Ischemia/reperfusion injury : a metabolic meltdown

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IV

Defective postreperfusion metabolic recovery directly associates with incident delayed graft function

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ABSTRACT

Delayed graft function (DGF) following kidney transplantation affects long-term graft function and survival and is considered a manifestation of ischemia/reperfusion injury. Preclinical studies characterize metabolic defects resulting from mitochondrial damage as primary driver of ischemia/reperfusion injury. In a comprehensive approach that included sequential establishment of postreperfusion arteriovenous concentration differences over the human graft, metabolomic and genomic analysis in tissue biopsies taken before and after reperfusion, we tested whether the preclinical observations translate to the context of clinical DGF. This report is based on sequential studies of 66 eligible patients of which 22 experienced DGF. Grafts with no DGF immediately recovered aerobic respiration as indicated by prompt cessation of lactate release following reperfusion. In contrast, grafts with DGF failed to recover aerobic respiration and showed persistent adenosine triphosphate catabolism indicated by a significant persistently low post reperfusion tissue glucose-lactate ratio and continued significant post-reperfusion lactate and hypoxanthine release (net arteriovenous difference for lactate and hypoxanthine at 30 minutes). The metabolic data for the group with DGF point to a persistent post reperfusion mitochondrial defect, confirmed by functional (respirometry) and morphological analyses. The archetypical mitochondrial stabilizing peptide SS-31 significantly preserved mitochondrial function in human kidney biopsies following simulated ischemia reperfusion. Thus, development of DGF is preceded by a profound post-reperfusion metabolic deficit resulting from severe mitochondrial damage. Strategies aimed at preventing DGF should be focused on safeguarding a minimally required post-reperfusion metabolic competence.

INTRODUCTION

Delayed graft function (DGF), the phenomenon of deferred functional recovery of a donor graft following transplantation, has detrimental effects on long-term graft function and graft survival.[1–3] The incidence of DGF is steadily rising, a fact thought to reflect increased use of so-called marginal organs in an era of donor shortages. DGF incidences up to 70% are reported for deceased donor grafts.[2,4] Incident DGF is thought to largely reflect ischemia/reperfusion (I/R) injury[5,6], the increase of tissue damage following reperfusion of previously ischemic tissue. A range of pharmaceutical interventions that target I/R such as antioxidants and anti-inflammatory and immune-modulatory drugs successfully quench I/R injury in preclinical models, but efforts to translate these experimental findings to the human situation have been unsuccessful.[5,7] Therefore there is currently no intervention that alleviates DGF and other forms of clinical I/R injury, a notion that points to an impaired translatability of preclinical findings[5]

Although DGF is common in deceased donor grafts, it is rare in the context of living donor kidney transplantation. This is a notable observation, because these grafts are also exposed to several hours of ischemia prior to reperfusion.[8] It was thus reasoned that differences in the response to I/R between living and deceased donor grafts provide critical clues towards the mechanism(s) driving DGF. Living donor procedures were used as comparators. We and others previously excluded commonly implicated causative factors such as oxidative damage[9]; neutrophil[10], thrombocyte or complement activation[11–15]; and inflammation[12,16,17] as main drivers of clinical I/R injury in the context of kidney transplantation. These observations imply that clinical I/R injury is driven by factors beyond those commonly brought forward.

There is accumulating evidence for a role of metabolic dysfunction as driver of I/R injury particularly from the context of myocardial I/R.[18–21] However, it is unclear whether and how these observations translate to the context of kidney transplantation, and in particular to incident DGF.

We here show that incident DGF is associated with profound and persistent post-reperfusion metabolic deficit caused by severe mitochondrial damage. The consequent severe metabolic shortfall interferes with processes critical for cell homeostasis and recovery such as gene transcription. Importantly, it was observed that mitochondrial damage could be partially rescued by the archetypical mitochondria stabilizing peptide SS-31.[22] The potential of this cardiolipin-binding peptide is extensively shown in preclinical studies[22,23] and the compound has now entered clinical evaluation.[24]

MATERIALS & METHODS

PATIENTS

The study protocol was approved by the local medical ethics committee of the Leiden University Medical Center, and written informed consent was obtained from each patient.

A total of 85 transplant recipients were enrolled. Twelve patients refused to give informed consent and 7 patients were excluded due to cancelled surgery (i.e. positive crossmatch, recipient deemed unfit for surgery or discarded organ). Patient enrolment is described in Supplementary Figure 1 and patient characteristics are described in Table 1. Renal allograft transplantations were performed according to the local standardized protocol.[10] In living donors, open minimal access nephrectomy was performed and Custodiol® HTK (histidine–tryptophan–ketoglutarate) solution was used for cold perfusion and storage of the kidney. Deceased donor kidneys were perfused and stored with University of Wisconsin solution. All included kidney transplants were preserved by means of static cold storage, none of the grafts received machine perfusion. The immunosuppressive regimen was based on induction therapy with basiliximab on day 0 and 4, and tacrolimus or cyclosporine A in addition to mycophenolate mofetil and steroids as maintenance therapy.

Delayed graft function (DGF) is the clinical readout of I/R injury upon kidney transplantation and comprises recipients who need dialysis in the first week after transplantation.[25]

ARTERIOVENOUS SAMPLING

Arteriovenous concentration differences over the transplanted kidney were measured in 24 patients undergoing kidney transplantation. Seven of these patients received a kidney from a living donor and 9 patients received a deceased donor graft. Reference arteriovenous samples for a normal kidney were obtained prior to the induction of renal ischemia during donor nephrectomy from living donors (n=4).

Prior to implantation of the graft, a 5 French umbilical vein catheter was positioned in the lumen of the renal vein through one of its side branches. At 30 s, and 3, 5, 10, 20 and 30 min after reperfusion (i.e. moment of reperfusion t=0), 10 mL blood aliquots were sampled. Paired arterial blood samples were obtained at 0, 10 and 30 min after reperfusion (Sup.Fig.7). The abdominal wall was closed about forty-five minutes after reperfusion, and the endpoint of sampling was reached 30 minutes after reperfusion. Blood samples were collected in precooled containers and immediately placed on melting ice. The validity of the arteriovenous sampling method was validated earlier measuring oxygen saturation.[10]

Blood gas analysis - Arterial and venous lactate levels and pH were measured in a certified clinical chemistry lab within 30 minutes of sampling.
Blood was collected in heparin-coated capillaries (Siemens, RAPIDLyte Multicap capillaries, 140 µL, Siemens Healthcare, The Hague, The Netherlands), mixed, and stored on melting ice. Analyses were performed on a certified clinical blood gas analyser (Siemens RAPIDLab 865, Siemens Healthcare) within 30 minutes after sample collection.

HYPOXANTHINE AND ALLANTOIN ANALYSIS IN PLASMA

Hypoxanthine and allantoin in plasma were measured using an in-house developed ultra performance liquid chromatography–mass spectrometry/mass spectrometry method. Briefly 30 µl plasma was mixed with 30 µl of a solution containing stable isotope labelled internal standards. Samples were deproteinized with 500 µl acetonitrile. After centrifugation (10 min, 12000 rpm, 4 °C) the supernatant was evaporated under nitrogen and reconstituted in 500 µl 50 mM ammonium formiate (pH 4.00).
Purine and pyrimidine metabolites were separated using a Waters Acquity UPLC system (Waters, Etten-Leur, The Netherlands) equipped with an Acquity HSS T3 (2.1 * 100 mm, df 1.8 µm). Separation of the compounds of interest was achieved by a 0.01 M ammonium formiate (pH 4.00) / acetonitrile gradient. Compounds were quantified using a Waters XEVO TQS tandem mass spectrometer (Waters, Etten-Leur, The Netherlands), both in negative or positive electrospray ionization using specific Multiple Reaction Monitoring transitions.

HIGH-RESOLUTION MAGIC-ANGLE-SPINNING NUCLEAR MAGNETIC RESONANCE (HR MAS NMR) SPECTROSCOPY

Renal cortical biopsies of 18 patients were obtained immediately prior to and 45 min after reperfusion. Eight patients received a kidney from a living donor and 12 patients received a kidney from a deceased donor. Tissue was snap frozen in liquid nitrogen and stored at -80°C. Metabolic profiling of the tissue biopsies was performed by HR MAS NMR spectrometry. Samples were prepared on ice and fitted in a leak-proof insert (30 µL, Bruker: Kel-F, Bruker, Delft, The Netherlands) used in a zirconium HR-MAS rotor (4 mm). The insert was filled with 3 µL cold (4°C) phosphate-buffered saline (PBS in D_2O) containing 4.5 mM TSP-d4 (trimethylsilyl-tetradeuteropropionic acid) and 25 mM sodium formate ($CHNaO_2$) as internal standards. HR-MAS spectra were recorded on a 14.1 T (600MHz, 1H) Bruker Avance III NMR spectrometer equipped with a TCI cryoprobe. All experiments were acquired at 4°C while spinning the samples at a rate of 5 kHz. Metabolite signals were quantified using the BATMAN R package.[26] Spectra were normalized based on the tissue weight used.

