before collection. Kidneys were then immediately flushed and perfused as described earlier in the ex vivo kidney perfusion section above. At the end of the perfusion, both kidneys were transplanted sequentially onto the vena cava and aorta using a 6-0 running suture. After 2 h of reperfusion, pigs were sacrificed using 100 mEq of potassium chloride intravenously.

Histopathologic Analysis of Biopsies

Cortical kidney biopsies were obtained at baseline, after 60min of warm ischemia, after 4 h of ex vivo perfusion, and at 2 h after autotransplantation. Biopsies were immediately flash frozen or formalin fixed and embedded in paraffin. Fixed kidney biopsies were cut into sections of 3 µm thickness and stained with silver Jones and Periodic Acid-Schiff. Slides were scanned using a Axio Scan z1 slide scanner (Zeiss). Histopathologic analysis score was performed based on those described by Goujon et al[25,26] using Zen software (Zeiss). Whole biopsies were assessed and blinded to group assignment. The following categories were assessed: glomerular integrity, tubular dilatation, brush border integrity, cellular debris in lumina of tubules, interstitial edema, and tubular cell vacuolization. Briefly, to assess glomerulus integrity, >10 glomeruli were randomly selected from the section and assigned a score of 0 to 3. The same procedure was followed in the remaining categories. After that, the score for each category was converted to a percentage. The final score was converted to a final scale from 0 to 5 according to the percentage of damage: 0% to 15% (0), 15% to 30% (1), 30% to 45% (2), 45% to 60% (3), 60% to 75% (4), and >75% (5) using the following formula: ($Category_{Final\ Score}$/3) *100. The final score for each biopsy ranged from 0 to 30, with 30 the highest score corresponding to more severe damage. Scoring was performed blindly by 2 independent researchers.

RT-qPCR Analysis

Kidney biopsy powder was homogenized in Tripure Isolation Reagent (Roche, Switzerland). Total RNA was extracted as previously described.[27] cDNA was synthesized by random hexamer priming with the Verso cDNA kit (Prime Script RT reagent, Takara). RT-qPCR was performed with Power SYBR Green Master Mix (Ref: 4367659, Applied Biosystems, Thermo Fisher Scientific AG, Switzerland) in a ViiA 7 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific AG, Switzerland). Amplification data were analyzed using the QuantStudio 1.3 software (Thermo Fisher Scientific AG, Switzerland). Fold changes were calculated using relative standard curves methods, using ribosomal protein RPL27 genes as standards. Inflammatory gene expression was quantified 2 h after reperfusion and compared with their respective baseline. Primers' sequences are indicated in Table S1 (SDC, http://links.lww.com/TXD/A437).

Metabolite Analysis

Tissue samples were preextracted and homogenized by the addition of 150 µL of MeOH:H2O (4:1) in the Cryolys Precellys 24 sample homogenizer (2 × 20 s at 10 000 rpm, Bertin Technologies, Rockville, MD, United States) with ceramic beads. The bead beater was air-cooled down at a flow rate of 110 L/min at 6 bar. Homogenized extracts were centrifuged for 15 min at 4000 *g* at 4 °C (Hermle, Gosheim, Germany). The resulting supernatant was collected and analyzed by hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry (HILIC-MS/MS). Proteins were extracted using 20 mmol/L Tris-HCl (pH 7.5), 4M guanidine hydrochloride, 150 mmol/L NaCl, 1 mmol/L Na2EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L betaglycerophosphate, 1 mmol/L Na3VO4, and 1 µg/mL leupeptin using the Cryolys Precellys 24 sample Homogenizer (2 × 20 s at 10 000 rpm, Bertin Technologies, Rockville, MD, United States) with ceramic beads. BCA Protein Assay Kit (Thermo Scientific, Massachusetts, United States) was used to measure (A562 nm) total protein concentration (Hidex, Turku, Finland), and samples were normalized based on the tissue weight before the LC-MS/MS analysis by extracting with different volumes of MeOH:H2O (4:1, v/v). Extracted samples were analyzed by HILIC-MS/MS in both positive and negative ionization modes using a 6495 triple quadrupole system (QqQ) interfaced with a 1290 UHPLC system (Agilent Technologies). Raw LC-MS/MS data were processed using the Agilent Quantitative analysis software (version B.07.00, MassHunter Agilent technologies). Relative quantification of metabolites was based on extracted ion chromatogram areas for the monitored MRM transitions. Peak areas of detected metabolites were analyzed in "R" software, and signal intensity drift correction and noise filtering (if necessary, using CV [QC features] >30%) was done within the MRM PROBS software.

Statistical Analysis

Data are presented as mean ± SEM, and differences are considered significant when $P < 0.05$. Comparisons between groups were analyzed using ANOVA and post hoc Tukey tests or Sidak's test when indicated. Tukey's and Sidak's test were used to test for differences between 22 °C + O_2 and 4 °C perfusions. Two-group comparisons were performed using Student *t* tests (Prism 9.2, GraphPad Softwares, San Diego, CA, United States). Fitting curves of the metabolites concentration over time were computed using R (version 4.1, https://cran.r-project.org).

