Received: 24 November 2019 | Revised: 23 January 2020 | Accepted: 24 January 2020

DOI: 10.1111/ajt.15800

ORIGINAL ARTICLE

AJT

Brief O_2 uploading during continuous hypothermic machine perfusion is simple yet effective oxygenation method to improve initial kidney function in a porcine autotransplant model

Tom Darius[1,2] | Martial Vergauwen[2] | Thomas Smith[3] | Isabelle Gerin[4] | Virginie Joris[5] | Matteo Mueller[6] | Selda Aydin[7] | Xavier Muller[6] | Andrea Schlegel[8] | Jay Nath[9] | Christian Ludwig[3] | Chantal Dessy[5] | Marie-Christine Many[10] | Guido Bommer[4] | Philipp Dutkowski[6] | Pierre Gianello[2] | Michel Mourad[1,2]

[1]Surgery and Abdominal Transplant Unit, University Clinics Saint Luc, Université Catholique de Louvain, Brussels, Belgium

[2]Pole de Chirurgie Expérimentale et Transplantation, Université Catholique de Louvain, Brussels, Belgium

[3]The Institute of Metabolism and Systems Research (IMSR), University of Birmingham, Birmingham, UK

[4]Walloon Excellence in Lifesciences and Biotechnology (WELBIO), Laboratory of Physiological Chemistry, de Duve Institute, Université Catholique de Louvain, Brussels, Belgium

[5]Pole of Pharmacology and Therapeutics, Experimental and Clinical Research Institute, Université Catholique de Louvain, Brussels, Belgium

[6]Department of Surgery and Transplantation, Swiss HPB Center, University Hospital Zurich, Zurich, Switzerland

[7]Department of Pathology, University Clinics Saint Luc, Université Catholique de Louvain, Brussels, Belgium

[8]Liver Unit, Queen Elizabeth Hospital Birmingham, University Hospitals Birmingham, Birmingham, UK

[9]Department of Renal Transplantation, Southmead Hospital Bristol, Bristol, UK

[10]Department of Morphology, Experimental and Clinical Research Institute, Université Catholique de Louvain, Brussels, Belgium

Correspondence
Tom Darius
Email: tom.darius@uclouvain.be

Funding information
Astellas Pharma; Fonds De La Recherche Scientifique - FNRS; Fondation Recherche Clinique; Fondation Saint Luc; Organ Recovery Systems

With oxygenation proposed as a resuscitative measure during hypothermic models of preservation, the aim of this study was to evaluate the optimal start time of oxygenation during continuous hypothermic machine perfusion (HMP). In this porcine ischemia-reperfusion autotransplant model, the left kidney of a ±40 kg pig was exposed to 30 minutes of warm ischemia prior to 22 hours of HMP and autotransplantation. Kidneys were randomized to receive 2 hours of oxygenation during HMP either at the start (n = 6), or end of the perfusion (n = 5) and outcomes were compared to standard, nonoxygenated HMP (n = 6) and continuous oxygenated HMP (n = 8). The brief initial and continuous oxygenated HMP groups were associated with superior graft recovery compared to either standard, nonoxygenated HMP or kidneys oxygenated at the end of HMP. This correlated with significant metabolic differences in perfusate (eg, lactate, succinate, flavin

Abbreviations: ^{1}H-NMR, proton nuclear magnetic resonance; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ANOVA, analysis of variance; AST, aspartate transaminase; ATP, adenosine triphosphate; AUC, area under the curve; CAC, citric acid cycle; FET, forward electron transport; FMN, flavin mononucleotide; Fr ex Na, fractional excretion of natrium; HIF-1α, hypoxia-inducible factor 1α; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; HO-1, heme oxygenase 1; KPS-1, kidney perfusion solution-1; LC-ESI-MS, liquid chromatography electrospray ionization mass spectrometry; LDH, lactate dehydrogenase; MMRM, mixed model for repeated measures; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; NMR, nuclear magnetic resonance; Nrf2, nuclear factor erythroid 2-related factor 2; pO_2, partial pressure of oxygen; RET, reverse electron transfer; ROS, reactive oxygen species; SD, standard deviation; SOD1, superoxide dismutase 1.

mononucleotide) and tissues (eg, succinate, adenosine triphosphate, hypoxia-inducible factor-1α, nuclear factor erythroid 2-related factor 2) suggesting superior mitochondrial preservation with initial oxygenation. Brief initial O_2 uploading during HMP at procurement site might be an easy and effective preservation strategy to maintain aerobic metabolism, protect mitochondria, and achieve an improved early renal graft function compared with standard HMP or oxygen supply shortly at the end of HMP preservation.

KEYWORDS

animal models: porcine, autotransplantation, ischemia-reperfusion injury (IRI), kidney (allograft) function/dysfunction, kidney transplantation/nephrology, organ perfusion and preservation, organ procurement and allocation, translational research/science

1 | INTRODUCTION

Several preclinical studies have shown a protective effect of supplemental oxygenation (continuous or end preservation) during hypothermic machine perfusion (HMP).[1-5] However, the optimal protocol to deliver oxygen during cold perfusion remains unclear. Our group compared different concentrations of partial oxygen pressure (pO_2) in the perfusate during continuous HMP in a porcine ischemia-reperfusion autotransplant model.[6,7] Although no differences in functional outcomes were observed between the low (30%) and the high (90%) perfusate pO_2 groups, aerobic mechanisms were better supported under high oxygen concentrations,[7] confirming previous preclinical studies.[2,8,9] Such preimplant metabolic optimization could help to abrogate the harmful effects of ischemia-reperfusion injury (IRI), with particular benefit in an era of frequent high-risk donor organ utilization, with associated morbidity.[10]

Furthermore, it remains unclear if additional oxygen is required during the entire preservation period. Reducing the duration of oxygen delivery to a short period either following organ retrieval or prior to implantation could confer significant logistical benefits. This may be particularly important where airplane travel is required for organ.

We therefore evaluate here the impact of start time and duration of active oxygenation during continuous HMP on early graft function in a pig kidney ischemia-reperfusion autotransplant model. We further measure renal flow, resistance and perfusate pO_2 during machine perfusion, and assess tubular function. Biomarkers and metabolic profiles are obtained from perfusate and renal biopsies.

2 | MATERIALS AND METHODS

2.1 | Animals

Female Belgian Landrace pigs of approximately 40 kg were used. Experiments were performed according to international guidelines, after approval by the local ethical committee for animal care. The animals were housed and cared for according to Belgian laws on experimental animal welfare.

2.2 | Study design

A standardized ischemia-reperfusion porcine kidney autotransplant model was used as previously described.[6]

2.3 | Anesthetic and surgical protocol

Anesthetic and surgical protocols were followed as described previously.[6,7] The left kidney was removed after 30 minutes of warm ischemia induced by vascular clamping of the renal vessels and subsequently flushed with 200 mL of Kidney Perfusion Solution (KPS-1, Organ Recovery Systems, Diegem, Belgium). The kidney was randomized to one of the following preservation strategies: (1) 2-hour oxygenated HMP (90% oxygen) ($HMPO_2$) + 20-hour HMP with no additional oxygen (n = 6) or (2) 20-hour HMP followed by 2-hour $HMPO_2$ (n = 5). Historical control groups were 22-hour standard HMP (n = 6) with no additional oxygen in the perfusate and 22-hour continuous oxygenated HMP ($HMPO_2$) (n = 8) (90% oxygen). Following preservation, the left kidney was autotransplanted after a right nephrectomy.[6]

The surviving animals were euthanized on day 13 after transplantation by an intravenous injection of T61® (Intervet International, MSD Animal Health, Madison, NJ) under general anesthesia.

2.4 | Machine perfusion

The LifePort Kidney Transporter® (Organ Recovery Systems) was used for all machine perfusion groups. In the active oxygenated study groups, the circuit was modified to include an oxygenator (Dideco Kids D100 neonatal oxygenator, Sorin Group, Saluggia, Italy) and

heat exchanger as described previously.[6,7] The KPS-1 solution was pumped through the kidney at a pressure of 30 mm Hg. Carbogen (95% O_2/5% CO_2) and not 100% oxygen was used for oxygen supplementation at 90% in the experimental groups to have identical conditions as compared with the historical oxygenated group. The temperature of the kidney and the circulating perfusate was maintained at 2-8°C.

2.5 | Outcome measures

The primary outcome measure was early graft function (measured as daily serum creatinine). The secondary outcome measures were tubular function (measured by fractional excretion of sodium [Fr ex Na] and protein creatinine ratio in the urine), serum biomarkers (lactate dehydrogenase [LDH] and aspartate transaminase [AST]), renal flow, resistance and pO_2 during machine perfusion, metabolic perfusate and tissue profile and graft histology of the corresponding preimplantation kidney graft biopsy.