RENAL TRANSCRIPTOME ANALYSIS

Pre- and post-reperfusion transcriptome was assessed in 24 patients undergoing renal allograft transplantation. Ten patients received a kidney from a living donor and 14 patients received a kidney from a deceased donor. Eight of the 14 deceased donor kidneys developed DGF.

A renal cortical biopsy was obtained at the end of cold storage before transplantation, and a post-reperfusion biopsy was taken 45 min after reperfusion. Biopsies were immediately snap frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted from renal tissues using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads.[27] The integrity of each RNA sample was examined by Agilent Lab-on-a-chip technology using the RNA 6000 Nano LabChip kit and a Bioanalyser 2100 (Agilent Technologies, Amstelveen, The Netherlands). RNA preparations were considered suitable for array hybridization only if samples showed intact 18S and 28S rRNA bands, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number ›8.0). Subsequent microarray analysis was performed using Illumina whole-genome gene expression BeadChips (Illumina BeadArray®, San Diego, USA) according to the instructions of the manufacturer at Service XS (Leiden, The Netherlands). The probe-level, background subtracted expression values were used as input for Illumina package to perform quantile normalization. Results were analyzed by biostatistical methods using average replicate values for each group of samples. Log2 ratios were computed and one value per gene was calculated for the average expression of probes with the same Entrez Gene ID. This resulted in 15,093 unique gene profiles. These genes were next inputted for pathway analysis through the Ingenuity Pathway Analysis suite (Redwood City, CA; *http://www.ingenuity.com*). As we are unable to differentiate between passive decay and active down-regulation for the down-regulated pathways, we only included all significantly up-regulated pathways. The top 25 up- regulated pathways from Ingenuity "Biological Function" collection are shown in Supplementary Figure 3. Values provided are composite P-values based on the o-hypothesis for each given pathway. Composition of these pathways is defined in the Ingenuity "Bio- logical Function" collection. For clarity, P-values are expressed as –log P-value.[28]

HISTOLOGY

Renal cortical biopsies were taken at the end of the cold ischemic period and compared to biopsies collected 45 min after reperfusion. Tissue of living and deceased donor grafts were compared.

Immunohistochemistry

Pre- and post-reperfusion kidney biopsies (n=18: Living (n=5), –DGF (n=5), +DGF

(n=8)) were formaldehyde-fixed, and paraffin-embedded. Sections (2 µm) were incubated overnight with an anti-cytochrome C antibody (1:200 diluted, room temperature) (Upstate Biotechnologies, Lake Placid, USA) and visualized using anti-mouse peroxidase-conjugated EnVision (DAKO, Glostrup, Denmark). Sections were counterstained with hematoxylin (Merck, Darmstadt, Germany). Healthy, non-ischemic human renal tissue was used as positive control/reference.

Transmission Electron Microscopy and Reflection Contrast Microscopy
Sections of the kidney biopsies (n=26: Living (n=9), –DGF (n=7), +DGF (n=10)) were immediately fixed in glutaraldehyde (2-3%) with paraformaldehyde (PFA 1-2%), followed by OsO_4 fixation, dehydration in graded ethanol and embedded in resin media. Mitochondrial morphology was evaluated by transmission electron microscopy and reflection contrast microscopy.[29]

RESPIROMETRY
The influence of warm and cold ischemia on mitochondrial respiration, and the ability of the mitochondrial (cardiolipin) membrane-stabilizing peptide SS-31 (D-Arg-2',6'-Dmt-Lys-Phe-NH_2) to preserve mitochondrial integrity was tested in a high-resolution respirometer (Oxygraph-2k; Oroboros Instruments, Innsbrück, Austria). SS-31 was synthesized, and checked for quality and purity by the LUMC peptide synthesis facility.
Experiments were performed on human kidney biopsies (n=6) that were collected during elective nephrectomy for oncologic indications. Kidney biopsies from a healthy kidney segment were taken immediately prior to clamping of the renal vasculature and stored on ice. Due to unavoidable transfer from the operating room to the research facility, all human biopsies were exposed to 60 min cold ischemia (2°C).
Warm ischemia was simulated by maintaining biopsies in a warm water bath (37°C) for 60 minutes. The effect of the mitochondrial membrane-stabilizing peptide SS-31 on renal tissue was tested by adding SS-31 (100 nM) to the storage and incubation medium.
Upon simulating cold and warm ischemia, the tissue was washed and permeabilized in saponin for 30 min at 4°C. Subsequently, the tissue was washed in mitochondrial respiration solution (MiR05, Oroboros Instruments, Innsbrück, Austria), containing 0.5 mM ethylene glycol tetraacetic acid, 3 mM magnesium chloride, 60 mM K-lactobionate, 20 mM taurine, 10 mM potassium dihydrogen phosphate, 20 mM HEPES, 110 mM sucrose and 1 $g.L^{-1}$ fatty acid-free BSA (pH 7.1). Tissue was then weighed and placed in the measurement chamber of the high-resolution respirometer and incubated at 37°C. To avoid oxygen diffusion limitation, oxygen concentration was increased to 400 µM and maintained above

270 µM throughout the experiment by adding pure oxygen.
The integrity of the outer-mitochondrial membrane was tested by adding 10 µM cytochrome C; samples with a ›15% increase in respiratory rate were excluded from further analysis. Leak respiration was assessed by adding the Krebs cycle intermediates sodium glutamate (10 mM), sodium malate (2 mM), and sodium pyruvate (5 mM). ADP-stimulated respiration was measured in 2.5 mM ADP. Maximal respiration, with simultaneous input of electrons through complex I and II, was measured through addition of 10 mM succinate. Maximal uncoupled respiration was measured after stepwise addition of 0.01 µM carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP). Subsequently, complex I was blocked by rotenone (0.5 µM). Finally, antimycin A was added to inhibit complex III, and residual oxygen consumption measured (non-mitochondrial respiration), which was subtracted from all values. All measurements were performed simultaneously and subsequently averaged. Values were mass-normalized, and reported as pmol $O2^{s-1.mg-1}$. The contribution of complex I function to the uncoupled maximal respiration was assessed by the relative decline of respiration after adding rotenone.

STATISTICS

Samples size was chosen based on previous studies on ischemia/reperfusion injury in human kidney transplantation. These studies validated our sample sizes to be of sufficient power to differentiate between groups.[9,10,13]
SPSS 22.0 (SPSSinc, Chicago, III) was used for statistical analysis.
For the plasma lactate and pH measurements, the area under the curve (AUC) was estimated and compared through a linear mixed model analysis for arterial and venous measurements for the total of 30 min. The model contained as independent variables time as categorical, the group, and the interaction between group and time. The covariance model was specified as unstructured. The delta AUC was calculated (venous minus arterial) and the null hypothesis (AUC=0) was tested by a Wald test based on the estimated parameters of the linear mixed model.
Raw data of the microarray were analyzed by biostatistical methods using average replicate values for each group of samples. Log2 ratios were computed and one value per gene was calculated for the average expression
Maximal mitochondrial respiration values were compared between t=0 min and prolonged warm ischemia and the effects of SS31 were tested with ANOVA.

RESULTS

Characteristics of the patient groups are summarized in Table 1 and Supplementary Figure 1. This report is based on sequential studies in which a total of 85 patients were enrolled. Twelve patients refused to give informed consent and 7 patients were excluded due to cancelled surgery. Postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. All living donor procedures showed immediate renal functional recovery. More than 50% of the deceased donor grafts developed DGF.

Table 1. Patient characteristics

	Donor			Recipient		
Group	Donortype (%)	Age (yr)	Male (%)	Age (yr)	Male (%)	CIT (min)
Living (n=27)	Living=100	53.3±1.7	44	50.7±2.8	63	217.3±3.7
-DGF (n=17)	DBD=58	53.3±4.6	53	58.1±2.4	58	791.9±45.3
	DCD=42					
+DGF (n=22)	DBD=27	53.4±3.2	73	56.6±2.8	59	996.9±60.0
	DCD=73					

Ages and cold ischemia time are expressed as mean±SEM. Patients were included in 3 recruiting rounds (see flow chart in Supplementary Figure S1). Recipients of living donor grafts were taken as a reference because DGF is rare in this group. Deceased donor grafts were classified based on outcome after transplantation. DGF is the status in which the transplant recipient is in need of dialysis in the first week(s) after transplantation and is primarily caused by ischemia/reperfusion injury. Duration of cold ischemia was significantly different between groups (analysis of variance: P < 0.01).