RESULTS

Ex Vivo Kidney Perfusion Improves Kidney Perfusion and ATP Generation

To mimic DCD, kidneys underwent 60 min of warm ischemia before procurement. Kidney grafts were then perfused in a homemade MRI-compatible pulsatile perfusion machine at 22 °C with active oxygenation (22 °C + O_2), at 4 °C with active oxygenation (4 °C + O_2), or 4 °C without additional oxygen (4 °C, Figure 1A). After 4 h at 22 °C + O_2, cortical and medullary flows were improved compared with 4 °C + O_2. This was reflected by an increase in the perfusion-descending slope (+35% cortex +26% medulla, Figure 1B).

Next, mean ATP levels were measured by averaging pMRSI voxels containing graft tissue resulting in a single spectrum (Figure 2A). In fact, we previously demonstrated that mean ATP levels (average AUC of the β- and γ-ATP peaks) are reduced by warm ischemia and correlate with (IR) injuries.[10] Using pMRSI, α-, β-, and γ-ATP and PME containing AMP were only detected during ex vivo perfusion with active oxygenation at 4 °C and 22 °C (Figure 2A). In kidneys perfused at 22 °C + O_2, ATP and PME levels were 3 times higher than at 4 °C + O_2 perfusion (5.5 mmol/L versus 2.1 mmol/L and 0.79 mmol/L versus 0.26 mmol/L, Figure 2B). This increase of AMP and ATP levels at 22 °C + O_2 was confirmed by liquid chromatography-mass spectrometry (LC-MS, +59%, +36% and +45%, respectively; Figure 2C). Surprisingly, PME and ATP concentrations tended to be higher in the medulla independently of the perfusion conditions, as demonstrated by voxel mapping of the metabolites (Figure S1, SDC, http://links.lww.com/TXD/A437).

Kidney ATP Levels Increased up to 10 h During Ex Vivo Subnormothermic Kidney Perfusion

In healthy kidneys perfused at 4 °C + O_2, α-, β-, and γ-ATP remained stable for up to 22 h of perfusions.[10] To determine the effect of 22 °C perfusion on ATP production over time in DCD grafts, PME and α-, β-, and γ-ATP concentrations were monitored for 42 h (time course experiment). Kidney α-, β-, and γ-ATP concentrations were 2 times higher after 10 h of perfusion compared with baseline (0.5 mmol/L to 1 mmol/L for β-ATP, 0.75 mmol/L to 1.5 mmol/L for γ-ATP; Figure 3A) and 4 °C + O_2.[10] The PME concentration was 4 times higher than ATP at the beginning of the perfusion (4 mmol/L of PME versus 1 mmol/L of α-, β-, and γ-ATP), and rapidly decreased to reach a plateau at 2 mmol/L. This is consistent with the hypothesis that the PME containing the AMP is consumed over time to generate ATP. Finally, after 10 h of perfusion at 22 °C + O_2, ATP levels gradually decline to ultimately reach 0 mmol/L after 42 h of perfusion (Figure 3B). PME concentration remains stable for up to 42 h (Figure 3B).

Ex Vivo Kidney Perfusion at 22 °C Reduces Kidney Ischemia and Reperfusion Injuries

To evaluate the benefit of 22 °C ex vivo perfusion before transplantation, we examined the histological damage using a modified Goujon score (described in the methods section), shown to reflect kidney function.[10,25] Kidney biopsies were analyzed at baseline, after 60 min of warm ischemia, at the end of the ex vivo perfusion, and at 1 h after transplantation. Surprisingly, no significant damage was observed after warm ischemia (Figure 4A,B). Consistent with previous findings, histological damages were significantly increased at the end of the

