Supplemental Digital Content (SDC)

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Methods S1: Detailed methods.

Ethics

Ethics approval for this project was obtained from the Western Sydney Local Health District human research ethics committee. All prospective donors' families were consented for the potential research use of kidneys for research purposes prior to the procurement process. Further project support was obtained from the NSW Organ and Tissue Donation Service (OTDS), and collaboration was also established with the Australian Red Cross Blood Service (ARCBS).

Inclusion and exclusion criteria

Kidneys were obtained for the purposes of this research from any deceased donor under the following circumstances – (i) they were deemed unsuitable for transplantation for any reason during or after procurement, or (ii) in the event of a planned liver-only donor whereby the kidneys had been deemed medically unsuitable prior to retrieval. Kidneys were only excluded from subsequent NMP when autologous or allogeneic blood was not available for perfusion.

Kidney procurement

Retrieval was undertaken in a standard fashion, after aortic cannulation and cold perfusion with Soltran; University of Wisconsin (UW) solution was also used if the liver and/or pancreas were also procured. If autologous blood was to be utilized for subsequent NMP, the inferior vena cava (IVC) was dissected and immediately accessed using a 28-32 Fr intercostal catheter at the commencement of cold perfusion. Vented blood was collected into a customized blood bag (LivaNova Australia, Dandenong, Australia) containing anticoagulant-citrate-dextrose solution A (ACD-A) (Aurora Bioscience, Bella Vista, Australia) and saline-adenine-glucose-mannitol solution (SAGM) (Macopharma, Chatswood, Australia), and stored on ice. Kidneys were stored in the final flush solution (UW or Soltran), surrounded by 0.9% sodium chloride ice slush, prior to transportation to our center.

Donor and retrieval details that were recorded included age, sex, comorbidities, donation

pathway (DBD or DCD), ABO blood group, kidney donor profile index (KDPI)[1, 2], donor cause of death (COD), intended and actual organs retrieved, reason for kidney discard/nonutilization, cross-clamp time, warm ischemic time (WIT), cold ischemic time (CIT), and kidney anatomy.

Kidney preparation

Kidneys underwent standard back-table preparation. The renal artery was cannulated with a blunt-tipped vascular cannula connected to a ¼ inch luer lock adaptor (Medtronic, MN, USA and LivaNova Australia, Dandenong, Australia), and the cannula was secured with a silk tie. The ureter was cannulated with a shortened heparin tip (Medtronic), which was also secured using a silk tie. Kidneys remained on ice slush until the commencement of NMP.

Blood preparation

In the event that autologous blood was used, collected donor whole blood was centrifuged at 3500 RPM for 15 minutes, and the supernatant discarded. The residual packed red blood cell (PRBC) mass was washed with Hartmann's solution, re-centrifuged for 10 minutes, and the supernatant was once again discarded. PRBCs were then passed through a leukocyte filter (Terumo Pty Ltd, Tokyo, Japan) and collected into a new blood bag (total PRBC volume approximately 250 ml).

The ARCBS provided all PRBC units for the NMP cases in which banked blood was utilized (O+ or O- units only; total PRBC volume approximately 250 ml). All subsequent simulated transplantation experiments were conducted using whole banked blood (O+ or O-), also obtained from the ARCBS. Total volume of each whole blood unit was approximately 500 ml, with 250 ml of this used for each paired kidney (see below).

Ex vivo perfusion set-up

The NMP system was assembled as previously described.[3] In brief, NMP was undertaken using autologous or banked PRBCs, to which was added Hartmann's solution (150 ml), gelofusine (250 ml), 10% mannitol (50 ml), 10% calcium gluconate (5 ml), 8.4% sodium bicarbonate (15 ml), sterile water for injection (25 ml), and heparin (2000 units). Continuous infusions of nutrient (M199 with ultraglutamine) solution (20 ml/hr), 5%

dextrose (5 ml/hr), and verapamil (5 mg in 2 ml, run at 5 ml/hr) were also run during NMP. Creatinine was added to the circuit (700 μmol in 5 ml 0.9% NaCl, to give an approximate concentration of 1000 μmol/L; Merck, Darmstadt, Germany) to enable subsequent quantification of creatinine clearance (CrCl). The kidney was placed in a customized, 3D-printed perfusion chamber.[4] Only the renal artery was cannulated, with the renal vein left open to drain into the reservoir via the perfusion chamber. Urine was collected and output replaced with Hartmann's solution. NMP was undertaken at a temperature of 37°C, with flow rates adjusted to maintain at a mean arterial pressure (MAP) of 75-85 mmHg.