TISSUE AND PLASMA METABOLITES

We first assessed net lactate release from the reperfused graft as read-out of metabolic competence. Figure 1 shows arterial (red) and venous (blue) lactate levels over the reperfused kidney. Reperfused living donor grafts and deceased donor grafts without delayed graft function (-DGF grafts) show an almost instantaneous converging of arterial and venous plasma lactate levels, indicating immediate cessation of lactate release following washout of accumulated lactate. In contrast, persistent net lactate release (net graft lactate release 30 minutes after reperfusion (*mean (±SEM)): 1.7 (±0.67) mmol in grafts with DGF (+DGF) vs. 0.0 (±0.04) and 0.0 (±0.05) in resp. living and –DGF grafts (P<0.000038*)), and persistent metabolic acidosis (*venous pH: 7.22 (±0.06) in +DGF grafts vs. 7.33 (±0.02) and 7.33 (±0.0215) in resp. living and –DGF grafts (P<0.004*), were observed for grafts with delayed graft function (+DGF grafts) (Sup.Fig.2).

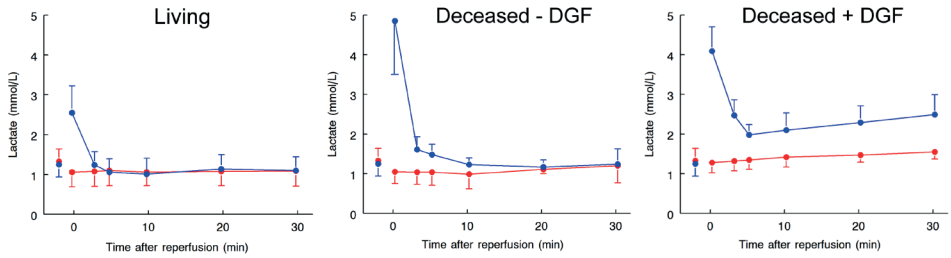


Figure 1. Persistent post reperfusion lactate release from +DGF grafts.
Arterial (red) and renal vein (blue) lactate levels (mean(±SEM)).
Left) Grafts from living donors (n=11) show absence of lactate release after a brief period of lactate washout upon reperfusion, which indicates an almost instantaneous recovery of aerobic respiration.
Middle) There is an increase of accumulated lactate in –DGF grafts (n=5), but instant recovery of aerobic respiration.
Right) Persistent (P‹0.000038) lactate release from +DGF grafts (n=8) during the full 30-minute measurement window indicates persistent anaerobic respiration. (Lactate release measured 30 minutes after reperfusion: 1.7(±0.67) mmol in +DGF grafts vs. 0.0(±0.04) and 0.0(±0.05) in resp. living and – DGF grafts)
Data points left of the curve reflect references from healthy kidneys.

Data from the renal tissue biopsies followed these observations with recovery of tissue glucose content and negligible lactate in biopsies taken 45 minutes after reperfusion in living donor grafts (*glucose/lactate ratio before: 0.19 (±0.03) vs. after 0.90 (±0.16) reperfusion (P‹0.0039) and –DGF grafts: 0.28 (±0.07) vs. after 0.87 (±0.24) reperfusion (P ‹0.026),* Fig.2A). In contrast, +DGF grafts showed persistent high tissue lactate and absent glucose recovery (*before: 0.21 (±0.04) vs. after 0.22 (±0.06*) reperfusion, Fig.2A) resulting in a persistent low glucose/lactate ratio. These observations for lactate and glucose may indicate a temporal dominance of glycolysis as the dominant source of adenosine triphosphate (ATP) in +DGF grafts or alternatively (and non-exclusively), a situation of metabolic exhaustion. To test the latter, we assessed arteriovenous (AV)-differences for hypoxanthine, the end product of ATP catabolism. Figure 2B shows the hypoxanthine washout and immediate cessation of hypoxanthine release in living donor grafts and –DGF grafts, but persistent hypoxanthine release (*P‹0.0024) from +DGF grafts: 12.1 (±4.63) µmol hypoxanthine in +DGF grafts vs. 0.0 µmol (±0.67) and 0.6 µmol (±0.53) in living and. –DGF grafts*). This observation points to ongoing post-reperfusion ATP catabolism in +DGF grafts.

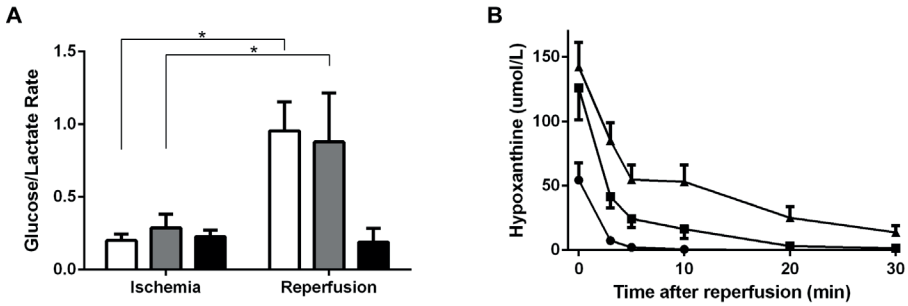


Figure 2. Readouts of tissue metabolic competence.
A) Recovery of tissue glucose/lactate ratio (mean(±SEM)) in living (n=6) and –DGF grafts (n=6) (Resp. living: glucose/lactate ratio before 0.19(±0.0) vs. after 0.90(±0.16) reperfusion (P‹ 0.0039) and –DGF grafts: before 0.28(±0.07) vs. after 0.87(±0.24) reperfusion (P‹0.026)) within 45 minutes of reperfusion, but persistently low ratio in +DGF grafts (n=6) (P‹0.927) (before 0.21(±0.04) vs. after 0.22(±0.06) reperfusion). Glucose/lactate ratio postreperfusion was significantly higher in –DGF than in +DGF grafts (P‹0.0019).
White bars represent living donor grafts, gray represent –DGF and black represent +DGF grafts.
2B) Increased washout of accumulated hypoxanthine (mean(±SEM)) in –DGF (n=5) and + DGF grafts (n=8) versus living donors (n=11). There is a cessation of hypoxanthine release in living and –DGF grafts, but persistent release from +DGF grafts (P‹0.0024) (12.1(±4.63) µmol hypoxanthine in +DGF grafts vs. 0.0(±0.67) µmol and 0.6(±0.53) µmol in living vs. –DGF grafts).
Circles represent living donor grafts, squares represent –DGF and triangles represent +DGF grafts.

GENE EXPRESSION PROFILES

From the above findings, the picture emerges of failing metabolic recovery as a critical determinant of later DGF. Given the high metabolic demands of gene transcription[30] we used the postreperfusion transcriptome as a readout of metabolic competence. We first established early reperfusion-related changes in transcriptome of living donor grafts (i.e. gene expression profiles in paired kidney biopsies taken immediately before and 45 minutes after reperfusion) as a reference. Functional analysis (ingenuity pathway analysis platform) showed that early reperfusion of living donor grafts is dominated by up-regulation of redox response networks and a broader, more moderate up-regulation of predominantly metabolism-associated gene networks (Sup.Fig.3A). Findings for the deceased donor grafts showed clearly reduced gene transcription with a less outspoken

up-regulation of redox response networks and no up-regulation of metabolic networks (Sup.Fig.3A).
A comparison of baseline gene expression profiles of living donor versus deceased donor grafts showed that these differences in the transcriptome were reperfusion related (Sup.Fig.3B).
Collectively, these findings imply graded postreperfusion metabolic responses in the different graft types. In living donor grafts an almost immediate metabolic and functional recovery of reperfused grafts was seen: immediate reinstatement of aerobic respiration, urine production and gene transcription. An intermediate metabolic recovery (rapid normalization of tissue glucose and lactate levels, but impaired functional recovery (delayed recovery of urine production and minimal gene transcription) was found in –DGF deceased donor grafts. +DGF grafts are characterized by an inadequate metabolic and functional recovery: persistent anaerobic respiration, absence of urine production necessitating dialysis and minimal gene transcription or even transcriptional anergy.[31]

MITOCHONDRIAL FUNCTION AND MORPHOLOGY

Persistent anaerobic respiration and impaired functional recovery in the presence of an adequate nutrient and oxygen supply in reperfused deceased donor grafts is suggestive of mitochondrial failure following reperfusion. Cytochrome C decompartmentalization has been described as a marker of loss of mitochondrial integrity after experimental I/R injury.[32]
We performed cytochrome C staining on pre- and post-reperfusion human kidney biopsies. A discrete punctuate staining of cytochrome C was observed in pre- and post-reperfusion biopsies from living donors, as well as in prereperfusion biopsies from deceased donor kidneys (both –DGF and +DGF). Postreperfusion biopsies from deceased donor grafts on the other hand show diffuse cytochrome C staining in the cytoplasm, consistent with loss of mitochondrial integrity. (Fig.3A – Sup. Fig.4) These qualitative observations were confirmed by reflection-contrast microscopy and transmission electron microscopy. (Fig.3B) Subtle abnormalities in mitochondrial morphology were present at baseline, but exaggerated after reperfusion. Moreover, transmission electron microscopy for the living and – DGF donor grafts shows decrease of mitochondrial swelling and recovery of mitochondrial morphology 45 minutes after reperfusion. In contrast, transmission electron microscopy images of +DGF grafts reveal deterioration of mitochondrial morphology, with disrupted inner and outer membranes, fragmented mitochondria and mitochondrial loss 45 minutes after reperfusion (Fig.3C).