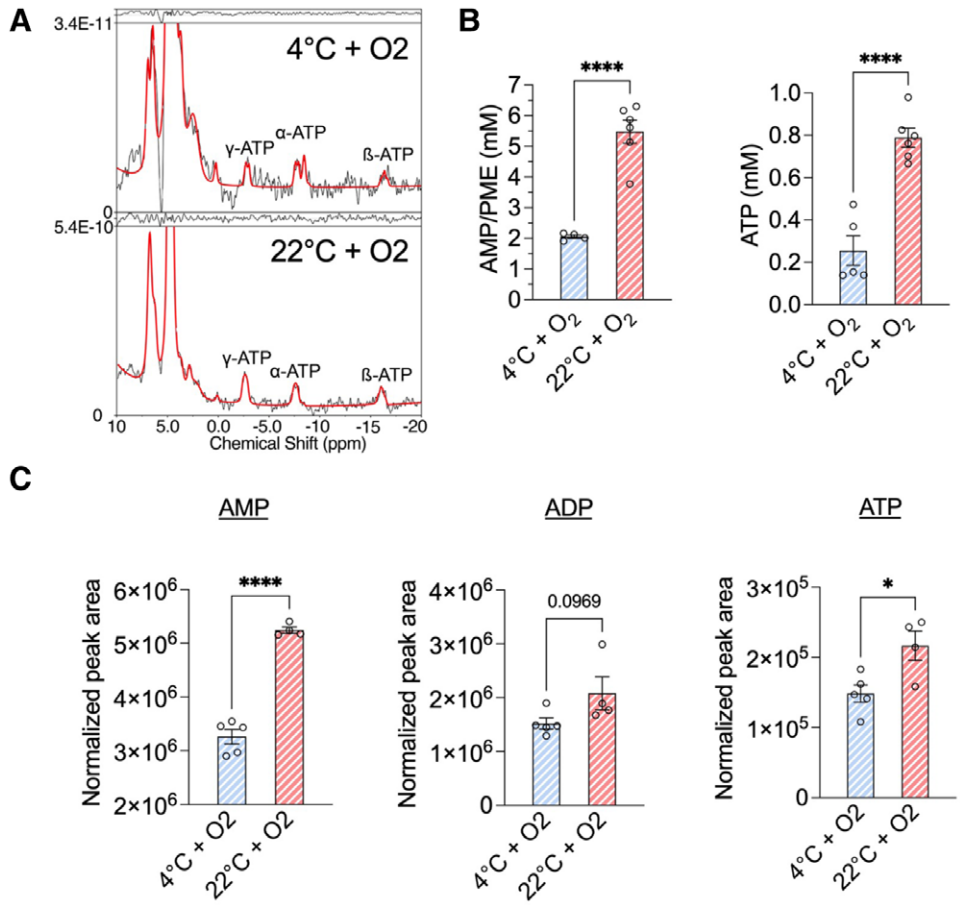

FIGURE 2. Energy metabolism is improved during subnormothermic perfusion. A and B, Representative of pMRSI spectra fitted with LCModel (A) and kidney PME and β, γ-mean ATP levels (B) during 4 °C + O_2 and 22 °C + O_2 perfusion before transplantation. C, Kidney AMP, ADP, and ATP levels measured by LC-MS. Bars indicate mean ± SEM, and asterisks indicate the significance of the difference between perfusions methods by Student's *t* test or 1-way ANOVA and Tukey test. ****$P < 0.0001$. n = 4 to 5 per group. ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; LC-MS, liquid chromatography-mass spectrometry; PME, phosphomonoesters; pMRSI, ^{31}P magnetic resonance spectroscopic imaging.