To provide a direct comparison between CS and NMP in the absence of the ability to transplant these kidneys, ex vivo reperfusion with whole blood was undertaken in paired kidneys to simulate transplantation. This system utilizes whole blood containing leukocytes, complement, and other inflammatory mediators; furthermore, the protective verapamil infusion was omitted. Ex vivo whole blood reperfusion was undertaken at a MAP of 85-95 mmHg (maintained by flow adjustment), at 37°C for 60 minutes, after a simulated second warm ischemic ('anastomotic') time of 30 minutes during which the kidney was left at room temperature. Perfusion parameters (pressure and flow) and urine output (UO) were sequentially recorded during NMP and whole blood reperfusion.

Perfusion experiments

(i) Single kidneys (n = 7) underwent NMP for 1-3 hours. These kidneys were used to (a) establish NMP system feasibility, functionality, and safety; (b) compare NMP using autologous and banked blood; and (c) investigate leukocyte extravasation from the graft during NMP.

(ii) Paired kidneys (n = 8, i.e. 4 kidney pairs) were randomly allocated to either the cold static storage ('CS') or 'NMP' groups. 'CS' kidneys underwent standard CS, a subsequent 30 minute simulated SWIT period at room temperature, and then ex vivo whole blood reperfusion for 60 minutes to simulate the immediate post transplant reperfusion period. 'NMP' kidneys underwent CS, followed by one hour of NMP, a simulated SWIT of 30 mins, and finally ex vivo whole blood reperfusion for 60 minutes (using the initial NMP circuit set-up).

Samples

Sequential kidney biopsies were taken at the end of CS, after each hour of NMP (if

applicable), and at the end of ex vivo whole blood reperfusion (if applicable). Biopsy samples were stored in 10% natural buffered formalin, RNALater solution (Ambion/Thermo Fisher Scientific, TX, USA), or snap frozen in dry ice with or without OCT media (Tissue-Tek, ProSciTech, Australia), for subsequent analyses. Blood samples were also taken from the circuit and the start and end of perfusion, as applicable, and sent to the hospital laboratory for quantification of hemoglobin, white cell counts, platelet counts, hematocrit, electrolytes, urea, creatinine, blood sugar level, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), albumin, and osmolality. Arterial and venous blood gas samples were taken during the start and end of perfusion, and analysed for lactate, pH, partial pressure of oxygen (pO_2) and carbon dioxide (pCO_2), bicarbonate (HCO_3), and base excess (BE) using the i-STAT Alinity machine (Abbott, IL, USA). Urine samples were taken at the end of perfusion and analysed for electrolyte, creatinine, and protein levels.

Measurements and analyses

Renal blood flow (RBF) and intra-renal resistance (IRR = *MAP/RBF*)[5] was recorded throughout perfusion and normalized to a kidney weight of 250 grams. Urine output (UO) was recorded every hour of perfusion (ml). CrCl (ml/min/100g/hr) during NMP and ex vivo whole blood reperfusion was calculated using the following formula – (urine Cr (μmol/L) x urine volume (L))/plasma Cr (μmol/L). Fractional excretion of sodium (FeNa) (%) was calculated as – (100 x plasma Cr (μmol/L) x urine Na (mmol/L))/(plasma Na (mmol/L) x urine Cr (μmol/L)). Renal oxygen consumption (mmHg*ml/min/g) at end-NMP or ex vivo reperfusion was determined using – [RBF (ml/min) x (PaO_2 – PvO_2) (mmHg)]/kidney weight (g).[6]

Renal histopathology

All biopsies underwent Periodic Acid-Schiff (PAS) staining according to standard methods. Each pre and post-NMP, and post ex vivo whole blood reperfusion was assigned a Remuzzi score by a blinded renal histopathologist.[7] The following parameters were assessed – number of glomeruli; glomerular sclerosis (%); chronic damage (tubular atrophy/interstitial fibrosis; %); arteriolar hyalinosis (0 – absent; 1 – present); intimal elastosis (0 – absent; 1 – less than medial thickness; 2 – more than medial thickness); and extent of acute tubular injury (0 – absent; 1 – loss of tubular cell brush

borders/vacuolization; 2 – cell detachment/casts; 3 – coagulation necrosis).