2.6 | Samples and analyses

2.6.1 | Renal flow and resistance measurement during HMP and perfusate analyses

The machine perfusion device continuously registers renal flow and resistance. Perfusate samples were taken at start, 15, 30, 60, 120 minutes, and at the end of machine perfusion. Perfusate pO_2 was continuously measured during perfusion by a micro fiber oxygen transmitter (Oxy-4 micro, Precision Sensing GmbH, Regensburg, Germany). Glucose, AST, and LDH analyses were performed by a dry chemistry analyzer Fui Dri-Chem NX500 (Fujifilm Corporation, Tokyo, Japan). For metabolite identification and quantification using nuclear magnetic resonance (NMR) spectroscopy, neat perfusate samples were mixed with a concentrated NMR buffer, granting end concentrations of 0.5 mmol/L DSS, 0.1 mol/L phosphate buffer, 25% D_20 and 2 mmol/L imidazole for each sample. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were acquired on a 600-MHz Bruker Avance III NMR spectrometer in 5 mm NMR tubes. The spectra were phase and baseline corrected with Metabolab software.[11] Metabolite identification and quantification was performed using Chenomx NMR Suite (Chenomx, Edmonton, Alberta, Canada). Metabolic profiles were assessed at the perfusion start, after 2 hours and at the end. The following metabolites were quantified: acetate, adenine, alanine, aspartate, formate, gluconate, glutamate, glutathione, glycine, hypoxanthine, isoleucine, lactate, leucine, mannitol, myo-inositol, and succinate. Perfusate samples were also analyzed with the spectrometer (fluorescence filter at 525 nm) to detect the amount of flavin mononucleotide (FMN) release at the beginning of perfusion, after 15, 30, 60, 120 minutes, and at the end of HMP.

2.6.2 | Blood and urine analysis

Venous blood samples were taken daily during follow-up. Urine samples were collected for biochemical analysis. Blood and urine analyses were determined using routine clinical methods.

2.6.3 | Tissue sampling and analysis

Kidney biopsies were taken at five different time points. Wedge biopsies were taken before the onset of preservation after ex vivo flush with KPS-1, after 22 hours of preservation, and before euthanasia. Needle biopsies of the kidney graft were taken at 5 and 45 minutes after in vivo reperfusion. Biopsies were stored both, fresh at −80°C pending metabolic analysis and also fixed in acidified formal alcohol, dehydrated, and embedded in paraffin wax for histology. Protein expression levels were determined by western blot analysis using start and end preservation and reperfusion samples of frozen kidney biopsies as previously described.[12] Levels of oxidative enzymes such as heme oxygenase 1 (HO-1) (Abcam ab13243), antioxidant enzymes such as superoxide dismutase 1 (SOD1) (Abcam ab13498), and Nuclear factor erythroid 2-related factor 2 (Nrf2) (ABcam ab92946) and reactive oxygen species (ROS) sensitive protein such as hypoxia-inducible factor 1α (HIF-1α) (BD transduction, BD 610959), have been detected and were normalized to the levels of β-actin. Metabolites (lactate, succinate, glutamate, adenosine monophosphate [AMP], adenosine diphosphate [ADP], adenosine triphosphate [ATP] and oxidized and reduced nicotinamide adenine dinucleotide [NAD^+ and NADH, respectively]) were extracted from frozen kidney biopsies by methanol-chloroform extraction and analyzed by liquid chromatography coupled to electrospray ionization mass spectrometry (LC-ESI-MS) using a method based on Coulier et al.[13] Metabolite quantification was performed as described by Veiga-da-Cunha et al.[14]

Sections of 4 μm were cut and stained with hematoxylin and eosin for evaluation using light microscopy. Sections were scored blindly assessing changes according to the histopathological consensus criteria for preimplantation kidney biopsies[15] and the acute tubular injury score described by Hosgood et al[16] to assess changes in four morphological categories: tubular dilatation, tubular debris, vacuolation, and infiltration.

2.7 | Statistical methods

The study follows a parallel design with four active groups. No imputation was done, so all analyses were based on the observed cases only.

The primary outcome measure of early graft function was assessed through analysis of serum creatinine over time using generalized linear model techniques. Serum creatinine values were normalized to 40 kg animal body weight prior to use in the analysis to correct for differences in animal and organ size. Serum creatinine was analyzed using mixed model for repeated measurements

(MMRM) with factors treatment, time-point, and interaction term to test for overall difference in graft function over time between groups. Individual comparison of groups were conducted using contrast analyses within the main MMRM model testing the null hypothesis that the least squares mean difference between two groups was zero (no mean difference). For the contrast analysis, *P* values (based on *t* test of least squares means) were reported along with a mean (SD) graph where appropriate. All secondary outcome variables were similarly modeled to the primary outcome measure. In case of variables without repeated measurement (eg, area under the curve [AUC]) the MMRM model was reduced to an analysis of variance (ANOVA) model with factor treatment. As this was a preclinical exploratory trial, no formal sample size calculations were performed and no correction for multiplicity was foreseen. All analyses were performed using SAS 9.4 (SAS Institute, Cary, NC) and Prism 8.2.0 (GraphPad Software, LaJolla, CA).

3 | RESULTS

3.1 | Operative data, major complications, and animal survival

Twenty-nine autotransplants were performed and four were excluded from the analysis due to early death or graft-interfering complications (sudden death at day 2 [n = 1], hydronephrosis of the right kidney diagnosed during the autotransplant procedure because of mechanical obstruction caused by the balloon of the bladder catheter [n = 1], respiratory arrest 30 minutes after extubation on day 0 [n = 1], necrotizing anal prolapse at day 5 necessitating euthanasia [n = 1]). Operative data of the remaining 25 autotransplantations are presented in Table 1. The number of pigs in the nonhistorical study groups was reduced to 11 during the project, based on an intermediate statistical analysis, instead of the foreseen 16 to limit animal suffering.

3.2 | Graft function

The AUC of the serum creatinine within 13 days after autotransplantation is shown in Figure 1A. Comparison of the groups based on the MMRM demonstrated that all oxygenated HMP groups, independent of the start time of oxygenation were superior to standard HMP without additional oxygen. Two hours of O_2 administration at the start of HMP was superior to end-ischemic O_2 supply during HMP ($P = .04$) and equivalent to the continuous oxygenation group (22-hour $HMPO_2$) ($P = .36$).

The daily evolution of serum creatinine after transplantation is shown in Figure 1B. No statistically significant difference was observed between the 22-hour $HMPO_2$ and the initial O_2 uploading group. However, both, the oxygen uploading and continuous oxygenation groups demonstrated significantly lower serum creatinine ($P < .05$), compared with the standard HMP group during the first 4 days after transplantation. This early functional benefit was not seen for the end-preservation O_2 approach.

TABLE 1 Operative data of 25 pigs in an ischemia-reperfusion autotransplant model according to the study group

Variable	2-h $HMPO_2$ + 20-h HMP (n = 6)	20-h HMP + 2-h $HMPO_2$ (n = 5)	22-h HMP (n = 6)	22-h $HMPO_2$ (n = 8)	*P* value
Weight pig, kg	44.28 ± 2.57	43.60 ± 1.96	39.23 ± 2.00	43.83 ± 7.30	.1906
Left nephrectomy procedure time, h:m	3 h 35 ± 0 h 21	3 h 35 ± 0 h 16	3 h 16 ± 0 h 31	3 h 31 ± 0 h 26	.5214
Weight left kidney, mg					
Before preservation	108 ± 8	113 ± 11	100 ± 13	109 ± 18	.4606
After preservation	149 ± 11	162 ± 27	151 ± 22	149 ± 30	
Perfusate pO_2, mm Hg					
(a) Inline measured at 4°C					
At start HMP	495 ± 111	148 ± 3	106 ± 3	NA	.0002
At end HMP	134 ± 3	495 ± 65	8 ± 1	NA	<.0001
(b) Perfusate samples analyzed and recalculated at 37°C					
At start HMP	617 ± 118	208 ± 21	165 ± 9	697 ± 84	<.0001
At end HMP	219 ± 13	789 ± 14	68 ± 3	717 ± 56	<.0001
Right nephrectomy and autotransplant procedure time, h:m	3 h 30 ± 0 h 18	3 h 00 ± 0 h 04	3 h 52 ± 0 h 54	2 h 54 ± 0 h 45	.0135
Preservation time, h:m	22 h 14 ± 0 h 17	22 h 04 ± 0 h 20	22 h 40 ± 0 h 36	20 h 51 ± 0 h 57	.1777
Anastomotic time, min	24 ± 2	24 ± 2	32 ± 4	26 ± 3	.0017

Note: Data are given as the mean ± SD.

Abbreviations: HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion.