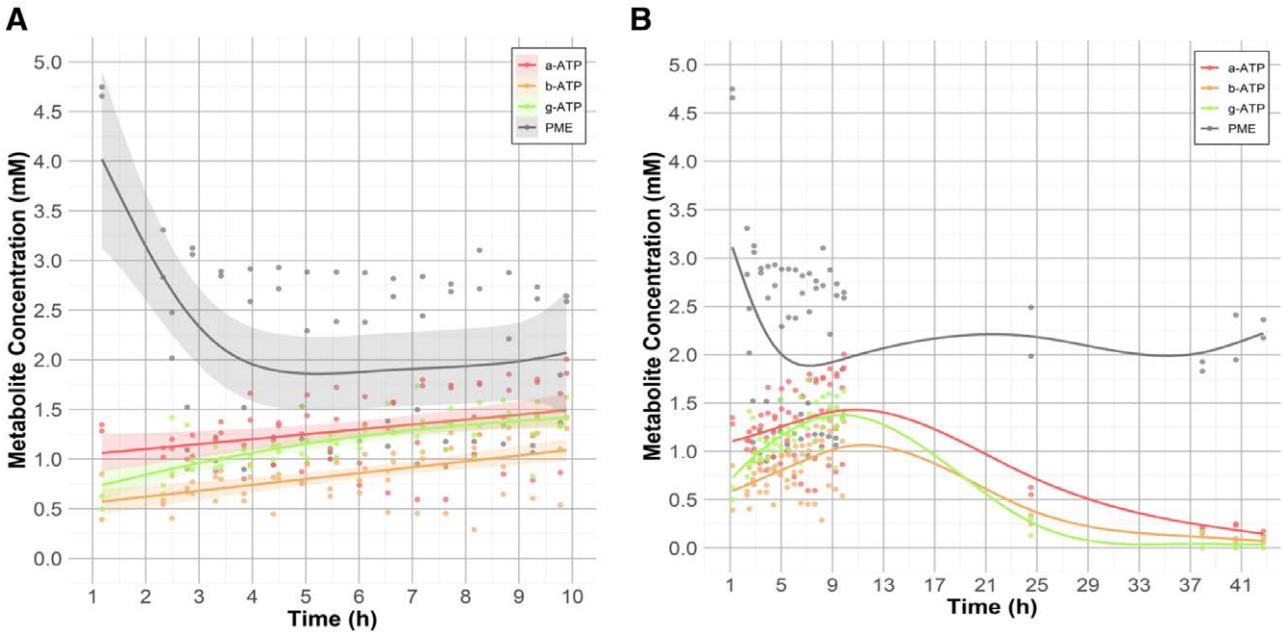

FIGURE 3. Kidney ATP levels increased up to 10 h during ex vivo subnormothermic kidney perfusion. A, Monitoring of ATP levels during 22 °C + O_2 perfusion (A) up to 10 h and (B) up to 42 h. Concentration (mM) of the indicated metabolites over time, in kidney, during 22 °C + O_2 perfusion. n = 4 per group. Fitting curves are generalized additive model (formula: y ~ s(x)) with a basis size of k = 15, n = 3. ATP, adenosine triphosphate; PME, phosphomonoesters.

ex vivo perfusion and after reperfusion in vivo (Figure 4A,B; Table S2, SDC, http://links.lww.com/TXD/A437). Importantly, 22 °C perfusion led to the greatest protection from IR injury (score of 22 °C + O_2, 4 °C + O_2, and 4 °C: 10.5 ± 2.3, 19.25 ± 3.9, and 18.3 ± 2.5, ± SD, Figure 4A,B). Perfusion of 22 °C significantly reduced tubular dilatation and luminal cell debris and protected the brush border (Figure 4A; Figure S2, SDC, http://links.lww.com/TXD/A437). In the 22 °C ex vivo perfusion group, interleukin (IL)-6 and IL-10 gene expressions were upregulated after transplantation (log2 fold-change of 22 °C + O_2, 4 °C + O_2, and 4 °C: 6.5 ± 0.8, 2.4 ± 0.9, and 3.9 ± 0.4 and 3.9 ± 0.4, 1.4 ± 0.9, 2.6 ± 0.7, respectively), whereas the expression of TNFalpha and Arg1 remained unaffected (Figure 4C). In addition, 2 h after kidney implantation, flow in the cortex and medulla improved at 22 °C + O_2 (+18% and +4% cortex, +17% and +11% medulla compared with 4 °C and 4 °C + O_2 respectively; Figure S2, SDC, http://links.lww.com/TXD/A437). Finally, ATP and, to a lesser extent, PME levels were significantly higher after transplantation in organs that were previously perfused 22 °C + O_2 (Figure 4D). Altogether, ex vivo perfusion at 22°C improved kidney metabolism and reduced (IR) injuries during transplantation.

DISCUSSION

Here, we found that kidney graft perfusion at 22 °C with an oxygenated MP-Belzer solution, without oxygen carrier, increased ATP production and minimized IR injuries during transplantation compared with perfusion at 4 °C. Of interest, active oxygenation did not increase ATP production at 4 °C. In addition, the simplicity of subnormothermic perfusion machine, without the need for a heating unit or oxygen carrier, could be easily used in a clinical setting and lower the costs. Altogether, perfusion of kidney graft at 22 °C could translate into greater utilization of kidney allograft.

Previously, the benefits of normothermic perfusion (37 °C) were linked to an increase in fatty acid metabolism and oxidative phosphorylation.[12] Similarly, kidney perfusion at 22°C improved mitochondrial ATP production, consistent with our hypothesis that, at 22 °C, kidneys are metabolically active.[22,28] Interestingly, in cold-stored organs, it has also been shown that gradual rewarming from hypothermia to normothermia before transplantation improves kidney function,[29,30] highlighting the importance of restoring metabolism before implantation. At 22 °C, we observed an increase in PME and ATP levels during the first 10 h of perfusion. After 10 h of perfusion at 22 °C, ATP level gradually declined to reach 0 mmol/L at 42 h of perfusion. We previously reported that, at 4 °C, in the absence of warm ischemia, ATP levels remained stable up to 22 h of perfusion but at significantly lower levels (0.26 mmol/L).[10] Similarly, ATP levels decrease during cold storage in the kidney[28] and liver[31] and correlate with the degree of injury. In humans, ATP predicted immediate graft function,[10,31] and ATP is often used as a marker of viability during ischemia.[27,32] Although long-term perfusion at 22 °C using MP-Belzer solution might not by viable, it is a promising strategy to recondition organs and improve initial graft function. Future studies should investigate the advantages of short-term (<10 h) reconditioning at 22 °C of previously cold-stored organs.