Immunofluorescence

Renal tubular epithelial cell death was compared between paired kidneys (NMP versus CS pairs, using cryosections cut from samples taken at the end of ex vivo whole blood reperfusion) using TUNEL staining. A commercial in situ cell death detection kit was utilized for this purpose (Sigma-Aldrich/Merck, MO, USA). Slides were co-stained with DAPI (1:25,000) for 2 minutes. TUNEL staining was quantified using confocal microscopy.

Renal tissue oxidative stress was quantified and compared in paired NMP/CS samples using dihydroethidium (DHE) (Thermo Fisher Scientific), an indicator of tissue superoxide levels. Unfixed cryosections were thawed; DHE (10 μM) was applied to the surface of each section (incubated at 37°C for 22 mins). Slides were co-stained with DAPI as above. Confocal microscopy was utilized for visualization of DHE staining. Integrated densities were quantified using ImageJ software (National Institutes of Health, USA). Each section had 4 images taken, with mean densities for each image calculated from a further 4 regions of interest.

Complement (C9) staining was also performed in paired samples. Cryosections were fixed, blocked, and thence stained with C9 primary antibody raised in rabbits (1:250 dilution; Abcam, Cambridge, UK), and incubated for one hour at room temperature. This was followed by staining with goat anti-rabbit secondary antibody conjugated to Alexa Fluor 647 (1:400 dilution; Invitrogen, CA, USA) for a further one hour at room temperature. DAPI co-staining was performed. Sections were visualized using confocal microscopy; C9 staining intensity was quantified using ImageJ software, with 4 regions of interest utilized for each section image.

Flow cytometry analysis for leukocyte effluent from the graft during NMP

Blood samples were taken from the circuit at different time points (n = 3 kidneys) to analyze leukocyte extravasation from the graft. Samples were taken from the PRBC blood bag, and then at ‘start’ NMP (5 minutes after the commencement of NMP), one hour post commencement of NMP, and 1.5 and 2 hours post commencement of NMP. Briefly,

samples were spun and equivalent amount of “PRBCs” were used for staining. 50 μL of the graft circuiting “PRBCs” and control baseline “PRBCs” were added into a Trucount tube (BD Biosciences) and blocked with pure Fc1.3070 (BD Biosciences), followed by staining with an antibody cocktail and cell lysis/fixation with BD FACS lysing solution (BD Biosciences) according to the manufacturer's instructions and as described previously.[8] Fluorochrome-coupled anti-human antibodies to CD45, CD3, CD11c, CD14, CD16, CD19, CD56, CD123, CD141, HLA-DR, lineage cocktail (CD3, CD14, CD19, CD20, CD56) (BD Biosciences), and CD303 (Miltenyi Biotec) were used. Potential dendritic cell detection was performed using the following markers: HLA-DR+CD3-CD14-CD19-CD20-CD11c+CD141 and/or HLA-DR+CD3-CD14-CD19-CD20-CD11c-CD303+CD123+. Flow cytometric analysis was performed on a BD-LSR Fortessa (BD Biosciences) and Diva software (BD Biosciences) for evaluation of absolute numbers of granulocytes, monocytes, NK cells, B cells, T cells, NKT cells, and dendritic cells. Data was analyzed using FlowJo V10.

RNA expression by next-generation sequencing

Targeted whole transcriptome RNA expression[9, 10] was analyzed using paired kidneys undergoing NMP or CS alone, followed by ex vivo whole blood reperfusion. Kidney biopsies from each group were taken at end-CS, end-NMP (if applicable), and end-ex vivo reperfusion. RNA extraction was conducted using an ISOLATE II Mini-kit (Bioline Australia). For Ampliseq transcriptome analyses, libraries were prepared using Ion AmpliSeq Transcriptome Human Gene Expression Kit (Thermo Fisher Scientific) following the manufacturer’s protocol using 10 ng of total RNA and quantified by qPCR with Ion Library TaqMan Quantitation Kit (Thermo Fisher Scientific). Libraries with concentration ranging from 1,515 pM to 6,629 pM were obtained and normalized to 100 pM. Seven to eight normalized libraries were pooled together, templated on the Ion Chef System, then sequenced on Ion 540 Chips using the Ion S5 XL system. Reads were aligned back to the manufacturer’s supplied target reference with built in mapping software Tmap. The aligned data was TMM normalized using the edgeR package.[11]

Statistical analyses

Unless otherwise indicated, data is presented in the format mean ± standard deviation (SD). Continuous parametric variables were compared using the unpaired Student’s t-test, whilst

nonparametric continuous variables have been compared using the Mann-Whitney U test. The paired t-test was used for comparison of baseline and end-NMP data for each individual kidney, or functional data for each paired kidney at the end of ex vivo reperfusion. RBF and IRR graphs were compared by first calculating the area under the curve (AUC) for each parameter plotted on the graph. GraphPad Prism v. 7.02 was used for all of these statistical analyses. For all data comparisons, a p-value <0.05 was considered as statistically significant.