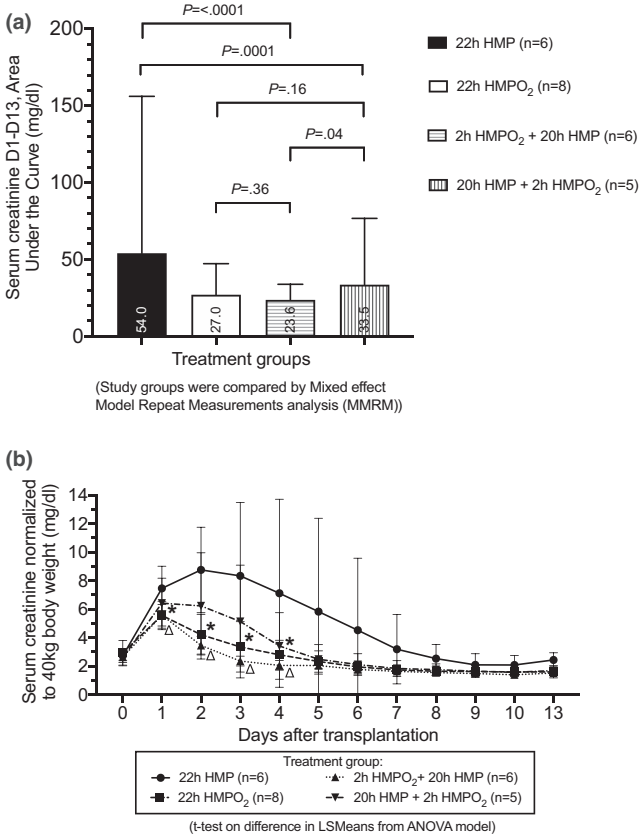

FIGURE 1 A, Area under the curve analysis of serum creatinine from day 1 until 13 after transplantation according to the study group demonstrating that oxygenated HMP, independent of the start time of oxygenation is superior to standard HMP. B, Evolution of daily postoperative serum creatinine in all study groups demonstrating superiority of the 22-h $HMPO_2$ and 2-h $HMPO_2$ + 20-h HMP group compared with standard HMP during the first 4 d after transplantation. Significance of $P < .05$ between 22-h HMP and 22-h $HMPO_2$ group is indicated with *. Significance of $P < .05$ between 22-h HMP and 2-h $HMPO_2$ + 20-h HMP group is indicated with $^{\Delta}$. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion

Tubular reabsorption of sodium was transiently disturbed in all study groups after transplantation but demonstrated a faster decrease to baseline values especially during the first three postoperative days in all oxygenated machine perfusion strategies compared with standard HMP (Figure 2A). The AUC of the fractional excretion of sodium demonstrated no significant differences between the study groups (Figure 2B). Similar findings were observed for the urinary protein creatinine ratio (Figure 2C,D).

3.3 | Evolution of postoperative serum AST and LDH

In the study groups, the peak of serum AST was observed at day 1 after kidney transplantation and dropped immediately afterwards showing no differences between the study groups. The peak of serum LDH in the study groups was also observed at day 1 after transplantation and dropped immediately afterwards into a normal range 6 days after transplantation, except for the initial O_2 uploading group where serum LDH was significantly higher during follow-up compared with all other study groups (Figure S1).

3.4 | Impact of perfusate pO_2 levels on renal flow and resistance during machine perfusion

Continuous pO_2 measurements during HMP are shown in Figure 3. Active oxygenation independent of the start time during HMP results in a significantly higher renal flow during machine perfusion compared with standard HMP without additional oxygenation (Figure 4A,B). Accordingly, the inverse findings were observed for renal resistance (Figure 4C,D).

3.5 | Perfusate glucose, lactate dehydrogenase, and aspartate transaminase

No differences in perfusate glucose and LDH were detected at the end of machine perfusion between the study groups. Perfusate AST at the end of HMP was significantly higher in the 2-hour $HMPO_2$ + 20-hour HMP group compared with the 22-hour HMP ($P = .003$) and 22-hour $HMPO_2$ group ($P = .01$) (Figure S2).

3.6 | Quantification of metabolite concentrations in perfusate

^{1}H-NMR analysis was used to determine the concentrations of metabolites in the circulating perfusate at the perfusion start, at 2 hours, and at the end in both experimental groups and only at the end of machine perfusion in both historical control groups (Tables S1-S3). All oxygenation strategies resulted in significantly lower concentrations of circulating metabolites lactate and succinate at the end of machine perfusion, compared with nonoxygenated HMP. These effects were most pronounced for the continuous and initial oxygenation groups with lower concentrations of lactate ($P = .01$) and succinate ($P = .03$) compared to oxygenation at the end of perfusion (Figure 5A,B).

FMN release during machine perfusion detected by fluorescence was significantly lower during the first 2 hours of machine perfusion in the initial O_2 uploading HMP group compared with the standard HMP group and end preservation-oxygenated HMP group (Figure 6).

3.7 | Oxidative stress and metabolic evaluation on preservation and reperfusion biopsies

Western blot analysis was used to determine protein expression of HO-1, SOD1, Nrf2, and HIF-1α in both experimental groups

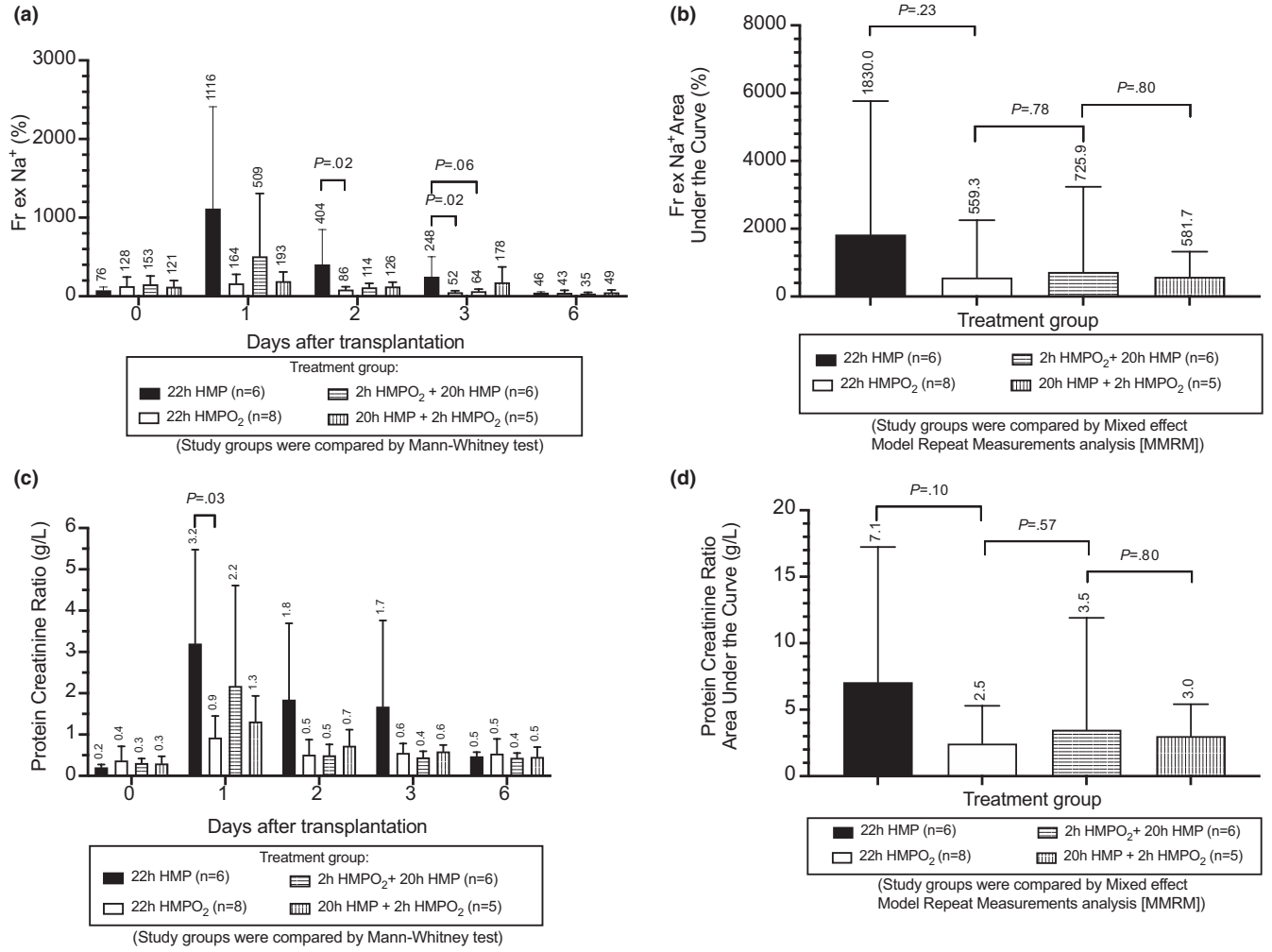

FIGURE 2 The daily evolution and the area under the curve analysis of the urinary fractional excretion of sodium and protein creatinine ratio according to the study groups (A, B, C, and D, respectively). Fr ex Na^+, fractional excretion of natrium; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion

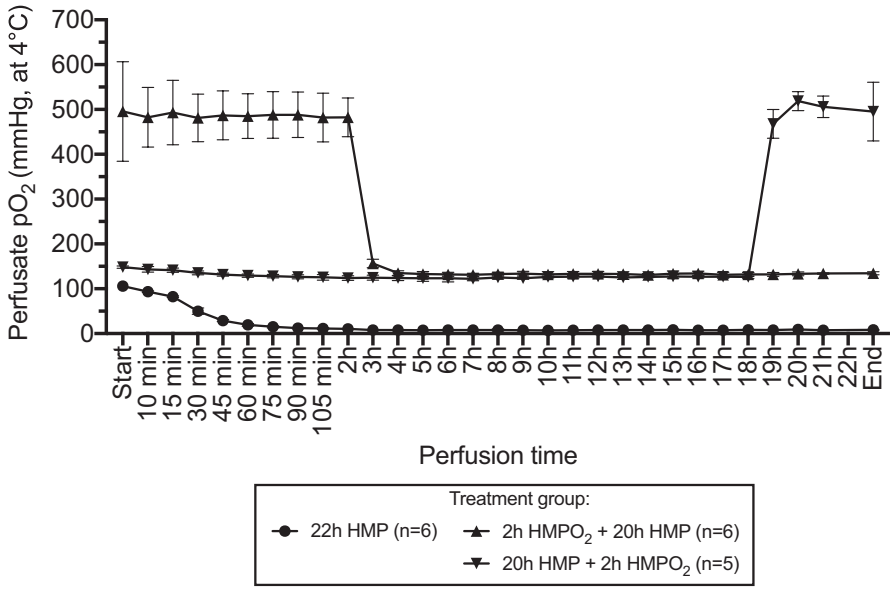

FIGURE 3 The evolution of perfusate oxygen concentration during machine perfusion measured continuously at 4°C by a microfiber oxygen transmitter added in series to the machine perfusion device and the external membrane oxygenator. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; mmHg, millimeter of mercury

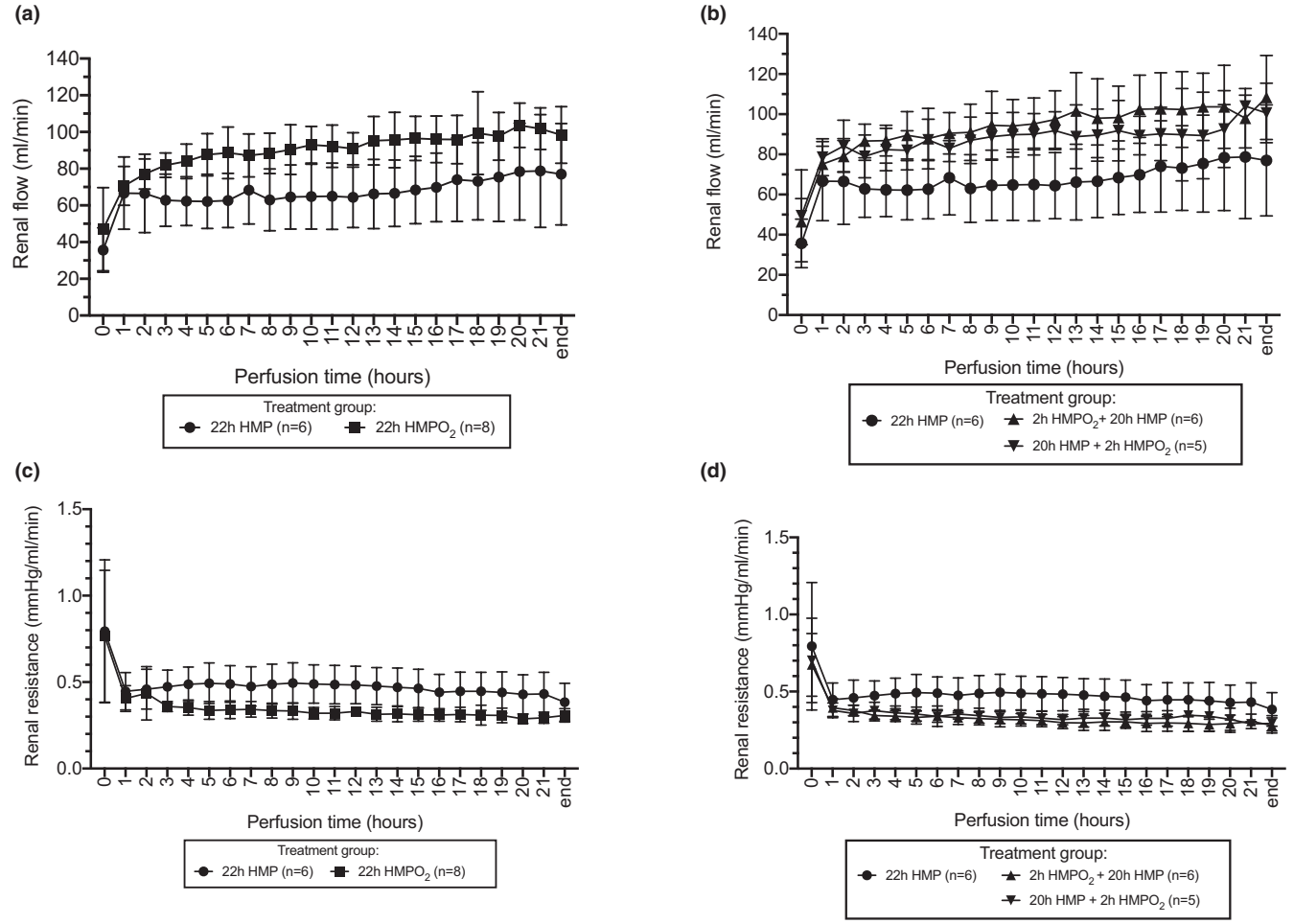

FIGURE 4 Influence of oxygen supply on ex vivo renal flow and renal resistance during continuous oxygenated HMP (A and C, respectively) and initial or end-ischemic O_2 uploading during HMP (B and D, respectively) compared with standard HMP. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion compared with standard HMP

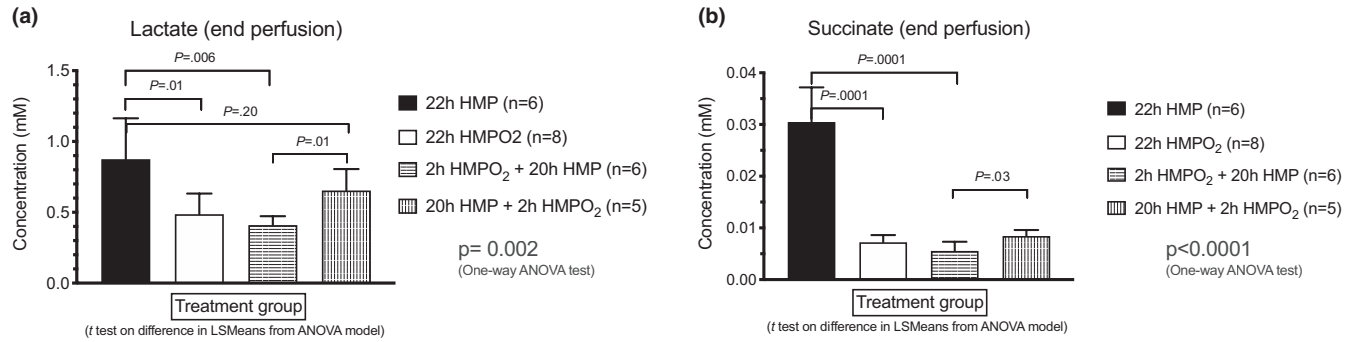

FIGURE 5 Concentrations of lactate and succinate in the circulating perfusion fluid measured by proton nuclear magnetic resonance, demonstrating the influence of oxygen independent of start time in all oxygenated machine perfusion groups compared with standard HMP. HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion compared with standard HMP

compared with the standard HMP group (Figure 7). We did not observe any difference in SOD1 and HO-1 expression comparing the study groups at the end of cold preservation and after 5 minutes of reperfusion. The SOD1 expression in the O_2 uploading group was significantly higher after 45 minutes of reperfusion compared with both other study groups. The Nrf2 expression was significantly lower after preservation in the O_2 uploading HMP group compared with the standard HMP group (P = .001). The Nrf2 expression after 5 minutes of reperfusion in situ after implantation was significantly lower in the initial O_2 uploading and the end-ischemic O_2 uploading group compared with the standard HMP group (P = .004 and .002, respectively). No difference of HIF-1α expression was

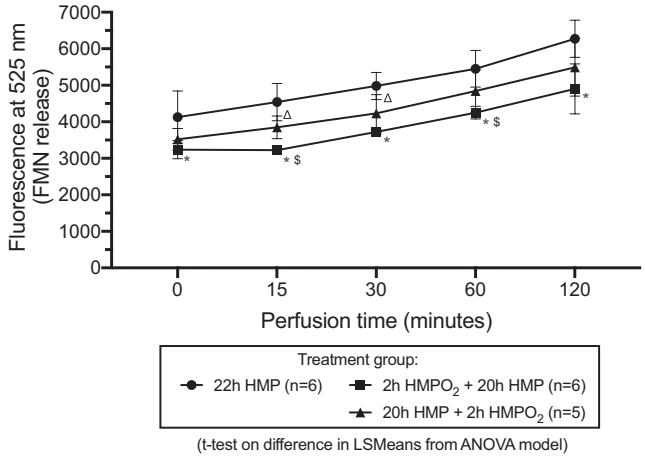

FIGURE 6 Flavin mononucleotide detection in the perfusate by fluorescence at 525 nm demonstrating significantly lower values during the first 2 h of machine perfusion inversely correlated with the perfusate oxygen concentration according to the study group. FMN, flavin mononucleotide; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion. Significance of $P < .05$ between 22-h HMP and 2-h $HMPO_2$ + 20-h HMP group is indicated with *. Significance of $P < .05$ between 22-h HMP and 20-h HMP + 2-h $HMPO_2$ group is indicated with $^{\Delta}$. Significance of $P < .05$ between the 2-h $HMPO_2$ + 20-h HMP and 20-h HMP + 2-h $HMPO_2$ is indicated with $^{\$}$

observed between study groups at the end of preservation and after reperfusion.