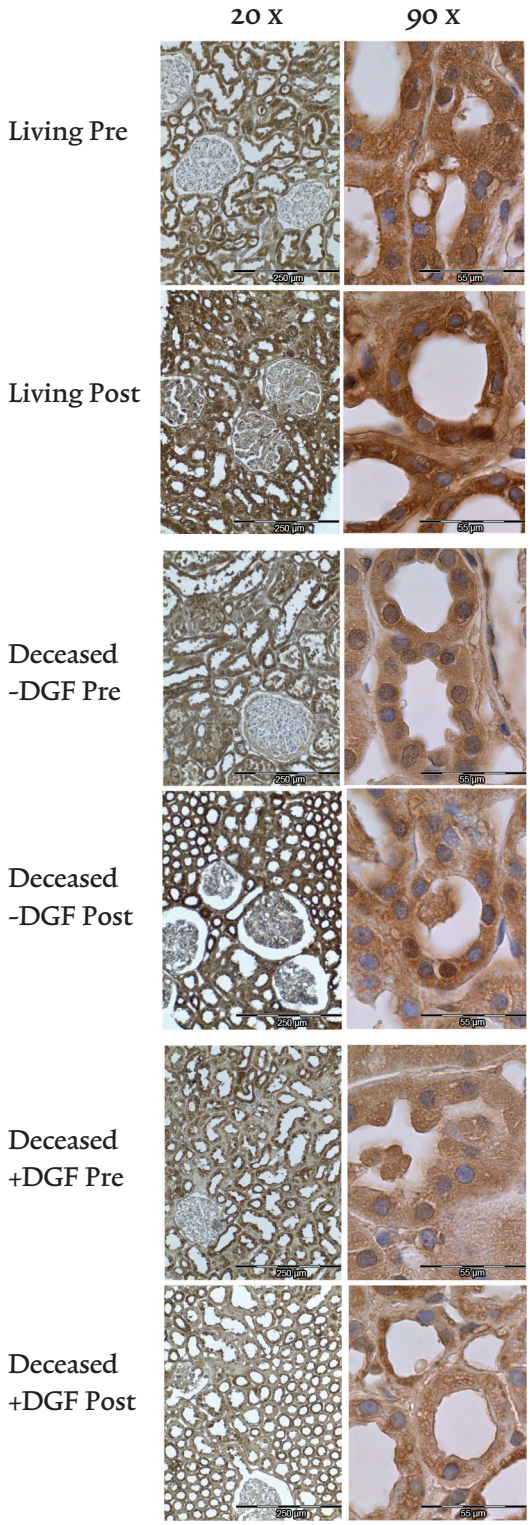
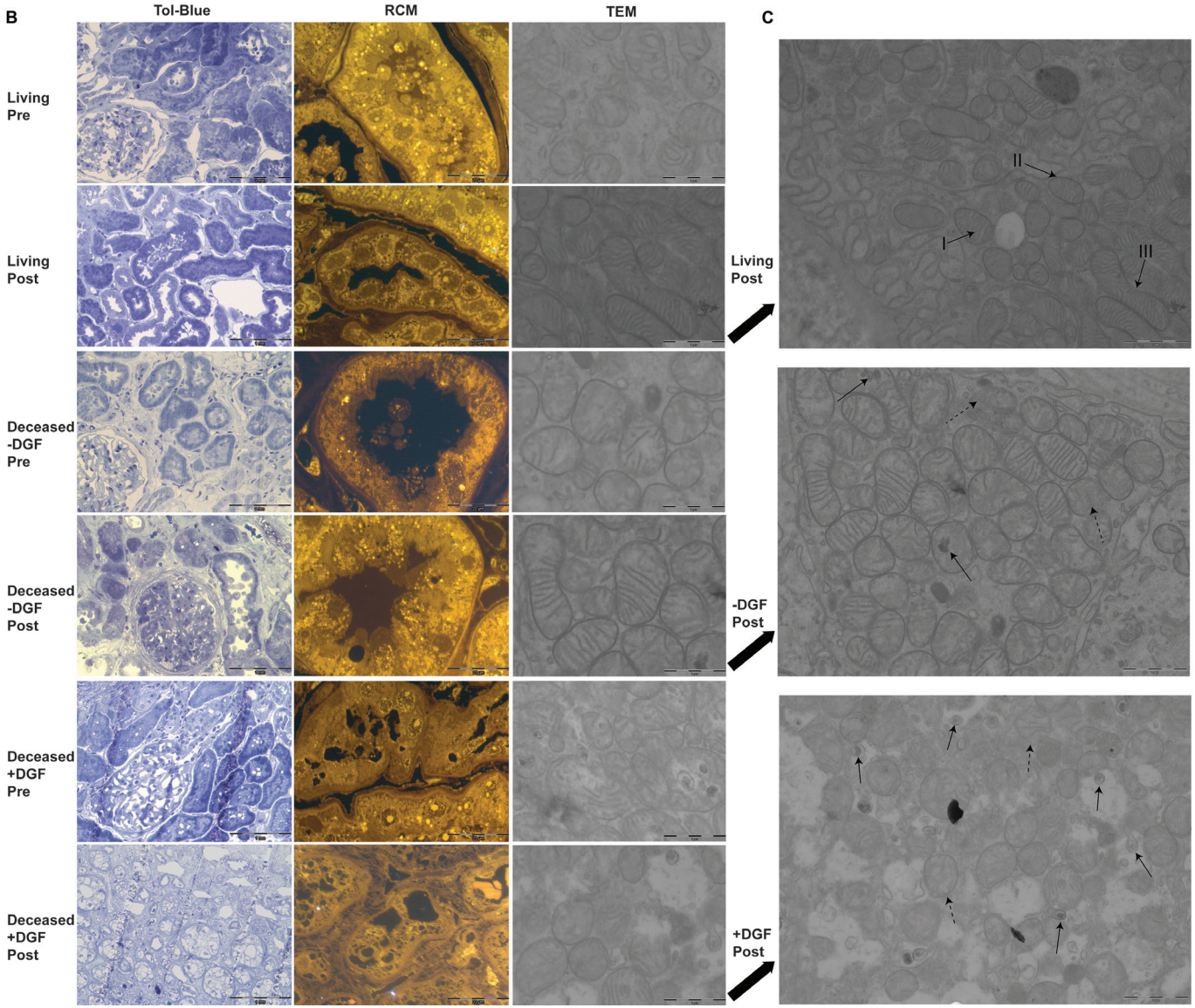


Figure 3. Overview of mitochondrial morphology in pre- and postreperfusion biopsies

A) Cytochrome C immunohistochemistry (Overview (left): 20-fold; detail (right): 90-fold). Cytochrome C staining in biopsies taken at the end of the ischemic period and 45 minutes after reperfusion shows loss of the punctuate staining pattern in –DGF and +DGF grafts. Loss of the punctuate pattern reflects cytochrome C decompartmentalization as a result of loss of mitochondrial integrity.[32] (Higher resolution images are shown in Supplementary Figure 4). (Pre- and postreperfusion kidney biopsies (n=18: Living (n=5), -DGF (n=5), +DGF (n=8))


B
Tol-Blue
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Living Pre
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II
III

Figure 3. Overview of mitochondrial morphology in pre- and postreperfusion biopsies