Differential expression analysis was performed using voom.[12] For all comparisons, changes in gene expression were deemed significant if they had a Benjamini-Hochberg adjusted p-value < 0.05. Pathway analysis was performed using a hypergeometric test to test if any Gene Ontology or Reactome categories were enriched for differentially expressed genes.[13-15] Wilcoxon-rank-sum tests with directional alternative hypotheses were used on the test statistics to test if any of the pathways were significantly up or downregulated. Further pathway analyses were conducted through the use of Ingenuity Pathway Analysis (IPA) (Qiagen Inc.).[16]

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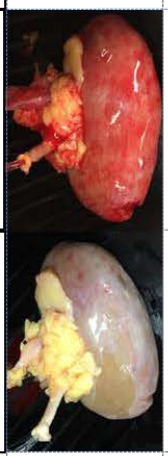
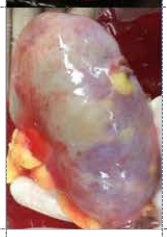
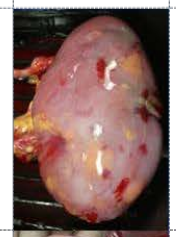
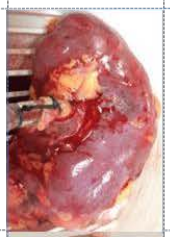
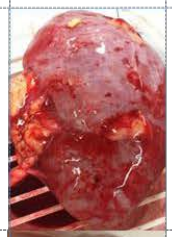
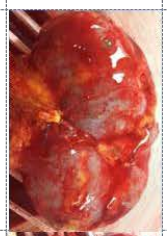
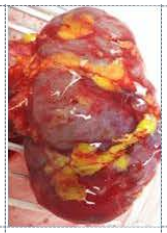
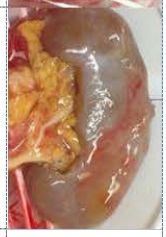
Table S1: Pictorial representation of all donor kidneys at the end of cold (static) storage (CS), NMP, and ex vivo whole blood reperfusion (RFN), as appropriate.

Donor #	Left Kidney			Right Kidney		
	End-CS	End-NMP	During *ex vivo* RFN	End-CS	End-NMP	During *ex vivo* RFN
1						
2						
3						
4						
5						

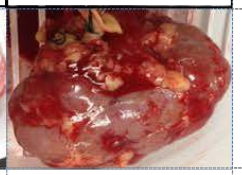
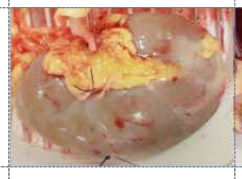
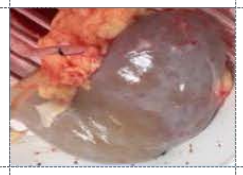
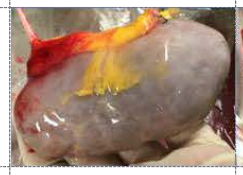
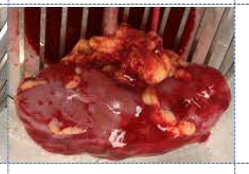
	Left Kidney			Right Kidney		
Donor #	End-CS	End-NMP	During *ex vivo* RFN	End-CS	End-NMP	During *ex vivo* RFN
6						
7						
8						
9						
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Table S2: Renal tubular injury scores at selected time-points in kidneys undergoing NMP.