No differences in lactate level were observed in biopsies obtained during preservation and after reperfusion comparing both experimental groups and the standard HMP group (Figure 8A) by LC-ESI-MS analysis. Succinate and glutamate levels measured at the end of the cold preservation were significantly lower in initial and end preservation-oxygenated groups compared with the standard 22-hour HMP group with no additional oxygen ($P = .04$ and $P = .04$ and $P = .02$ and $P = .02$, respectively for succinate and glutamate) (Figure 8B,C). The ATP levels, detected at the end of cold perfusion were significantly higher in kidneys receiving the oxygen initially compared with the standard 22-hour HMP ($P = .02$). No differences were observed between the standard HMP and the 20-hour HMP + 2-hour $HMPO_2$ group ($P = .18$) (Figure 8D). Brief initial or end-preservation O_2 uploading during HMP resulted in an 8- and 2-fold ATP level increase at the end of cold preservation compared with standard HMP, respectively. No significant differences were observed between both study groups and the standard HMP group regarding ADP, AMP, and NADH levels at the end of cold perfusion (Figure 8E-H). At 5 and 45 minutes after in situ reperfusion, NAD^+ was significantly higher in kidneys treated with oxygen initially when compared to standard 22-hour HMP group ($P = .001$ and $P < .0001$, respectively) (Figure 8H).

3.8 | Histology: light microscopy

Kidney biopsies were assessed at the beginning and the end of cold perfusion and also 13 days after implantation according to the Banff criteria[15] and the acute tubular injury score of SA Hosgood.[16] No differences were observed between all study groups (data not shown) at each biopsy time point.

4 | DISCUSSION

This ischemia-reperfusion porcine kidney autotransplant model is the first animal study to evaluate the impact of the start time of active oxygenation during continuous HMP on early graft recovery. The aim was to determine the optimal conditions for hypothermic kidney preservation to provide first an effective oxygenation and secondly an easy approach to not complicate logistics during organ donation and transport. Two-hour perfusate oxygenation, right at the start of HMP led to significantly improved early renal function when compared with 22-hour standard HMP without additional oxygen supply. Interestingly, perfusate oxygenation at the start of HMP achieved comparable outcomes as seen with continuous oxygenation during HMP. The established metabolic profile obtained in perfusate (ie, lactate, succinate, and FMN) and kidney tissues (ie, succinate, glutamate, ATP, Nrf2, and HIF-1α) suggests that a brief O_2 uploading at the start of HMP could be an easy and more beneficial preservation strategy to protect mitochondria and improve early graft function as compared with standard HMP without additional oxygenation and O_2 supply only at the end of the HMP period.

There is considerable evidence that mitochondrial succinate accumulation originating from the citric acid cycle (CAC) during ischemia is one main contributor for superoxide generation by reverse electron transport (RET) during subsequent in vivo reperfusion under normothermic conditions.[17-19] Dutkowski et al[20] described this mechanism assessed during different human liver perfusion strategies. In addition to the functional benefits of cold oxygenation, our study provides corroborative metabolic evidence in both perfusate (ie, lactate, succinate, and FMN) and tissue analysis (ie, succinate, glutamate, and ATP levels) that similar pathological processes occur during kidney perfusion that can be improved via oxygenation. The curious observation that massive changes in end preservation ATP levels between the different HMP groups did not lead to reciprocal changes in AMP levels might warrant further investigation.

The effects of oxygen during different cold perfusion strategies on mitochondria are illustrated in Figure 9. The main source of in vivo cellular ATP is mitochondrial oxidative phosphorylation, which is dependent on oxygen as the final electron acceptor (Figure 9A).[19,21-25] Complex I composed of 45 subunits contains one molecule of tightly, but non-covalently bound FMN and eight different iron-sulfur clusters.[24] Flavin serves as the primary electron acceptor from NADH, and a series of iron-sulfur clusters provide a pathway for the forward electron transfer to the bulk ubiquinone (Q).[24] During hypoxia, forward electron transport (FET) is interrupted with a number of pathological sequelae due to RET (accumulation of NADH, succinate and purine metabolites, FMN release [$FMNH_2$], and ATP/ADP decrease) (Figure 9B).[19,20,24-26] Cold machine preservation solutions were originally developed

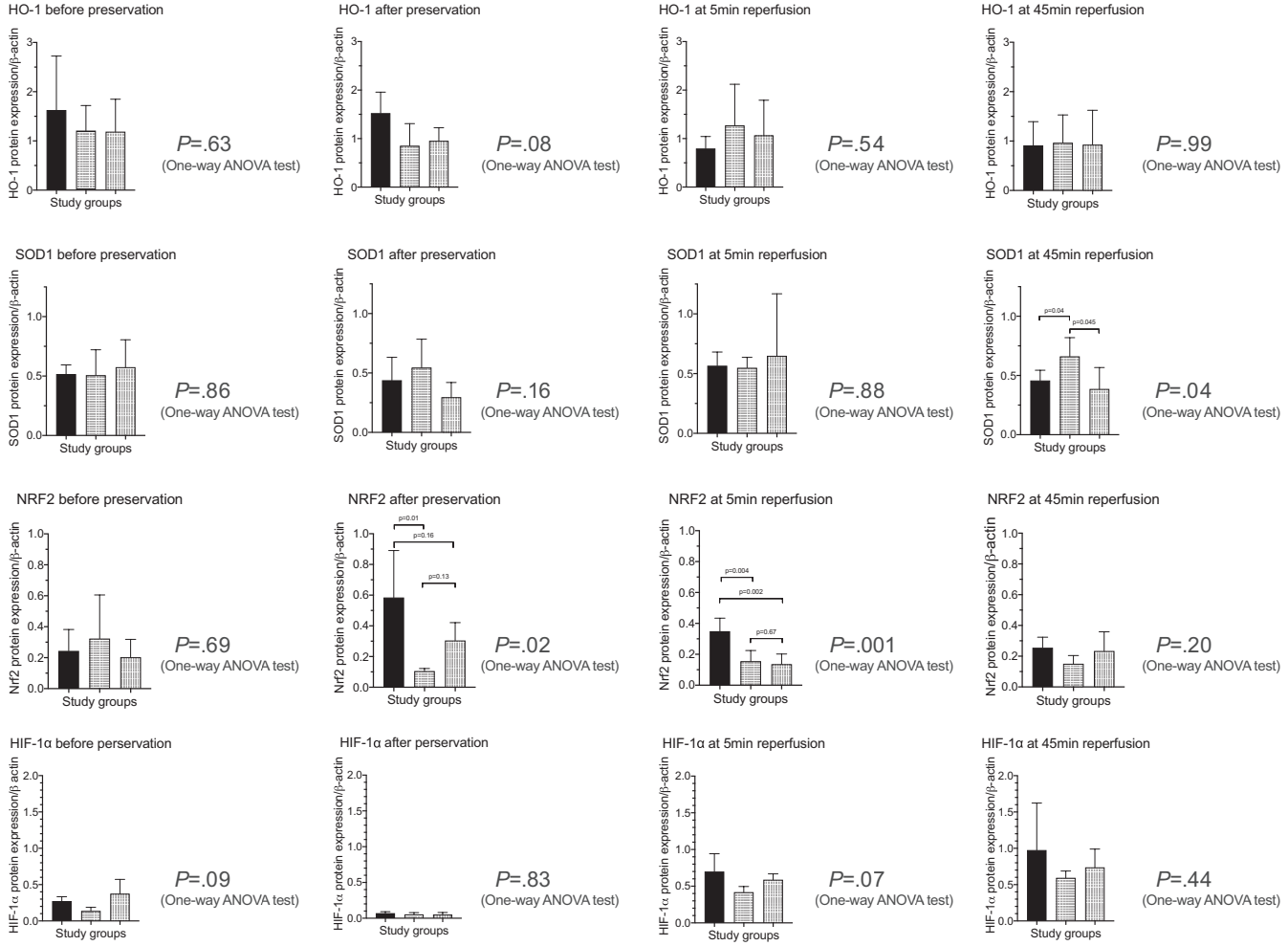

FIGURE 7 Oxidative stress-related protein expression analysis. Oxygenation during HMP reduces Nrf2 expression at the end of preservation and during early reperfusion compared to standard HMP. HIF-1α, hypoxia-inducible factor 1α; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion; HO-1, heme oxygenase 1; Nrf2, nuclear factor erythroid 2-related factor 2; SOD1, superoxide dismutase 1