In this study, LC-MS/MS was used to validate the accurate quantification of nucleotides by pMRSI.[27] Indeed, the fitting of α-ATP with a broad Gaussian might include the NAD+ and NADH signal.[10,33] Recent improvements using deep learning algorithms were used to reduce concentration estimation bias of metabolites with overlapping spectra.[34] pRMSI also suffers from a relative low sensitivity compared with liquid chromatography or 1H imaging at a constant magnetic field.[10] Thus, the acquisition is generally performed with higher voxel size to achieve enough signal to noise ratio while keeping an

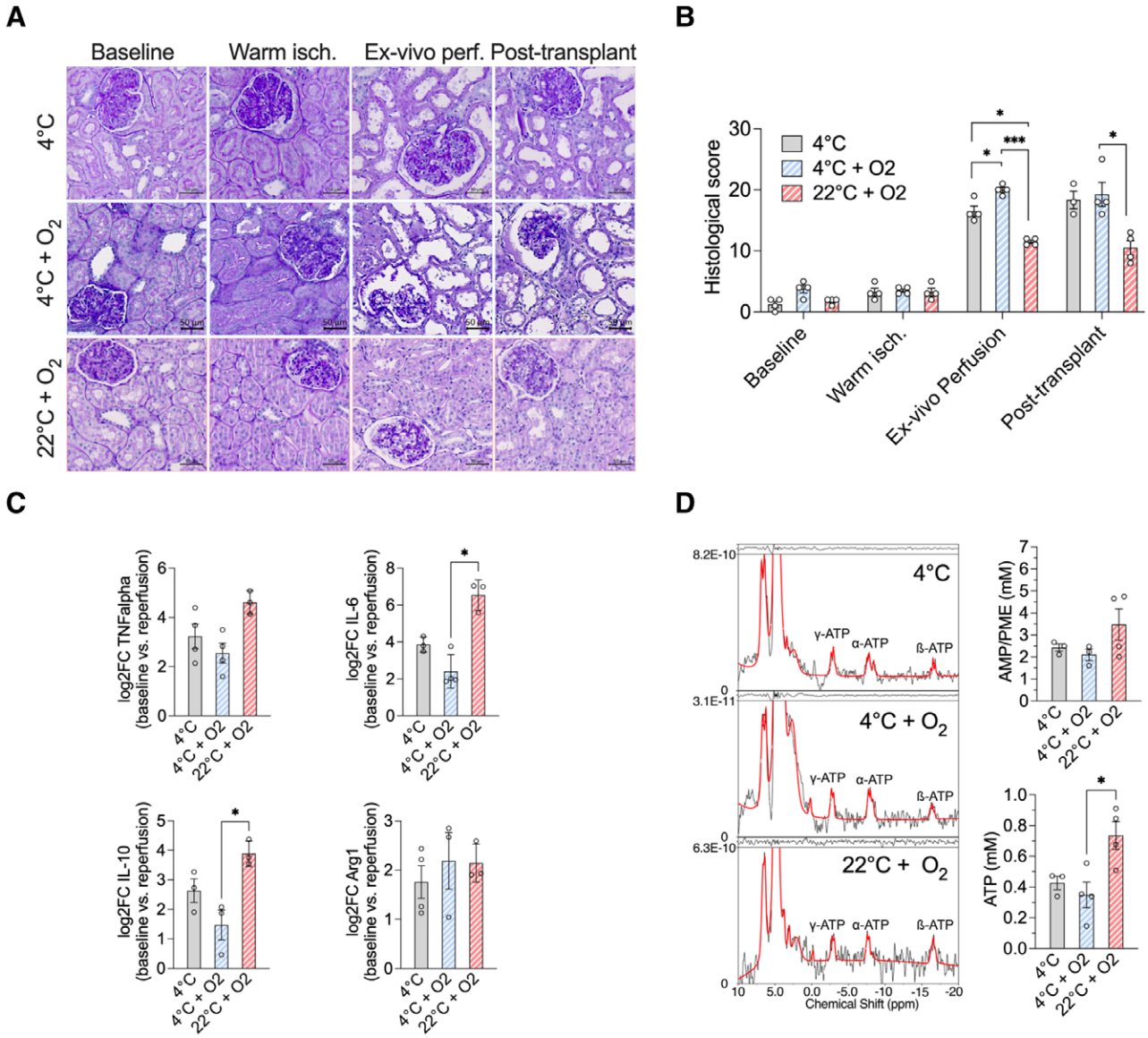

FIGURE 4. Subnormothermic perfusion reduces kidney damages. A and B, Representative cortical kidney sections (A) stained with PAS, and histological score (B) at the indicated time and conditions (4 °C, 4 °C+O_2, 22 °C+O_2). C, Expression of the indicated gene in kidney, analyzed by RT-PCR at baseline and after transplantation after 4 h of 4 °C, 4 °C+O_2, or 22 °C+O_2 perfusion. D, Representative of pMRSI spectra (left), and quantification (right) of kidney AMP and ATP levels during 4 °C, 4 °C+O_2, or 22 °C+O_2 perfusion after transplantation. The spectra are fitted using LCModel. Bars indicate mean ± SEM and median ± IQR. Asterisks indicate the significance of the difference between perfusions methods by 2-way ANOVA and Sidak post hoc test. *P < 0.05, ***P < 0.001, n = 4 per group. AMP, adenosine monophosphate; ATP, adenosine triphosphate; IQR, interquartile range; PAS, periodic acid-Schiff; PME, phosphomonoesters; pMRSI, ^{31}P magnetic resonance spectroscopic imaging.