Kidney	***End CS***	***60 min NMP***	***120 min NMP***	***180 min NMP***
DBD-D1	1 (focal 2*)	2	1 (focal 2*)	1 (focal 2*)
DBD-D2-L	1 (focal 2**)	1 (focal 2*)	1 (focal 2*)	
DBD-D2-R	1 (focal 2*)	1 (focal 2**)		
DBD-D3-L	1	1		
DBD-D4-L	0-1	1		
DBD-D5	0	1***	1	
DBD-D6-R	1 (focal 2**)	1		
DCD-D1	1	1		
DCD-D2-L	1 (focal 2*)	1		
DCD-D3	0	0-1		
DCD-D4	1	1 (focal 2**)	1 (focal 2****)	

***** Sloughed cells
** Casts and sloughed cells
*** Few casts only
**** 90 min sample; occasional casts

Figure S1: Renal blood flow (RBF) and intra-renal resistance (IRR) in (A) DCD, and (B) DBD kidneys, arranged by cold (CIT) and/or warm (WIT) ischemia times, as applicable.

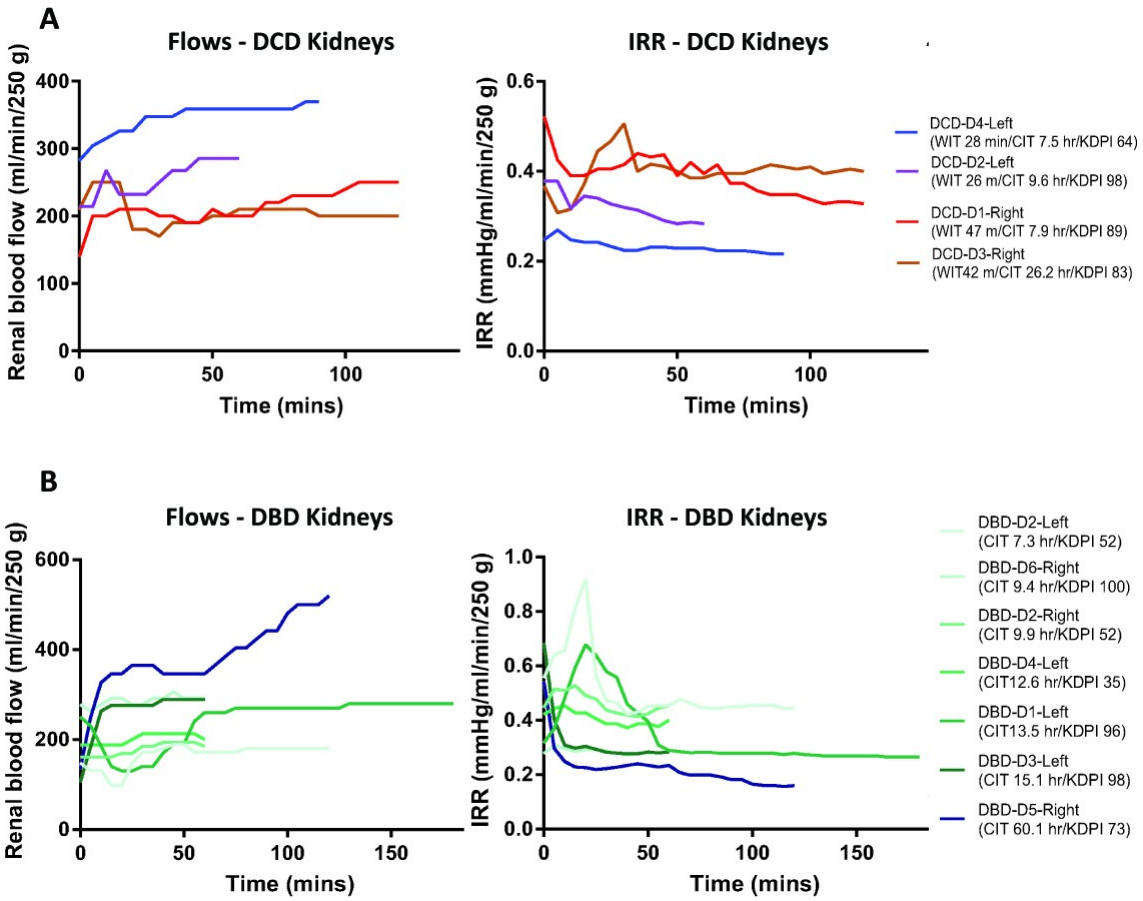

Table S3: Comparative perfusate baseline hematologic and biochemical parameters at the start of NMP in kidneys perfused with autologous or banked (allogeneic) blood.