for nonoxygenated HMP and do not contain an oxygen carrier. Therefore, tissue oxygenation is provided by diffusion. In this study we observed that the diffusion of oxygen into perfusion fluid early during the HMP promotes physiological mitochondrial processes with evidence of FET activity, as seen by lower NADH and succinate levels and subsequent less FMN release, and higher ATP resynthesis, compared with standard HMP or end-preservation O_2 supply during HMP (MODE 3 of mitochondrial operation according to Murphy) (Figure 9C).[20,25] During early in vivo reperfusion, FET generates ATP in the presence of oxygen. However, rapid consumption of accumulated succinate generates excessive ubiquinol (QH2), which impairs further FET. Such high succinate levels in combination with an acidotic milieu during ischemia result in RET at complex I with subsequent ROS production (Figure 9D).[19,27] Recent literature suggests that it is not the accumulated ischemic succinate but more likely the reduced or semireduced flavin located at the nucleotide-binding site that appears as the direct source of ROS production at complex I.[24] Reduced flavin ($FMNH_2$) can nonenzymatically react with molecular oxygen and generate ROS and oxidized flavin, which may rebind at complex I and recover the enzymatic activity during later reperfusion.[24] FMN determination during HMP by simple fluorescence is far easier and faster than any currently available succinate measurement and could therefore potentially be used as real-time surrogate marker to assess the metabolic status and predict IRI in solid organs. Indeed, Muller et al[28] described the use of FMN to predict liver function already during hypothermic oxygenated liver perfusion before implantation. We do realize that the perfusate FMN measurement by fluorescence detects only released FMN dislocated from complex I and does not detect the FMN that is still bounded in complex I. Because the proportion of released FMN originated from ischemic cell lyses is probably related to the

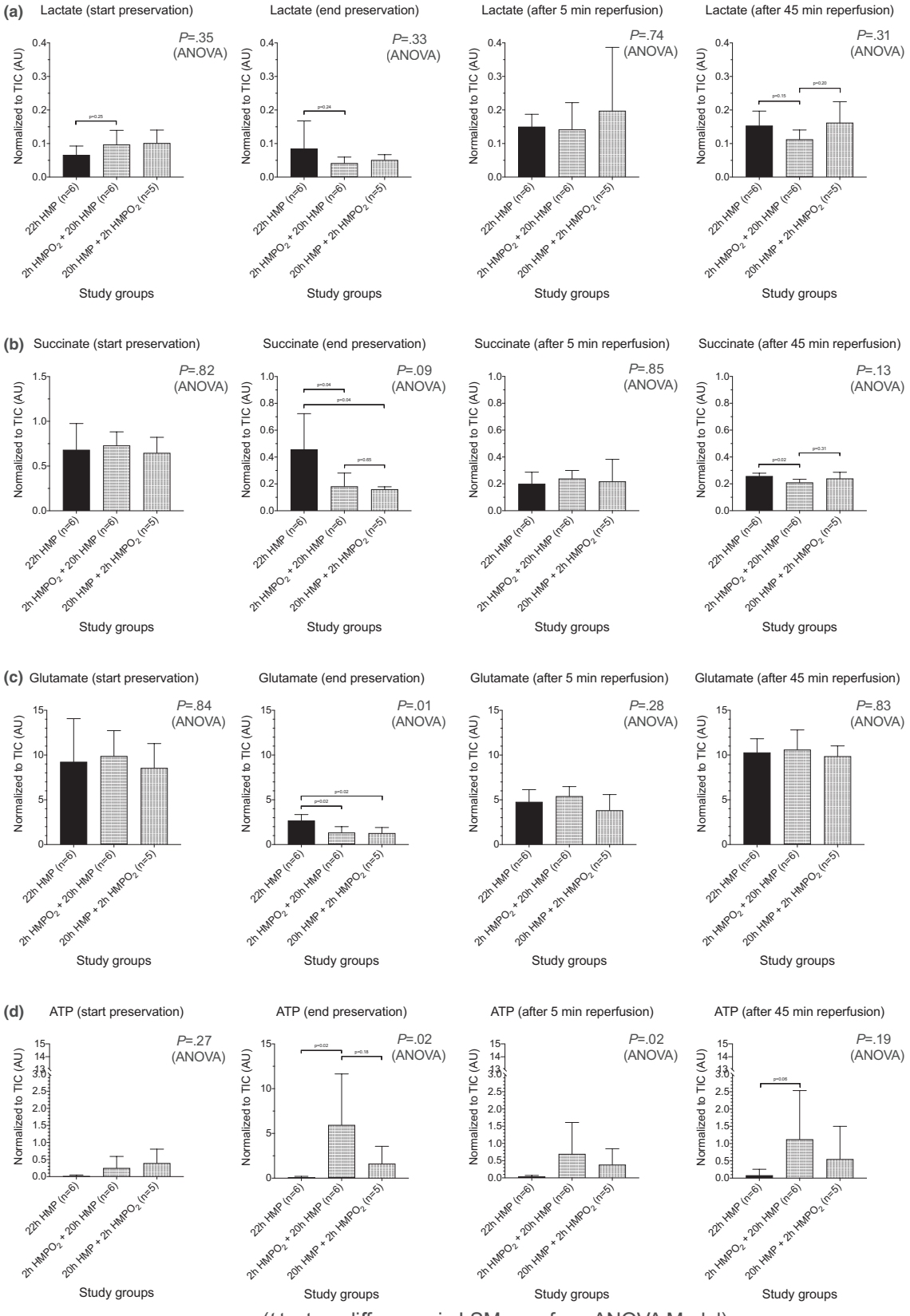

FIGURE 8 Metabolic profile analysis on preservation and reperfusion biopsies by liquid chromatography electrospray ionization mass spectrometry demonstrates that active oxygenation during HMP better supports aerobic metabolism, in particular the central major metabolites succinate and glutamate and the end product ATP. ADP, adenosine diphosphate; AMP, adenosine monophosphate, ATP, adenosine triphosphate; HMP, hypothermic machine perfusion; $HMPO_2$, oxygenated hypothermic machine perfusion