B) Overview of Toluidine-Blue staining (20-fold) (left), reflection contrast microscopy (900-fold) (middle) and Transmission electron microscopy (20.000-fold) (right) of biopsies taken before and after reperfusion.
Extensive reperfusion related qualitative changes with reduced mitochondrial content and loss of mitochondrial morphology in +DGF grafts.
(n=26: Living (n=9), -DGF (n=7), +DGF (n=10))
Living donor kidneys showed swollen ('donut-shaped') mitochondria with disorganized cristae and intact membranes at the end of the ischemic period. In the postreperfusion phase, mitochondrial recovery was found with decline of swelling, normal mitochondrial circumference, double membranes and well-organized cristae. (Fig.3C Living Post)
-DGF showed mitochondria with dysmorphological features at the end of the ischemic period (Pre) equal compared to the living donor kidneys. Mitochondria were swollen and cristae were disorganized, though membranes were intact. In biopsies taken 45 minutes after reperfusion (Post), decrease of mitochondrial swelling resulted in restoration of mitochondrial morphology. (Fig.3C –DGF post)
+DGF showed swollen mitochondria with disorganized cristae, electron dense granules (circular / convoluted cristae) organized in an irregular pattern. Opposite to the adequate functioning kidneys, postreperfusion biopsies did not show any repair, and mitochondrial morphology even deteriorated. After reperfusion, mitochondria were fragmented, with disrupted inner and outer membranes and mitochondria seemed almost diluted in the cytoplasm. (Fig.3C +DGF post)

C) Living Post. Details of proximal tubule cell of a living donor graft 45 minutes after reperfusion. Well-organized mitochondria with normal patterned cristae (I), intact double membranes (II) and a physiological variance of mitochondrial size (III). Transmission Electron Microscopy (TEM) image (20.000 fold) – scale bar: 1µm.
-DGF Post. Details of proximal tubule cells of a –DGF graft 45 minutes after reperfusion. Note presence of mitochondrial swelling, presence of mitochondrial convolutes (solid arrows) and disorganized christae (dashed arrows). TEM image (20.000 fold) – scale bar: 1µm.
+DGF Post. Details of proximal tubule cells of a +DGF graft 45 minutes after reperfusion showing profound loss of mitochondrial morphology with fragmented mitochondria, mitochondrial convolutes (solid arrows) and disorganized christae (dashed arrows). TEM image (20.000 fold) – scale bar: 1µm.

EFFECTS OF THE MITOCHONDRIAL STABILIZER SS-31

Whereas living donor grafts and deceased donor grafts are both exposed to a considerable period of cold ischemia, the 2 types of grafts clearly differ in their exposure to warm ischemia. Whereas living donor grafts experience minimally exposure to warm ischemia, grafts from deceased donors, in particularly grafts from donors donating after a circulatory death are exposed to considerable periods of warm ischemia (Sup.Table 1). Because the lactate and hypoxanthine data points to mitochondrial failure in –DGF grafts we reasoned that warm ischemia as the greatest common divisor could induce mitochondrial damage. Following this it was hypothesized that preventive interventions stabilizing mitochondria would preserve metabolic competence. These hypotheses were tested by simulating warm ischemia in human kidney biopsies, followed by reoxygenation of the biopsies in a high-resolution respirometer. Experiments were performed in the presence and absence of SS-31, an archetypical mitochondria-stabilizing peptide. Figure 4 shows that exposure of human kidney biopsies to 1 hour of warm ischemia results in a profound reduction of overall oxidative phosphorylation (Fig.4A))(*oxygen consumption dropping from: 24.8 (±3.77) vs. 11.2 (±2.41) pmol* $O_2^{s-1.mg-1}$*,(P<0.016,* Fig.4A) and reduced complex I activity (a*ctivity dropping from 40 (−7) to 31(±4)%, P<0.005* (Fig.4B). These effects are largely prevented by including SS-31 in the incubation medium (overall oxidative phosphorylation: *oxygen consumption before warm ischemia: 28.8 (±5.53) vs. 21.2 (±5.79) pmol* $O_2^{s-1.mg-1}$ *after 60 minutes of warm ischemia) (complex I activity resp. (54% (±5) vs. 52% (±4) before and after 60 minutes of ischemia*).[33,34]

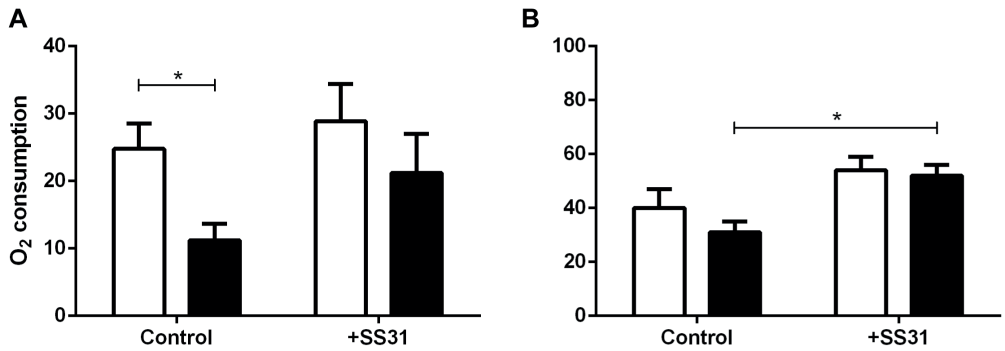


Figure 4. The mitochondrial stabilizing peptide SS-31 prevents ischemia-induced loss of mitochondrial function.
A) Maximal mitochondrial (uncoupled) respiration (pmol $O2^{s-1.mg-1}$). SS-31 preserves maximal oxidative capacity (mean(±SEM)) following 60 minutes of warm ischemia. With SS-31: 28.8(±5.53) before warm ischemia vs. 21.2(±5.79) pmol $O2^{s-1.mg-1}$ after 60 minutes of warm ischemia, compared to without SS-31: 24.8(±3.77) before warm ischemia vs. 11.2(±2.41) pmol $O2^{s-1.mg-1}$ after 60 min warm ischemia (P‹0.016). (n=6)
White and black bars, respectively, represent before and 60 minutes after warm ischemia maximal respiration.
B) Relative contribution (%) of complex I to maximal oxidative phosphorylation. SS-31 prevents loss of the contribution of complex I to the overall respiration (mean(±SEM)) following warm ischemia. With SS-31: 54(±5)% before warm ischemia vs. 52(±4)% after 60 minutes of warm ischemia, compared to without SS-31: 40(±7)% before warm ischemia vs. 31(±4)% after 60 minutes of warm ischemia (P‹0.005). (n=6)
White and black bars, respectively, represent before and 60 minutes after warm ischemia.

ROLE FOR ROS

All observations point to an association between mitochondrial failure-related metabolic incompetence and incident DGF. A critical question is how these observations relate to the perceived pivotal role for oxidative stress in general, and in particular to the recently proposed critical role for succinate-driven reactive oxygen species (ROS) formation[19] in the development of I/R injury.

The postperfusion transcriptomes of both living and deceased donors show activation of the NRF-2 redox-response pathway, indicative of redox stress during early reperfusion. Yet it is unclear whether the redox stress relates to excess ROS

formation. To that end, we quantified allantoin release (AV-differences) from the kidney grafts. In humans, allantoin is the stable end product of the antioxidative action of uric acid. As such, formed allantoin reflects the amount of ROS quenched by uric acid.[35] As shown in Figure 5 allantoin release was minimal and similar in the 3 donor groups, an observation that points to minimal radical scavenging by uric acid during reperfusion. These observations challenge a prime role for ROS in general and specifically a central role for succinate-driven ROS formation in the context of kidney transplantation/DGF.[19] In this light, we tested whether the apparent succinate accumulation during (warm) renal ischemia observed in rodents[19] translates to the context of human kidney transplantation. Whereas the data confirm progressive hypoxanthine accumulation during ischemia, ($P<0.012$, Sup.Fig.5A and Fig.2B) succinate levels do not increase during progressive ischemia and did not relate to incident DGF (Sup.Fig.5B). In fact it was observed that tissue succinate levels decline during progressive ischemia ($P<0.00061$).

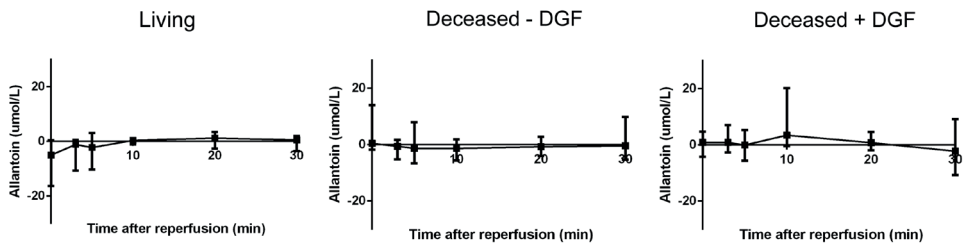


Figure 5. Absence of allantoin release from living, -DGF and +DGF grafts in the first 30 minutes after reperfusion.
Allantoin, the final end product when uric acid performs as reactive oxygen species (ROS) scavenger is not released following renal ischemia/reperfusion (mean(±SEM): +DGF (n=8), -DGF (n=5), living(n=11)).