acceptable scan time. As an example, this lack of sensitivity limitation hinders the measurement of ATP at 4 °C without oxygen. The application of machine learning[35] and neural network can further improve pMRSI sensitivity, spatial resolution, and computing time.[36] Indeed, ongoing improvement in pMRSI spatial resolution, in combination with spatial phase encoding, can provide multivoxels acquisition of the kidney graft that enables metabolite mapping over the full field of view (Figure S1, SDC, http://links.lww.com/TXD/A437[37]). Overall pMRSI remains a powerful, noninvasive tool to quantify ATP.[10]

During ex vivo perfusion at 22 °C, we did not compare passive versus active oxygenation of Belzer MPS UW, perfusion with Hb-based oxygen carrier, or packed red blood cells. Importantly, oxygenated machine perfusion at 22 °C lowers metabolic demand compared with organs perfused at 37 °C.[38] Thus, although still metabolically active, grafts maintained at 22 °C could be safely perfused with MP-Belzer without oxygen carriers. At 22 °C, Hb-based oxygen carrier achieved short-term kidney function equivalent to blood.[39] Similarly in human kidneys, compared with hemoglobin oxygen carrier, perfusion with packed red blood cells at 37 °C resulted in similar vascular flow, oxygen consumption, or ATP levels.[40] It is likely, that, although perfusion at 22 °C allows considerable recovery of energy metabolism compared with 4 °C, metabolism is significantly reduced (compared to 37 °C) so that passive oxygenation is sufficient for adequate oxygen delivery. Altogether, we hypothesize that adequate tissue oxygenation can be achieved at 22 °C without the use of packed red blood cells and complex blood perfusion machine.

Interestingly, we did not observe a benefit of active oxygenation at 4 °C, except for slightly improved graft perfusion. Consistently, the addition of oxygen to hypothermic machine perfusion did not significantly improved DCD porcine kidney function.[41] Similarly, a recent clinical trial failed to show 12-mo difference in eGFR between kidneys perfused at 4 °C with oxygen compared with hypothermic perfusion alone.[11] Previous studies comparing oxygenated perfusion used various flow rates (50–100 mL/min) and PO_2 levels (500–650 mmHg), complicating data interpretation.[11,42-44]

A potential disadvantage of normothermic preservation appears to be the generation of a proinflammatory milieu, with the accumulation of inflammatory mediators including cytokines and damage-associated molecular patterns.[45,46] Consistently, here both pro- and anti-inflammatory cytokines (IL-6 and IL-10, respectively) were increased after ex vivo perfusion at 22 °C. On the other hand, compared with 37 °C, ex vivo lung perfusion at 25 °C reduced the production of inflammatory mediators and was associated with reduced histologic graft injury after transplantation.[47] Altogether, cytokines profile and its relevance over time in kidney graft undergoing perfusion at 22 °C needs to be evaluated further.

Our study has several limitations that need to be acknowledged. First, the impact of perfusion at 22 °C and ATP levels on kidney function (serum creatinine, urea, and estimated glomerular filtration rate) or urine production after transplantation were not assessed. Consistent with previous reports, no urine output was recorded during the first hour following transplantation.[48] Interestingly, in human, urine production during normothermic ex vivo perfusion was not correlated with posttransplant kidney function.[49] Moreover, proper assessment of kidney function could not be performed because of local regulation, which did not allow survival surgery. Thus, the histological score, previously correlated with the degree of kidney injury,[10,24-26] was used as a surrogate endpoint of kidney function. Future clinical trials should help determine the benefits of perfusion at 22 °C on postoperative graft function. In addition, the benefits of subnormothermic perfusion should be tested in all forms of marginal donors, including kidneys from old donors, after acute kidney injury, and after prolonged cold preservation.

In conclusion, subnormothermic perfusion of porcine DCD kidneys improved ATP production and reduced IRI. Perfusion of DCD grafts at 22 °C should be tested in clinical trials to determine if it can improve posttransplant graft function and patient survival.

ACKNOWLEDGMENTS

We thank Jean-Pierre Giliberto from the department of surgery, University Hospitals of Geneva, as well as Hector Gallart Ayala from the Metabolomics Platform, Faculty of Biology and Medecine, University of Lausanne, Switzerland, for the excellent technical assistance.