Characteristic	**Autologous Blood (Mean, SD)**	**Banked Blood (Mean, SD)**	**p-value**
Hemoglobin (g/L)	43.8 (25.2)	65.3 (13.7)	0.094
White cell count (x 10^9/L)	0.15 (0.1)	0.06 (0.05)	0.071
Platelet count (x 10^9/L)	45 (19.9)	0.9 (1.5)	< 0.001
Hematocrit (%)	14.3 (8.7)	20.7 (5.2)	0.150
Sodium (mmol/L)	142 (1.4)	143.9 (4.0)	0.404
Potassium (mmol/L)	5.4 (0.4)*	7.6 (1.5)*	0.107
Bicarbonate (mmol/L)	13 (4.2)	14.3 (1.9)	0.491

* Values for 2 autologous samples and 1 banked sample missing due to sample hemolysis (post/during collection)

Figure S2: Comparison of NMP using autologous or banked (allogeneic) packed red blood cells (PRBCs). UPPER PANELS – Flow and intra-renal resistance (IRR) during NMP using each source of blood. LOWER PANELS – Comparative renal glomerular, tubular, and functional parameters after NMP with banked versus autologous PRBCs. AST – aspartate aminotransferase; CrCl – creatinine clearance; FeNa – fractional excretion of sodium; LDH – lactate dehydrogenase; UO – urine output.