DISCUSSION

This study shows a clear association between impaired postreperfusion metabolic recovery and incident DGF. Whereas reperfused living donor grafts and –DGF deceased donor grafts show an instantaneous recovery of aerobic metabolism following reperfusion, +DGF grafts do not. In fact, a persistent low tissue glucose/lactate ratio, and continued lactate and hypoxanthine release from +DGF grafts implies a profound postreperfusion metabolic deficit and failure to reinstate aerobic respiration. Altogether, these observations point to mitochondrial impairment in +DGF grafts, a notion that is confirmed by imaging studies and functional analysis. In vitro experiments on human kidney tissue show that mitochondrial integrity can be partially rescued by the cardiolipin-binding peptide SS-31.

Results from this study indicate metabolic recovery as a discriminative factor between –DGF and +DGF kidney grafts. Ideally, metabolic recovery would be assessed through tissue ATP content. Yet, ATP measurements require strict sampling conditions (clamp freezing) that cannot easily be met in the clinical transplantation setting.[36] Magnetic resonance spectroscopy is an alternative, indirect means of measuring tissue ATP content but again this technique cannot be applied during the actual transplantation procedure. In the past, 31P-magnetic resonance spectroscopy was used to establish the graft energy-status prior to transplantation.[37,38] These studies show that presence of high-energy phosphates strongly associates with a favorable outcome. Consequently, these observations hint at a close association between an adequate prereperfusion energy status and a favorable clinical outcome. Persistent hypoxanthine release from +DGF grafts extends these observations to the postreperfusion context, with continued hypoxanthine release reflecting persistence of ATP catabolism despite an apparent adequate oxygen and nutrient supply.[39] Plasma and tissue data for lactate show that this is at least partially related to a defective aerobic metabolism. Whereas living donor grafts and –DGF deceased donor grafts show an almost immediate cessation of lactate release upon reperfusion (AV-differences), and on the tissue-level reversal of tissue glucose/lactate ratio, +DGF grafts exhibited persistent lactate release during the full 30 minutes' postreperfusion measurement window and on the tissue level, a persistently low tissue glucose/lactate ratio. These observations for +DGF grafts imply failure to reconstitute aerobic metabolism.

It was realized that the +DGF group mainly comprised donors after circulatory death. An additional analysis on a larger series of timed AV samples (n=40) was performed that allowed us to differentiate between donortypes. Supplementary Figure 6 shows that absent recovery of aerobic respiration is linked to incident DGF rather than donortype.

We next tested whether these differences in metabolic recovery have functional consequences. Deceased donor grafts all experience a delayed functional recovery (clearance); as such we decided for changes in the transcriptome as functional readout of metabolic competence. Gene transcription comes at significant energy cost[30], and it was thus reasoned that positive changes in the transcriptome occurring during the early reperfusion phase constitute an indirect readout of energy reserve. Whereas the early reperfusion–related changes in the transcriptome of living donor grafts is dominated by up-regulation of the fast responding NRF-2 redox response pathway along with a broad, though less outspoken, but comprehensive up-regulation of (tissue) homeostatic pathways, changes in deceased donor grafts were limited to a more moderate (and dose-dependent) up-regulation of the NRF-2 pathway[40], findings that are in line with the conclusions of Kusaka et al.[21] Observed enrichment for stress-related networks such as cell trafficking, hemostasis and apoptosis in grafts from cadaveric donors presumably reflects influx of inflammatory cells in these grafts prior to removal of the organ from the donor.[41] On basis of these findings one could envision a graded metabolic status with adequate postreperfusion energy supplies in living donors (immediate functional recovery (clearance) and compensatory translational activity), an intermediate metabolic deficit in –DGF grafts (partial recovery of aerobic respiration, yet energy supplies insufficient to immediately sustain functional recovery and comprehensive gene expression), and a profound metabolic deficit (ATP catabolism) that interferes with organ function and recovery in +DGF donor grafts.

Failure to reinstate aerobic respiration in the +DGF grafts points to a mitochondrial deficit, which was indeed confirmed by histological quantification and functional measurements (respirometry) following simulated I/R. Data from the histological analysis indicates deterioration of mitochondrial morphology in +DGF grafts upon reperfusion. However, available imaging tools lack sensitivity to discriminate between a state of failing aerobic respiration (+DGF) and a state of minimal aerobic respiration that meets residual homeostatic metabolic requirements (cessation of lactate release and normalization of tissue glucose/lactate ratio) in –DGF grafts.

The cardiolipin-binding peptide SS-31 has been shown to preserve mitochondrial integrity upon ischemia and reperfusion. This peptide is designed to penetrate the mitochondria and accumulates in the inner mitochondrial membrane. SS-31's mechanism of action is believed to be ROS-scavenging mediated by the phenolic group present in tyrosine.[42] Although absence of uric acid oxidation (allantoin formation) during early reperfusion and our previous studies on release of biomarkers of oxidative damage (isoprostanes; nitrite, nitrate, nitrotyrosine)[9] challenge a dominant role for ROS in I/R-related tissue injury during kidney

transplantation, it is important to point out that these markers reflect gross ROS stress (allantoin) and damage, and as such do not exclude a role for ROS on the microscale level as would be the case with oxidative damage at the level of the mitochondrial inner membrane.

At present SS-31 is the only mitochondria-targeting peptide that has entered clinical evaluation.[24] Given its specificity and the abundance of preclinical data we chose to include this peptide for the proof-of-principle studies.[43] SS-31 has proven effective in cultured renal tubular cells in which it preserved mitochondrial structure and accelerated ATP recovery upon reperfusion.[22] We extended these findings to human kidney tissue and found that incubation with SS-31 partially preserved respiratory chain complex I integrity upon simulated I/R. A randomized trial [EMBRACE-STEMI][24] in which SS-31 was infused in patients with a ST-elevation myocardial infarction did not show improved outcome.[44] Similar conclusions were reached for cyclosporine, which is also shown to stabilize mitochondria, but that also failed as rescue treatment for myocardial infarction.[45] Although these results appear to rule out an effect of these compounds as rescue treatments in the context of myocardial infarction, they do not exclude an effect in the context of transplantation for which preventive treatment is possible. Clinical results for other mitochondrial-stabilizing strategies such as melatonin are currently awaited[20,46], and promising compounds such as meclizine await clinical evaluation.[47,48]

On basis of the above findings, the picture emerges of adequate metabolic recovery as a critical determinant of outcome after kidney transplantation. Mitochondrial failure as result of I/R results in a state of severe metabolic exhaustion that may not only severely impact cellular homeostasis, but also may lead to a state of transcriptional and translational anergy[31], paralyzing the cellular response networks, and rendering the cells insensitive to internal and external signals. These observations imply that the best strategy for alleviating I/R injury should be focused on mitochondrial preservation[49] or mitochondrial recuperation.[46,49] Our previous work,[9] and the current observations for allantoin and succinate challenge a role for succinate-controlled reperfusion injury[19] in the context of graft I/R injury. It was realized that I/R injury increases the antigenic load of the graft and therefore enhance allogenic response upon transplantation. However, results from our study and our earlier studies clearly raise DGF as reflection of I/R injury instead of an immunological issue.[9-11,13,16,50]