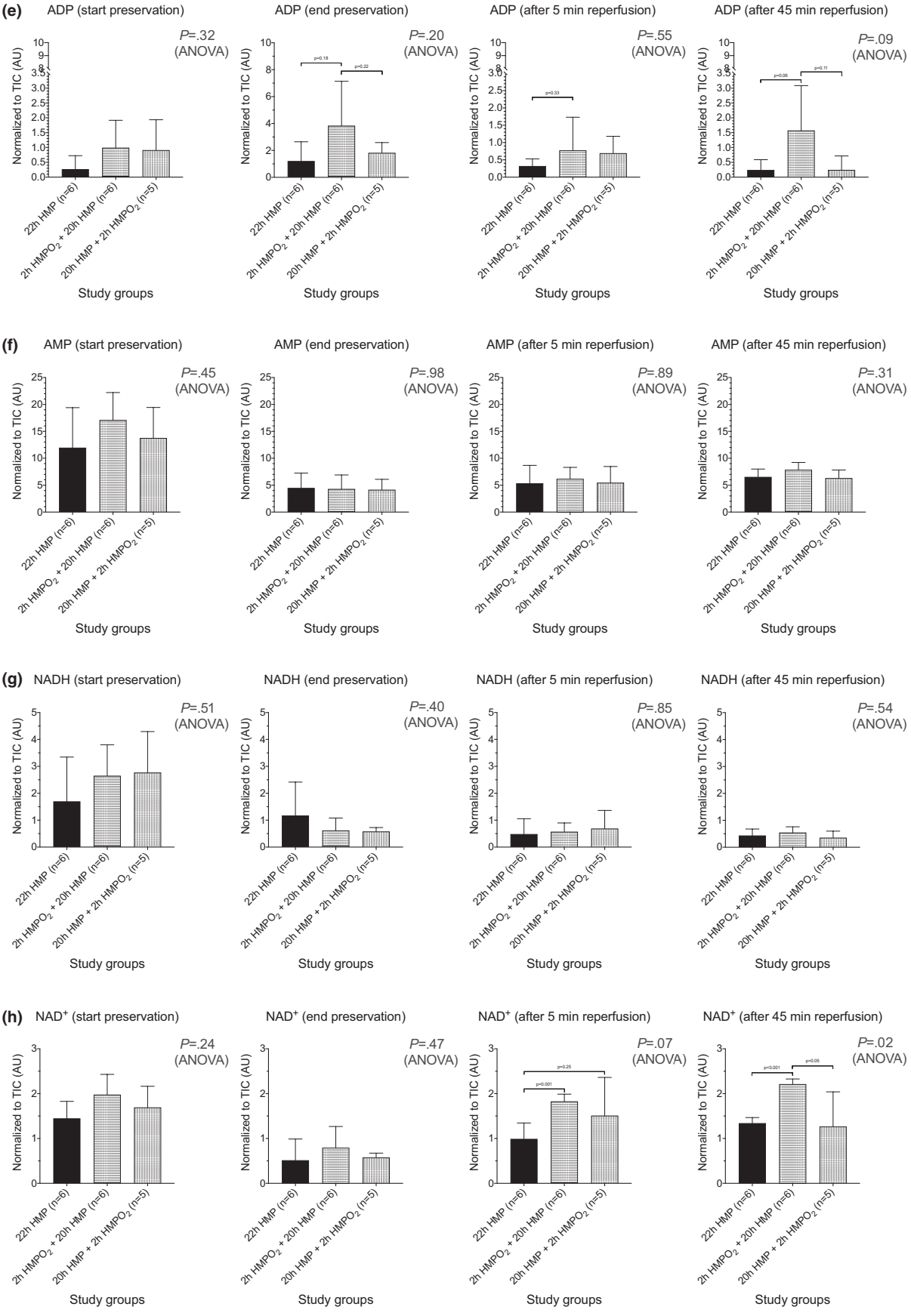

FIGURE 8 Continued

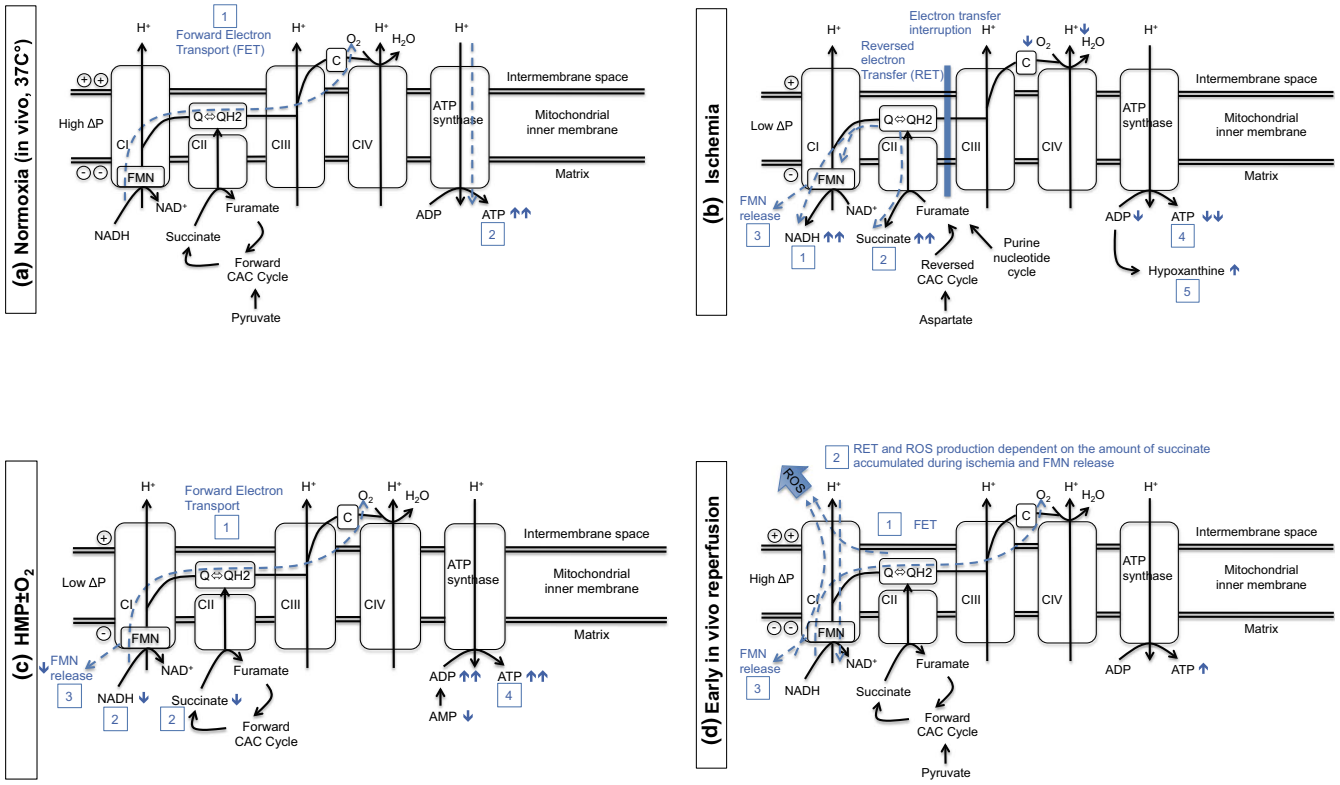

FIGURE 9 The effect of different kidney oxygenated HMP strategies. A, In vivo, during physiologic oxygenated conditions at 37°C, forward electron transport (FET) (dotted line) (1) drives the proton pumps (CI, CIII, CIV) against a charge gradient at the oxidative phosphorylation chain and proton back flow drives ATP synthase to generate ATP (dotted line) (2).[19,25] B, During ischemia, major metabolic alterations occur at the mitochondrial level in the kidney graft.[18,19] In the absence of oxygen, electron transfer is interrupted and first NADH (1) and second citric acid metabolite succinate (2) increase because both act now as electron carrier to allow CI continuously pump protons during ischemia. Also FMN release (3) at CI results from over-reduction of flavin via reversed electron transfer (RET).[24,26] Consequently, ATP and ADP decrease (4) and purine metabolites increase (5) (MODE 1 of mitochondrial operation according to Murphy).[19,25] C, During HMP conditions, mitochondrial respiratory function is significantly slowed down preventing the accumulation of electron overload at CI and CII. The degree of FET during HMP depends on the perfusate oxygen concentration (1), in particular during the first 2 h of HMP and results in a decrease of succinate and NADH (2) and less FMN release (3) and an increase of ADP/ATP regeneration (4) (MODE 3 of mitochondrial operation according to Murphy).[20,25] D, During early, in vivo reperfusion, FET (1) generates ATP. However, in case of too much accumulated mitochondrial succinate, rapid consumption of accumulated succinate generates too much ubiquinol (QH2), which impairs further FET. In combination with acidic pH from ischemia this results in a RET at CI (2).[19,27] Reduced or semireduced FMN located at the nucleotide-binding site is the direct source of ROS in CI during RET but reduced FMN can also nonenzymatically react with molecular oxygen producing ROS.[24] FMN, flavin mononucleotide; CI-IV, mitochondrial complex I-4 [Color figure can be viewed at wileyonlinelibrary.com]

duration of machine perfusion, we focused on FMN measurement during the first 2 hours of HMP, which correlated well with initial graft function in our study.

Nrf2 is a transcription factor that coordinates the basal and stress-inducible activation of antioxidants as well as cytoprotective genes and therefore acts as a protective transcription factor in IRI.[29,30] In our study, brief initial oxygenation resulted in the lowest Nrf2 expression at the end of HMP and following early in situ reperfusion, suggesting a reduced oxygen stress with O_2 uploading. Hypoxia-inducible factor is a heterodimeric protein composed of an oxygen-regulated alpha (α) and a constitutively expressed beta (β) subunit.[31-35] HIF-1α accumulation during renal ischemia has been widely reported.[36] In contrast, we observed a drop in HIF-1α expression in all conditions measured right after preservation attest of the high sensitivity to experimental procedures and precludes from any interpretation on subsequent variations. We found that HIF-1α levels were lowest in the initial oxygenation group following reperfusion. This might be explained by the difference in pO_2 during the first 2 hours of HMP comparing both experimental groups.

We recognize that there are a number of limitations inherent to this porcine autotransplant.[6] The use of historical control groups might have introduced a relevant bias due to slightly shorter anastomosis time in the experimental study groups compared with one of the historical groups but was recommended by the ethics committee to reduce animal numbers and suffering. However, because total preservation time was comparable between all study groups we do not expect to have an important influence on the outcome measures. Tubular injury measured as fractional excretion of urinary sodium and urinary protein-to-creatinine ratio is commonly used in

porcine animal models to evaluate the impact of IRI.[16,37] Despite efforts to ensure homogeneity in this animal model, several outcome measures (eg, urinary sodium and ATP) demonstrated significant variations and thus small differences between study groups may not have been identified.

An interesting observation was that even without oxygen supply to the external oxygenator a greater perfusate pO_2 was observed compared with standard HMP (Figure 3A). This mechanism is presumably due to passive exposure to room air through fibers of the membrane oxygenator. As a consequence the perfusate pO_2 in both initial and end oxygenation groups remained static during 20 hours without active oxygenation.

Practically, both brief initial and end-preservation oxygenation might be easy to implement in clinical practice. Kidneys originated from donors after circulatory death with more ATP debt at the start of preservation, compared with kidneys originated from brain death donors, probably benefit more from brief initial O_2 uploading at the donor hospital. This eliminates the need for an external oxygen source during transport. Concerning end-preservation oxygenation during HMP, it is unknown if the beneficial effect of oxygen maintains using shorter preservation periods as in most clinical programs compared with the model used in this study.