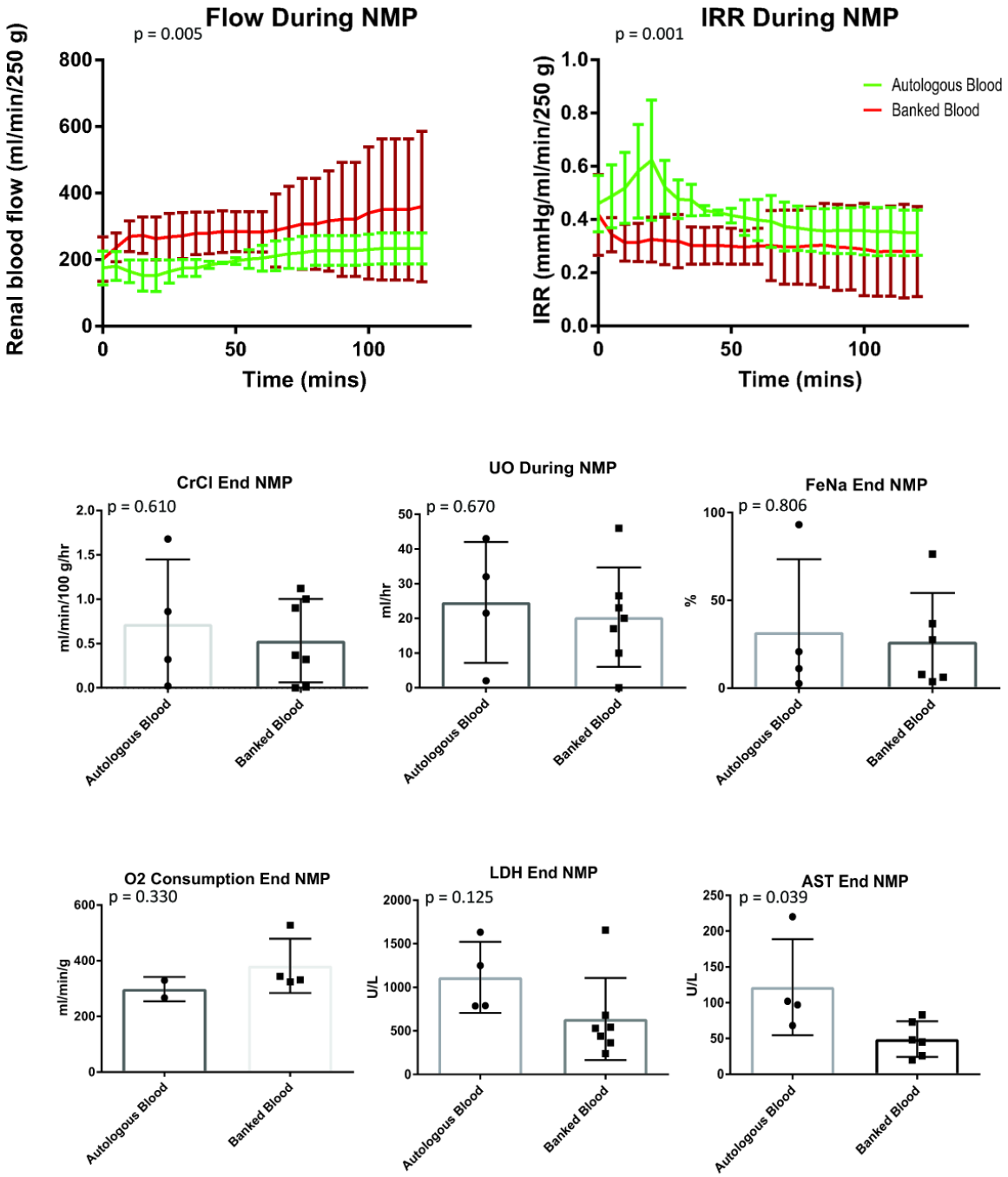

Figure S3: Principal component analysis (PCA) for all paired kidney samples that underwent whole transcriptome RNA sequencing.

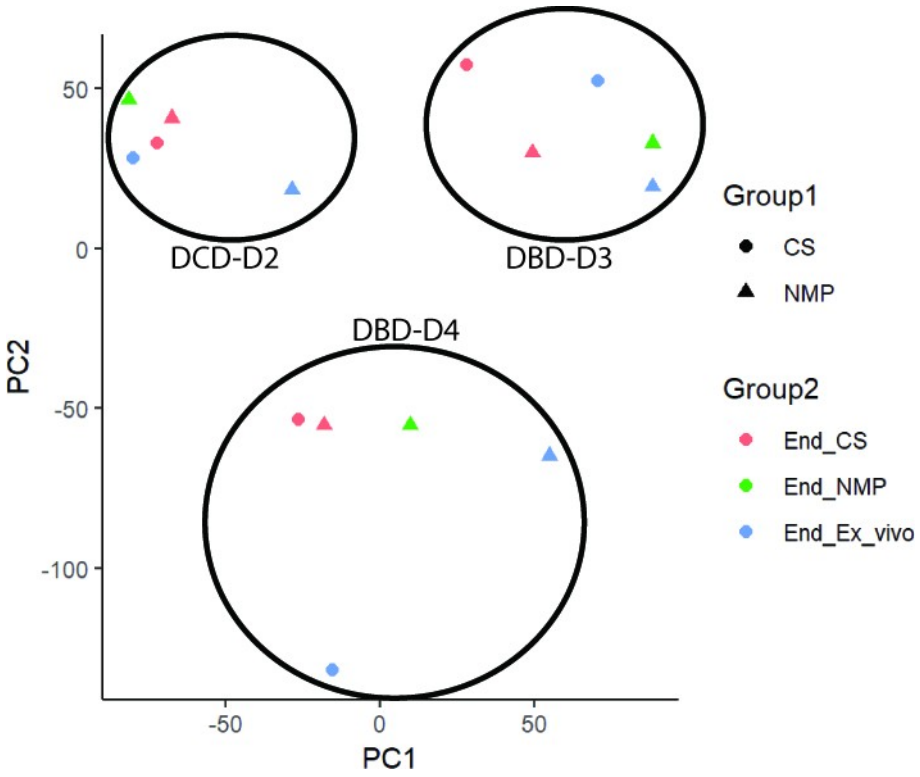

Table S4: Differentially expressed genes and pathways in paired kidneys after NMP (in comparison to the end-CS samples from the same kidneys [NMP group]).

Table S5: Differentially expressed genes and pathways after ex vivo whole blood reperfusion in paired kidneys having NMP (in comparison to the end-NMP samples from the same kidneys).

Table S6: Differentially expressed genes and pathways after ex vivo whole blood reperfusion in paired kidneys having NMP compared to CS alone.

Table S7: IPA – Tabulation of Diseases/Functions outlined in Figure S4.

Excel spreadsheets – accessible as separate attachments

Figure S4: IPA – Diseases/functions activated and/or repressed by NMP in comparison to paired kidneys having CS alone (sampled at the end of simulated transplantation). Each large box indicates a Disease/Function category, whilst each small box represents a distinct Disease/Function process (annotation). Boxes are colored based on z-score (orange indicates an increase in the predicted pathway activation state, and blue indicates a decrease).