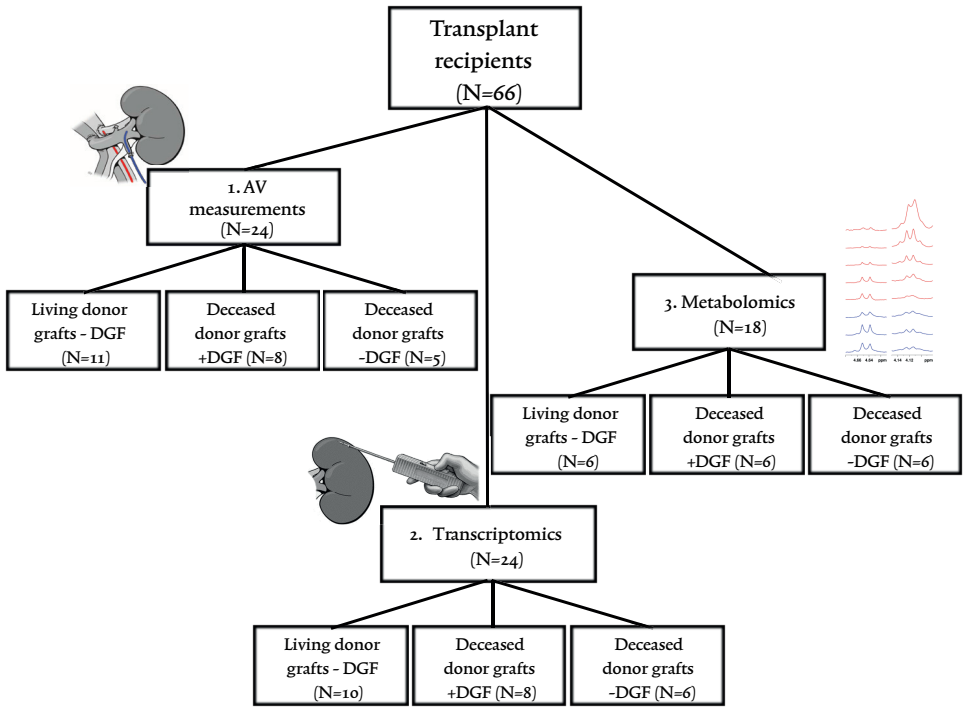
Limitations: This is a relative small study and as such we were unable to address specific points such as the influence of specific donor characteristics, types of anesthesia used during transplantation, or specific recipient characteristics. Moreover, no machine preservation was used in this cohort. However, despite the small sample sizes of our patient cohorts, this study allowed us to reveal

striking differences between the different study groups. A larger sample size would not change the conclusions of the study but would rather result in smaller confidence intervals. Finally, regarding the inherent problems with quantifying mitochondrial damage, we decided for a qualitative presentation of the imaging data.

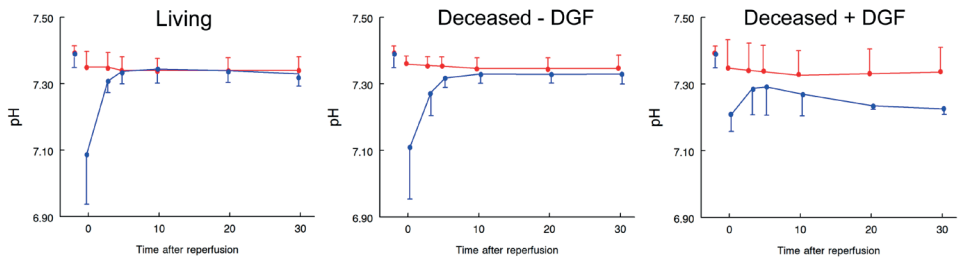
Although these studies have been performed in the context of kidney transplantation it came to our attention that observations from this study may also apply to other clinical situations of ischemia and reperfusion, such as myocardial infarction and cerebrovascular accidents. For the context of kidney transplantation, results from this study suggest that preserving mitochondrial integrity and metabolic competence[51] is critical in the prevention of DGF.

Supplementary Table 1 is published online

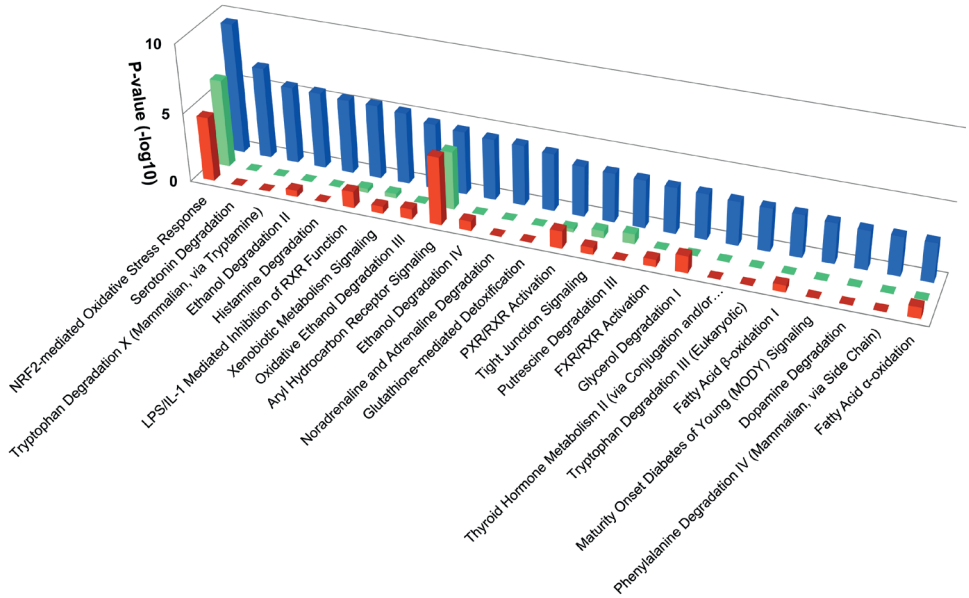
SUPPLEMENTARY FIGURES



Supplementary Figure 1. Flow chart describes the enrolment of patients. Patients were included in 3 recruiting rounds (1-2-3). Kidney transplants were divided based on outcome of transplantation. Living donor grafts functioned as reference group, deceased donor grafts were classified as +DGF when they were in need of dialysis in the first week(s) after transplantation. Characteristics of the patient groups are summarized in Supplementary Table 1.

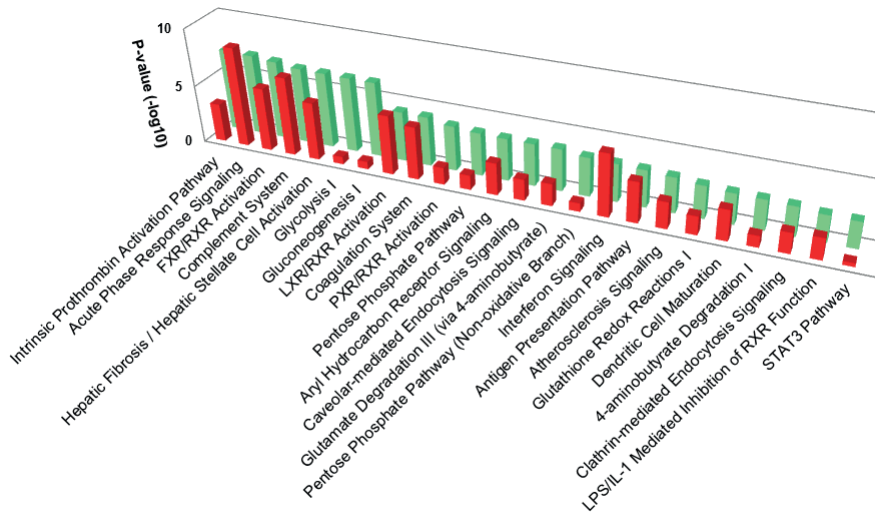


Supplementary Figure 2. Persistent acidosis upon reperfusion of +DGF grafts. Arterial (red) and renal vein (blue) pH (mean, SEM). Left + Middle) Rapid normalization of venous pH in living and –DGF grafts. Right) Persistent acidosis in +DGF grafts. (P‹ 0.004)



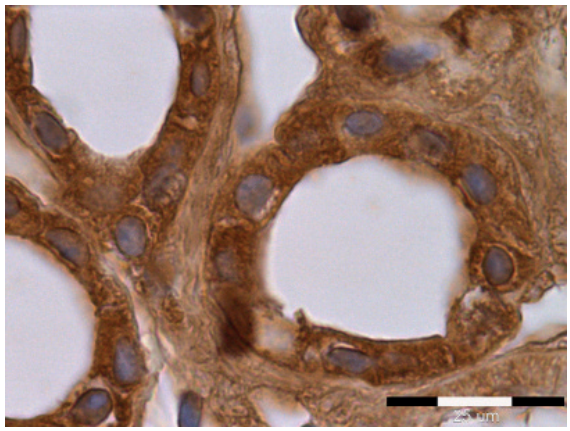
Supplementary Figure 3. Ingenuity Pathway Analysis–based analysis of gene expression.