REFERENCES

1. Assis-Borba L, Cristelli MP, Paula MI, et al. Expanding the use of expanded criteria donors in kidney transplantation. *Int Urol Nephrol*. 2014;46:1663–1671.
2. Summers DM, Watson CJ, Pettigrew GJ, et al. Kidney donation after circulatory death (DCD): state of the art. *Kidney Int*. 2015;88:241–249.
3. Tennankore KK, Kim SJ, Alwayn IP, et al. Prolonged warm ischemia time is associated with graft failure and mortality after kidney transplantation. *Kidney Int*. 2016;89:648–658.
4. Dragun D, Hoff U, Park JK, et al. Prolonged cold preservation augments vascular injury independent of renal transplant immunogenicity and function. *Kidney Int*. 2001;60:1173–1181.
5. Zhao H, Alam A, Soo AP, et al. Ischemia-reperfusion injury reduces long term renal graft survival: mechanism and beyond. *Ebiomedicine*. 2018;28:31–42.
6. Ponticelli C. Ischaemia-reperfusion injury: a major protagonist in kidney transplantation. *Nephrol Dial Transplant*. 2014;29:1134–1140.
7. Lee CM, Carter JT, Randall HB, et al. The effect of age and prolonged cold ischemia times on the national allocation of cadaveric renal allografts. *J Surg Res*. 2000;91:83–88.
8. Kayler LK, Magliocca J, Zendejas I, et al. Impact of cold ischemia time on graft survival among ECD transplant recipients: a paired kidney analysis. *Am J Transplant*. 2011;11:2647–2656.
9. Moers C, Smits JM, Maathuis MH, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med*. 2009;360:7–19.
10. Longchamp A, Klauser A, Songeon J, et al. Ex vivo analysis of kidney graft viability using 31p magnetic resonance imaging spectroscopy. *Transplantation*. 2020;104:1825–1831.
11. Jochmans I, Brat A, Davies L, et al; COMPARE Trial Collaboration and Consortium for Organ Preservation in Europe (COPE). Oxygenated versus standard cold perfusion preservation in kidney transplantation (COMPARE): a randomised, double-blind, paired, phase 3 trial. *Lancet*. 2020;396:1653–1662.
12. Urbanellis P, McEvoy CM, Škrtić M, et al. Transcriptome analysis of kidney grafts subjected to normothermic ex vivo perfusion demonstrates an enrichment of mitochondrial metabolism genes. *Transplant Direct*. 2021;7:e719.
13. Urbanellis P, Hamar M, Kaths JM, et al. Normothermic ex vivo kidney perfusion improves early DCD graft function compared with hypothermic machine perfusion and static cold storage. *Transplantation*. 2020;104:947–955.
14. Hosgood SA, Barlow AD, Hunter JP, et al. Ex vivo normothermic perfusion for quality assessment of marginal donor kidney transplants. *Br J Surg*. 2015;102:1433–1440.
15. Kaths JM, Hamar M, Echeverri J, et al. Normothermic ex vivo kidney perfusion for graft quality assessment prior to transplantation. *Am J Transplant*. 2018;18:580–589.
16. Nicholson ML, Hosgood SA. Renal transplantation after ex vivo normothermic perfusion: the first clinical study. *Am J Transplant*. 2013;13:1246–1252.
17. Parente A, Osei-Bordom DC, Ronca V, et al. Organ restoration with normothermic machine perfusion and immune reaction. *Front Immunol*. 2020;11:565616.
18. Eshmuminov D, Becker D, Bautista Borrego L, et al. An integrated perfusion machine preserves injured human livers for 1 week. *Nat Biotechnol*. 2020;38:189–198.
19. Kaths JM, Paul A, Robinson LA, et al. Ex vivo machine perfusion for renal graft preservation. *Transplant Rev (Orlando)*. 2018;32:1–9.
20. Bhattacharjee RN, Ruthirakanthan A, Sun Q, et al. Subnormothermic oxygenated perfusion optimally preserves donor kidneys ex vivo. *Kidney Int Rep*. 2019;4:1323–1333.
21. Bruinsma BG, Sridharan GV, Weeder PD, et al. Metabolic profiling during ex vivo machine perfusion of the human liver. *Sci Rep*. 2016;6:22415.
22. Lazeyras F, Buhler L, Vallee JP, et al. Detection of ATP by "in line" 31P magnetic resonance spectroscopy during oxygenated hypothermic pulsatile perfusion of pigs' kidneys. *MAGMA*. 2012;25:391–399.
23. Provencher SW. Automatic quantitation of localized in vivo 1H spectra with LCModel. *NMR Biomed*. 2001;14:260–264.
24. Longchamp A, Meier RPH, Colucci N, et al. Impact of an intra-abdominal cooling device during open kidney transplantation in pigs. *Swiss Med Wkly*. 2019;149:w20143.
25. Meier RPH, Piller V, Hagen ME, et al. Intra-abdominal cooling system limits ischemia-reperfusion injury during robot-assisted renal transplantation. *Am J Transplant*. 2018;18:53–62.
26. Goujon JM, Hauet T, Menet E, et al. Histological evaluation of proximal tubule cell injury in isolated perfused pig kidneys exposed to cold ischemia. *J Surg Res*. 1999;82:228–233.
27. Longchamp A, Mirabella T, Arduini A, et al. Amino acid restriction triggers angiogenesis via GCN2/ATF4 regulation of VEGF and H2S production. *Cell*. 2018;173:117–129.e14.