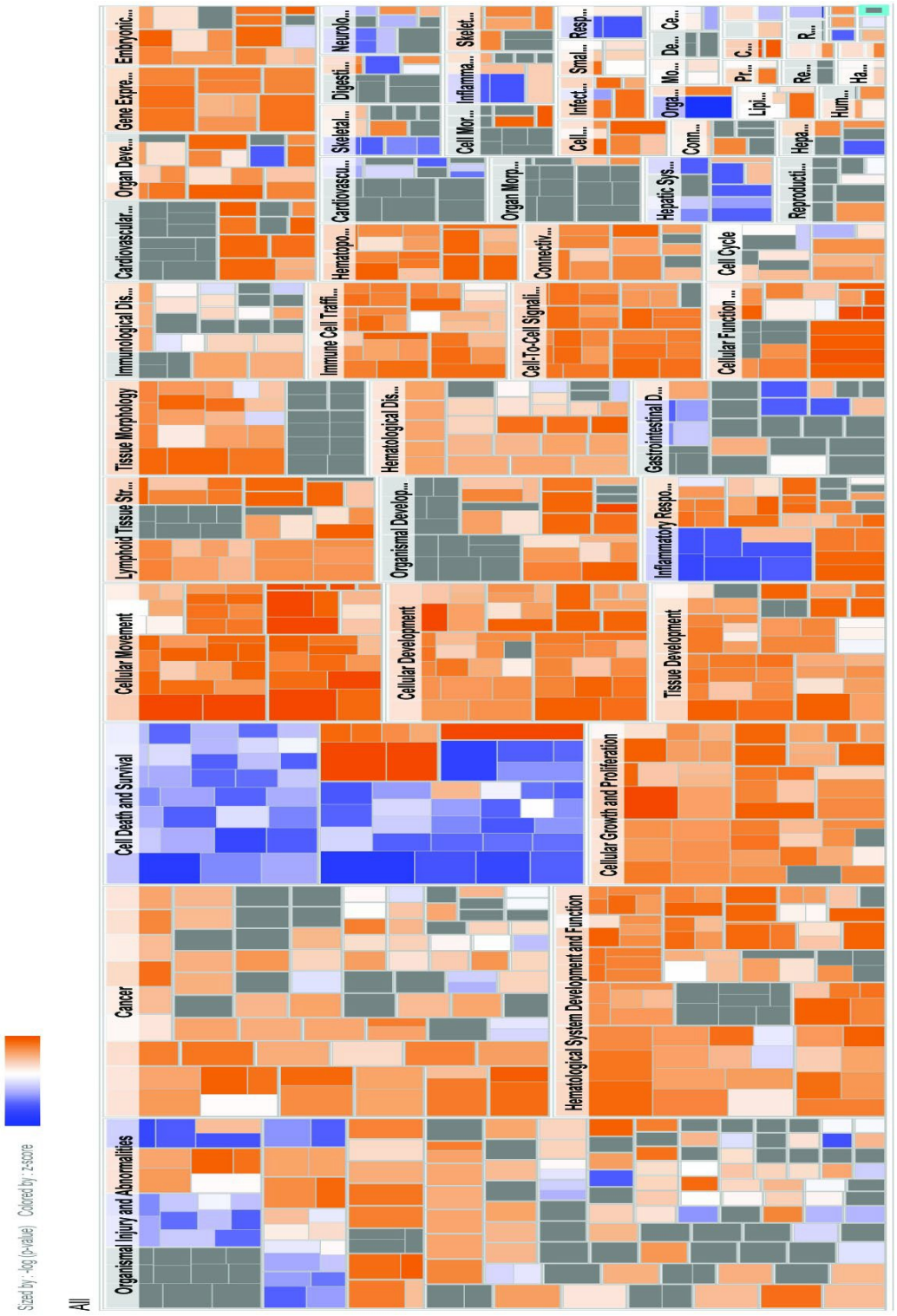

Table S8: Remuzzi scores (including tubular injury scores) after simulated transplantation in paired kidneys having CS alone or NMP.

Pair No.	*Treatment Group*	*No. Glomeruli*	*% Sclerosed*	*% Chronic Damage (Tubular atrophy/Interstitial fibrosis)*	*Arteriolar Hyalinosis*	*Intimal Elastosis*	*Acute Tubular Injury*
1	NMP	22	5	5	1	NA	1
1	CS	20	5	5	1	NA	1
2	NMP	40	5	5	1	2	1 (with focal 2)
2	CS	30	10	5	1	1	1-2
3	NMP	60	0	2	0	0	1 (with focal 2)
3	CS	35	2	2	1	NA	1
4	NMP	225	4	5	1	1	1-2
4	CS	280	3	3	1	2	1 (with focal 2)