Supplementary Figure 3A. Early reperfusion-related changes in the transcriptome. Differential gene expression in paired biopsies taken immediately before, and 45 minutes after reperfusion. The upper row shows the top 24 most significantly upregulated pathways (Ingenuity Pathway Analysis) in living donor grafts. Values provided are composite p-values based on the 0-hypothesis for each given pathway. Composition of these pathways is defined in the Ingenuity 'Biological Function' collection. For clarity reasons p-values are expressed as –log p-value.
Blue bars represent living donor grafts (n=10), green represent –DGF (n=6) and red represent +DGF grafts (n=8). Early reperfusion of living donor grafts is dominated by upregulation of genes belonging to the rapidly responding NRF2-redox response pathway ($P<10^{-9.46}$) and a broader, more moderate upregulation of predominantly metabolism-associated pathways. Findings for the deceased donor grafts were clearly distinct from the living donor grafts with a less outspoken upregulation of the NRF2 pathway ($P<10^{-6.33}$ and $<10^{-4.64}$, for –DGF and +DGF grafts respectively) and an exclusive upregulation of the aryl hydrocarbon receptor signaling pathway (a redox-stress responsive pathway)(Bhattacharya et al. Am J Physiol Lung Cell Mol Physiol 2014;30:516–23) but no upregulation of homeostatic networks that were upregulated in the living donor grafts.



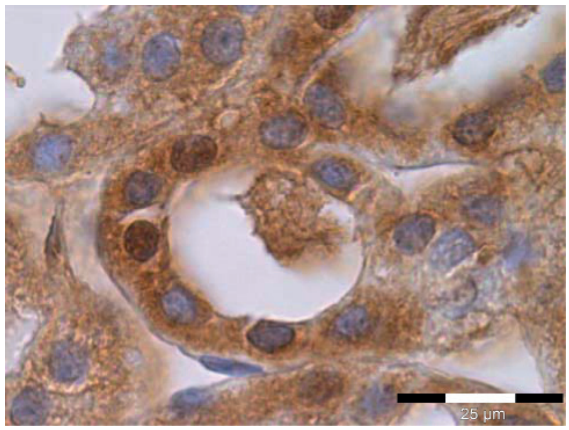
Supplementary Figure 3. Ingenuity Pathway Analysis–based analysis of gene expression.

Supplementary Figure 3B. Differences in the baseline (pretransplantation) transcriptome of –DGF (green) and +DGF (red) grafts versus the reference group of living donor grafts. Compared with reference (living donor grafts), prereperfusion transcriptomes of –DGF and +DGF grafts are hallmarked by increased expression of stress-related networks (e.g. inflammation, cell trafficking, and cell homeostasis). This observation presumably reflects influx of inflammatory cells in these grafts.[41] A further comparison of baseline gene expression in –DGF and +DGF deceased donor grafts showed similar baseline gene expression patterns, albeit with the notable exception of a more pronounced expression of genes belonging to glycolysis/gluconeogenesis pathways in –DGF grafts

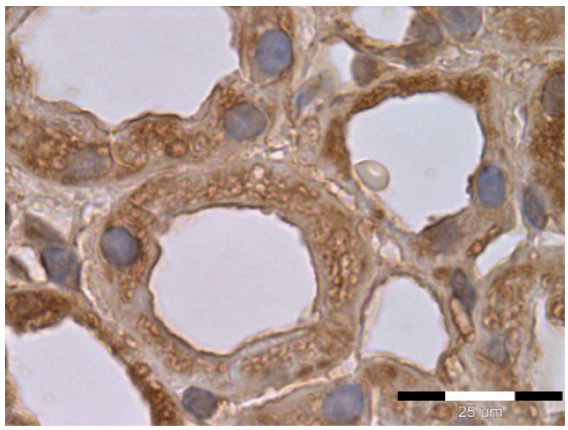


Supplementary Figure 4. Cytochrome C immunohistochemistry.

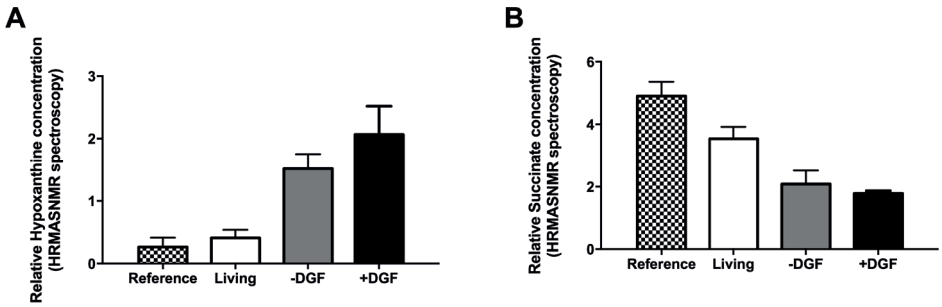
Supplementary Figure 4A. Cytochrome C staining (90 fold) of a living donor graft 45 minutes after reperfusion showing high density and punctuate stained pattern of Cytochrome C. (scale bar: 25 µm)



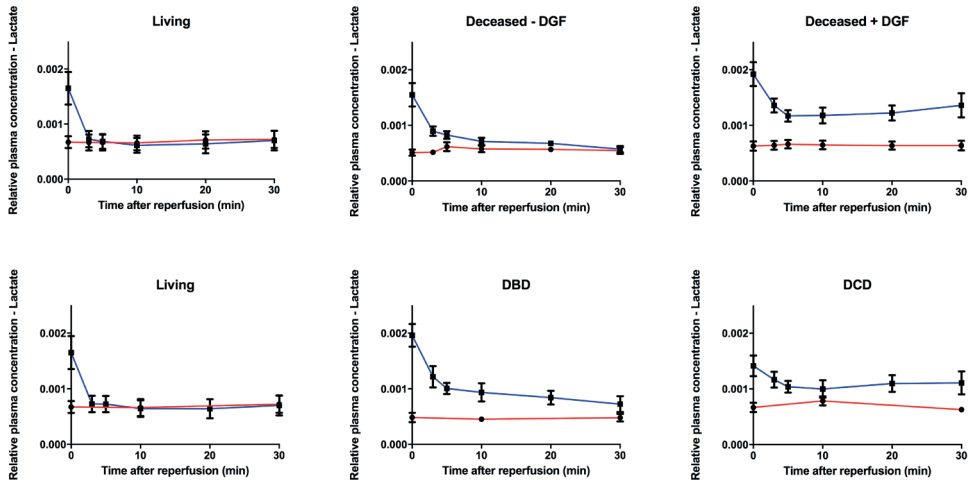
Supplementary Figure 4B. Cytochrome C staining (90 fold) of –DGF kidney 45 minutes after reperfusion showing sustained punctuate pattern of Cytochrome C. (scale bar: 25 µm)



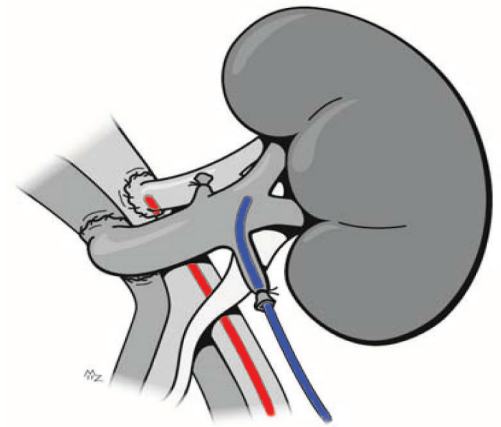
Supplementary Figure 4C. Cytochrome C staining (90 fold) of +DGF kidney 45 minutes after reperfusion showing loss of the punctate pattern of Cytochrome C. (scale bar: 25 µm)



Supplementary Figure 5. Tissue hypoxanthine (A) and succinate (B) content of non-ischemic references, and ischemic renal tissue.
Chequered bars represent non-ischemic references. White bars represent living donor grafts (ischemia time (mean(SEM): 217(4) min) grey and black bars represent deceased donor grafts: –DGF (grey) and + DGF (black bars) (ischemia times 792(45) min and 997(60) min resp.). Hypoxanthine content increases (P ‹ 0.012), whereas succinate levels decline (P ‹ 0.000061) during ischemia. Hypoxanthine and succinate contents in – and + DGF grafts were similar.



Supplementary Figure 6. Persistent lactate release relates to incident DGF, rather than donation status.
Plasma lactate levels (semi-quantitative NMR data) were analysed in plasma samples that were available from previous studies. The larger samples size (n=40), allowed for analysis based on donortype. Top row: persistent aerobic respiration (viz. continous lactate production) associates with incident DGF. These findings mirror those obtained by the quantitative analysis (Figure 1). The lower row shows similar lactate responses for DCD and DBD donor grafts. Hence, persistent lactate release associates with incident DGF rather than donor status.



Supplementary Figure 7. Arteriovenous blood sampling over the reperfused kidney. Arterial (red) venous (blue) concentration measurements were measured over the reperfused kidney. Arterial blood was sampled through cannulation of the epigastric artery and the effluent, venous renal blood was sampled through cannulation of the renal vein via one of its side branches (gonadal vein).

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