28. Buchs JB, Lazeyras F, Bühler L, et al. [The viability of kidneys tested by gadolinium-perfusion MRI during ex vivo perfusion]. *Prog Urol*. 2009;19:307–312.
29. Bruinsma BG, Yeh H, Ozer S, et al. Subnormothermic machine perfusion for ex vivo preservation and recovery of the human liver for transplantation. *Am J Transplant*. 2014;14:1400–1409.
30. Mahboub P, Ottens P, Seelen M, et al. Gradual rewarming with gradual increase in pressure during machine perfusion after cold static preservation reduces kidney ischemia reperfusion injury. *PLoS One*. 2015;10:e0143859.
31. González FX, Rimola A, Grande L, et al. Predictive factors of early postoperative graft function in human liver transplantation. *Hepatology*. 1994;20:565–573.
32. de Rougemont O, Breitenstein S, Leskosek B, et al. One hour hypothermic oxygenated perfusion (HOPE) protects nonviable liver allografts donated after cardiac death. *Ann Surg*. 2009;250:674–683.
33. Graveron-Demilly D. Quantification in magnetic resonance spectroscopy based on semi-parametric approaches. *MAGMA*. 2014;27:113–130.
34. Iqbal Z, Nguyen D, Thomas MA, et al. Deep learning can accelerate and quantify simulated localized correlated spectroscopy. *Sci Rep*. 2021;11:8727.
35. Lam F, Li Y, Guo R, et al. Ultrafast magnetic resonance spectroscopic imaging using SPICE with learned subspaces. *Magn Reson Med*. 2020;83:377–390.
36. Lee HH, Kim H. Deep learning-based target metabolite isolation and big data-driven measurement uncertainty estimation in proton magnetic resonance spectroscopy of the brain. *Magn Reson Med*. 2020;84:1689–1706.
37. Klauser A, Klauser P, Grouiller F, et al. Whole-brain high-resolution metabolite mapping with 3D compressed-sensing SENSE low-rank. *NMR Biomed*. 2022;35:e4615.
38. Raigani S, Carroll C, Griffith S, et al. Improvement of steatotic rat liver function with a defatting cocktail during ex situ normothermic machine perfusion is not directly related to liver fat content. *PLoS One*. 2020;15:e0232886.
39. Bhattacharjee RN, Patel SVB, Sun Q, et al. Renal protection against ischemia reperfusion injury: hemoglobin-based oxygen carrier-201 versus blood as an oxygen carrier in ex vivo subnormothermic machine perfusion. *Transplantation*. 2020;104:482–489.
40. Aburawi MM, Fontan FM, Karimian N, et al. Synthetic hemoglobin-based oxygen carriers are an acceptable alternative for packed red blood cells in normothermic kidney perfusion. *Am J Transplant*. 2019;19:2814–2824.
41. Venema LH, Brat A, Moers C, et al. Effects of oxygen during long-term hypothermic machine perfusion in a porcine model of kidney donation after circulatory death. *Transplantation*. 2019;103:2057–2064.
42. De Beule J, Jochmans I. Kidney perfusion as an organ quality assessment tool-are we counting our chickens before they have hatched? *J Clin Med*. 2020;9:E879.
43. Jochmans I, Akhtar MZ, Nasralla D, et al. Past, present, and future of dynamic kidney and liver preservation and resuscitation. *Am J Transplant*. 2016;16:2545–2555.
44. Hoyer DP, Gallinat A, Swoboda S, et al. Subnormothermic machine perfusion for preservation of porcine kidneys in a donation after circulatory death model. *Transpl Int*. 2014;27:1097–1106.
45. Scheuermann U, Zhu M, Song M, et al. Damage-associated molecular patterns induce inflammatory injury during machine preservation of the liver: potential targets to enhance a promising technology. *Liver Transpl*. 2019;25:610–626.
46. Sadaria MR, Smith PD, Fullerton DA, et al. Cytokine expression profile in human lungs undergoing normothermic ex-vivo lung perfusion. *Ann Thorac Surg*. 2011;92:478–484.
47. Gloria JN, Yerxa J, Kesseli SJ, et al. Subnormothermic ex vivo lung perfusion attenuates graft inflammation in a rat transplant model. *J Thorac Cardiovasc Surg*. 2021;164:e59–e70.
48. Juriasingani S, Ruthirakanthan A, Richard-Mohamed M, et al. Subnormothermic perfusion with H. *Biomolecules*. 2021;11:446.
49. Mazilescu LI, Urbanellis P, Kim SJ, et al. Normothermic ex vivo kidney perfusion for human kidney transplantation: first north American results [published online ahead of print, 2022 Mar 1]. *Transplantation*. 2022; doi:10.1097/TP.0000000000004098