In conclusion, brief initial O_2 uploading during standard HMP might be an easy and more effective preservation strategy to maintain the aerobic metabolism and protect mitochondria with subsequently improved early renal function, particularly, when compared with standard HMP or end-ischemic O_2 supply. Although initial oxygen supply was not superior compared to continuous oxygenation, such short cold perfusion with oxygen could be of interest because it eliminated the need for an oxygen source for HMP during kidney transport.

ACKNOWLEDGMENTS

Darius T received a grant from the “Fondation Saint Luc,” the “Fondation Recherche Clinique,” and Astellas Pharma BV, and logistic support and scientific collaboration from Organ Recovery Systems. Dessy C is a FNRS senior research associate. The authors are grateful to Vergauwen M and Beaurin G regarding the support during the pig experiments, to De Muylder P regarding the scientific collaboration and logistic support, to Druart S regarding the lab analysis and to Jansen B regarding the statistical analysis.

DISCLOSURE

The authors of this manuscript have conflict of interests to disclose as described by the *American Journal of Transplantation*. The LifePort Kidney Transporter, the Perfusion Circuits, and the oxygenators were supplied by Organ Recovery Systems (Diegem, Belgium).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Tom Darius https://orcid.org/0000-0002-8151-4286

REFERENCES

1. Koetting M, Frotscher C, Minor T. Hypothermic reconditioning after cold storage improves postischemic graft function in isolated porcine kidneys. *Transpl Int*. 2010;23(5):538-542.
2. Hoyer DP, Gallinat A, Swoboda S, et al. Influence of oxygen concentration during hypothermic machine perfusion on porcine kidneys from donation after circulatory death. *Transplantation*. 2014;98(9):944-950.
3. Thuillier R, Allain G, Celhay O, et al. Benefits of active oxygenation during hypothermic machine perfusion of kidneys in a preclinical model of deceased after cardiac death donors. *J Surg Res*. 2013;184(2):1174-1181.
4. Kron P, Schlegel A, de Rougemont O, Oberkofler CE, Clavien PA, Dutkowski P. Short, cool, and well oxygenated - HOPE for kidney transplantation in a rodent model. *Ann Surg*. 2016;264(5):815-822.
5. Gallinat A, Paul A, Efferz P, et al. Role of oxygenation in hypothermic machine perfusion of kidneys from heart beating donors. *Transplantation*. 2012;94(8):809-813.
6. Darius T, Gianello P, Vergauwen M, et al. The effect on early renal function of various dynamic preservation strategies in a preclinical pig ischemia-reperfusion autotransplant model. *Am J Transplant*. 2019;19(3):752-762.
7. Darius T, Vergauwen M, Smith TB, et al. Influence of different partial pressures of oxygen during continuous hypothermic machine perfusion in a pig kidney ischemia-reperfusion autotransplant model. *Transplantation*. 2019; https://doi.org/10.1097/TP.0000000000003051. [Epub ahead of print].
8. 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 (New York, N.Y.)*. 2012;25(5):391-399.
9. Patel K, Smith TB, Neil DAH, et al. The effects of oxygenation on ex vivo kidneys undergoing hypothermic machine perfusion. *Transplantation*. 2019;103(2):314-322.
10. Hamed MO, Chen Y, Pasea L, et al. Early graft loss after kidney transplantation: risk factors and consequences. *Am J Transplant*. 2015;15(6):1632-1643.
11. Ludwig C, Gunther UL. MetaboLab–advanced NMR data processing and analysis for metabolomics. *BMC Bioinformatics*. 2011;12:366.
12. Craps J, Wilvers C, Joris V, et al. Involvement of nitric oxide in iodine deficiency-induced microvascular remodeling in the thyroid gland: role of nitric oxide synthase 3 and ryanodine receptors. *Endocrinology*. 2015;156(2):707-720.
13. Coulier L, Bas R, Jespersen S, Verheij E, van der Werf MJ, Hankemeier T. Simultaneous quantitative analysis of metabolites using ion-pair liquid chromatography-electrospray ionization mass spectrometry. *Anal Chem*. 2006;78(18):6573-6582.
14. Veiga-da-Cunha M, Chevalier N, Stephenne X, et al. Failure to eliminate a phosphorylated glucose analog leads to neutropenia in patients with G6PT and G6PC3 deficiency. *Proc Natl Acad Sci USA*. 2019;116(4):1241-1250.
15. Liapis H, Gaut JP, Klein C, et al. Banff histopathological consensus criteria for preimplantation kidney biopsies. *Am J Transplant*. 2017;17(1):140-150.
16. Hosgood SA, Shah K, Patel M, Nicholson ML. The effect of prolonged of warm ischaemic injury on renal function in an experimental ex vivo normothermic perfusion system. *J Transl Med.* 2015;13:207.
17. Robb EL, Hall AR, Prime TA, et al. Control of mitochondrial superoxide production by reverse electron transport at complex I. *J Biol Chem*. 2018;293(25):9869-9879.
18. Chouchani ET, Pell VR, James AM, et al. A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury. *Cell Metab*. 2016;23(2):254-263.
19. Chouchani ET, Pell VR, Gaude E, et al. Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. *Nature*. 2014;515(7527):431-435.

20. Dutkowski P, Guarrera JV, de Jonge J, Martins PN, Porte RJ, Clavien PA. Evolving trends in machine perfusion for liver transplantation. *Gastroenterology*. 2019;156(6):1542-1547.
21. Wilson DF, Erecinska M, Drown C, Silver IA. The oxygen dependence of cellular energy metabolism. *Arch Biochem Biophys*. 1979;195(2):485-493.
22. Fuller BJ, Lee CY. Hypothermic perfusion preservation: the future of organ preservation revisited? *Cryobiology*. 2007;54(2):129-145.
23. Hosgood SA, Nicholson ML. Oxygen supplementation supports energy production during hypothermic machine perfusion in a model of donation after circulatory death donors. *Transplantation*. 2019;103(10):1980-1981.
24. Ten V, Galkin A. Mechanism of mitochondrial complex I damage in brain ischemia/reperfusion injury. A hypothesis. *Mol Cell Neurosci*. 2019;100:103408.
25. Murphy MP. How mitochondria produce reactive oxygen species. *Biochem J*. 2009;417(1):1-13.
26. Stepanova A, Sosunov S, Niatsetskaya Z, et al. Redox-dependent loss of flavin by mitochondrial complex I in brain ischemia/reperfusion injury. *Antioxid Redox Signal*. 2019;31(9):608-622.
27. Pell VR, Chouchani ET, Murphy MP, Brookes PS, Krieg T. Moving forwards by blocking back-flow: the yin and yang of MI therapy. *Circ Res*. 2016;118(5):898-906.
28. Muller X, Schlegel A, Kron P, et al. Novel real-time prediction of liver graft function during hypothermic oxygenated machine perfusion before liver transplantation. *Ann Surg*. 2019;270(5):783-790.
29. Vomund S, Schafer A, Parnham MJ, Brune B, von Knethen A. Nrf2, the master regulator of anti-oxidative responses. *Int J Mol Sci*. 2017;18(12):pii:E2772.
30. Tonelli C, Chio IIC, Tuveson DA. Transcriptional regulation by Nrf2. *Antioxid Redox Signal*. 2018;29(17):1727-1745.
31. Akhtar MZ, Sutherland AI, Huang H, Ploeg RJ, Pugh CW. The role of hypoxia-inducible factors in organ donation and transplantation: the current perspective and future opportunities. *Am J Transplant*. 2014;14(7):1481-1487.
32. Wang WW, Li ZZ, Wang W, et al. Enhanced renoprotective effect of HIF-1alpha modified human adipose-derived stem cells on cisplatin-induced acute kidney injury in vivo. *Sci Rep*. 2015;5:10851.
33. Rosenberger C, Pratschke J, Rudolph B, et al. Immunohistochemical detection of hypoxia-inducible factor-1alpha in human renal allograft biopsies. *J Am Soc Nephrol*. 2007;18(1):343-351.
34. Medina Villaamil V, Aparicio Gallego G, Santamarina Cainzos I, Valladares-Ayerbes M, Anton Aparicio LM. Searching for Hif1-alpha interacting proteins in renal cell carcinoma. *Clin Transl Oncol*. 2012;14(9):698-708.
35. Kellenberger T, Marcussen N, Nyengaard JR, Wogensen L, Jespersen B. Expression of hypoxia-inducible factor-1alpha and hepatocyte growth factor in development of fibrosis in the transplanted kidney. *Transpl Int*. 2015;28(2):180-190.
36. Nangaku M, Eckardt KU. Hypoxia and the HIF system in kidney disease. *J Mol Med*. 2007;85(12):1325-1330.
37. Gallinat A, Paul A, Efferz P, et al. Hypothermic reconditioning of porcine kidney grafts by short-term preimplantation machine perfusion. *Transplantation*. 2012;93(8):787-793.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Darius T, Vergauwen M, Smith T, et al. Brief O_2 uploading during continuous hypothermic machine perfusion is simple yet effective oxygenation method to improve initial kidney function in a porcine autotransplant model. *Am J Transplant*. 2020;20:2030–2043. https://doi.org/10.1111/ajt.15800