ISCHEMIA | REPERFUSION INJURY

A METABOLIC MELTDOWN

LEONIE WIJERMARS

Design: Leonie G.M. Wijermars
Cover: A. Willetts
Printing: Ridderprint
ISBN: 978-94-6299-880-3

Financial support by the Dutch Kidney Foundation, de Nederlandse Transplantatie Vereniging, Pfizer BV, Sanofi-Aventis and Chipsoft for printing this thesis is gratefully acknowledged.

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PROEFSCHRIFT
ter verkrijging van
de graad van Doctor aan de Universiteit Leiden
op gezag van Rector Magnificus Prof. Mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 21 maart 2018
klokke 11.15 uur

door
Leonie G.M. Wijermars
Geboren te Zwolle op 28-12-1989

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GENERAL INTRODUCTION

Leonie G.M. Wijermars

PROBLEM DEFINITION

Kidney transplantation is currently the only curative option in end-stage renal disease, with some 80,000 people globally receiving a kidney transplant each year. Thus the success rate of transplantation surgery is critical, but graft failure is often a major problem. The exact mechanisms that lead to graft failure for both the short- and longer-term are unknown, however the damage resulting from the period of graft ischemia and reperfusion (I/R) is a major contributor.
I/R injury following kidney transplantation leads to delayed graft function (DGF)[1] in the short term. DGF is defined as the situation in which the recipient of the kidney transplant is dialysis dependent for the first week(s) after transplantation.[2] Besides the negative short-term effects of DGF (increased morbidity for the graft recipient and higher costs of prolonged hospitalization), DGF also impairs the long-term transplantation outcome. For example, incident DGF increases the risk of chronic allograft nephropathy and impairs long-term graft survival.[1,3,4]
Donor organ shortages force the use of so-called 'expanded criteria donor' organs in kidney transplantation. These organs are either donated after a cardiovascular accident or donated by an older donor (›60 yrs. or 50-59 yrs. with comorbidities).[5] Use of these grafts leads to an higher incidence of DGF and the associated negative consequences.[1,6] Over the past decades millions of dollars have been spent on research focused on finding interventions to limit I/R injury. However, promising results from preclinical studies could not be translated to the clinical setting - this translational gap is yet to be bridged.

OBJECTIVE OF THIS THESIS

This thesis aims to increase the knowledge concerning (human) pathophysiological mechanisms of I/R injury. Unravelling the underlying mechanisms of I/R Injury is the first step to identifying targets for limiting or preventing injury and thereby improving transplantation success rates. To achieve this, this thesis describes an unbiased, genome-broad, metabolome-broad and epidemiological approach to the mechanisms of I/R injury in the clinical setting of kidney transplantation.

BACKGROUND

For decades, transplantations have been an area of major interest and research efforts have resulted in significant developments and new discoveries. Since the first successful living-related donor kidney transplantation in 1954, significant improvements have been achieved in both patient and graft survival. One of the first major breakthroughs was the discovery of the human leukocyte antigen (HLA) system - a gene complex encoding the major histocompatibility complex proteins. HLA typing instigated international HLA matching programs for donor organs.[7]

Later, the induction and maintenance immunosuppression regiments were introduced to reduce the risk of acute rejection.[8,9] Another important advance was the development and optimization of graft preservation fluid (i.e. UW, HTK).[10,11] However despite all these successes, incident DGF following kidney transplantation remains a major problem.

I/R INJURY IN KIDNEY TRANSPLANTATION

Several promising preclinical studies have been published on limiting I/R injury in kidney transplantation. The main objective has been suppressing the inflammatory response or the adaptation of the immune system of the recipient.[6] Much attention has been given to ischemic pre- and post-conditioning. It is hypothesized that pre-/post-conditioning activates anti-inflammatory, neuronal and humoral signalling pathways – potentially protecting the reperfused graft.[12] Other examples of widely studied anti-inflammatory interventions are the administration of vitamins[6] / antioxidants[13] (i.e. n-acetylcysteine[14]) and volatile anaesthetics[15].

Generally these interventions showed positive results in preclinical models of kidney transplantation, but evidence of clinical effectiveness is still lacking.[16,17] (i.e. ischemic pre- and post-conditioning[18–21]; anaesthetic preconditioning[22]; vitamins/antioxidants[23] (N-acetylcysteine[24]) and EPO administration[25]). It appears that the translation of interventions reducing I/R injury from bench to bedside is limited.[26–29]

In addition to interventions that are administrated after reperfusion, many studies focus on the preservation phase.[30,31] Currently, static cold storage and machine perfusion are the two main options for donor kidney preservation. Static cold storage has proven to be a consistent, reliable method; however as the composition of preservation fluids has not altered over the last decennium further innovations are not expected.[32] Machine perfusion has rapidly developed over the past years and recently a meta-analysis concluded that short-term outcomes of hypothermic machine perfusion are superior to those of static cold storage. However long-term outcomes are less clear[33] and results of new machine (varying the perfusion fluid, hypo- versus normothermic and oxygenated protocols) must be awaited.[33–36]

GENERAL MECHANISMS OF I/R INJURY

The mechanism of I/R injury is multifactorial and complex, comprising a metabolic imbalance and microvascular dysfunction during ischemia which leads to activation of innate and adaptive immune responses and cell death programs after reperfusion.[37] Several pathways have been suggested as culprit mechanisms including: vascular leakage (endothelial activation), no reflow phenomenon (endothelial/thrombogenic), complement activation, cell death programs

(apoptosis, necrosis, autophagy) and innate and adaptive immune activation.[37]
With regards to the translational challenges, the NIH Consortium for "preclinicAl AssESsment of CARdioprotective therapies" (CAESAR) wrote:
'*For 40 years, the National Heart, Lung, and Blood Institute (NHLBI) has invested enormous resources (at least several hundred million dollars) in preclinical studies aimed at developing infarct-sparing therapies, and several hundred (if not thousands) therapies have been claimed to limit infarct size in preclinical models. Unfortunately, due largely to methodological problems, this enormous investment has not produced any notable clinical application, and no cardioprotective therapy is currently available for clinical use.*'[17]
Consistent with the above, almost all therapies aiming to minimize I/R injury deducted from animal models lack proof in (sufficiently powered) randomized clinical trials (RCTs)[16,38,39] highlighting the lack of understanding of the pathophysiologic mechanism of I/R injury in humans.
Previous research by de Vries et al. on I/R injury in human kidney transplantation describes a systematic re-evaluation of the presumed mechanisms. De Vries' thesis concludes that mechanisms commonly implicated in experimental I/R injury do not play a key role in clinical kidney transplantation.[26–28,40] For example complement, thrombocyte and endothelial activation were all absent in I/R injury following kidney transplantation.
Cytokine release (IL-6, IL-9) was associated with I/R injury, but inhibition of these cytokines in an experimental model resulted in increased renal damage[28] implying a protective role for inflammatory processes in I/R injury. Remarkably the release of ROS (radical oxygen species)-mediated damage biomarkers after reperfusion could also not be demonstrated. This finding was rather controversial as in the field of I/R injury ROS generation is considered to be the major driver of I/R injury. The common concept of ROS being the major driver has led to extensive studies on antioxidants aiming to limit I/R injury. Despite finding antioxidants protection in experimental models, no antioxidant has proved itself to be effective in treating or preventing clinical I/R injury.[23,41] This supports our theory that the role of ROS is less important than commonly thought.

CONTENT OF THIS THESIS

This thesis contributes to bridging the translational gap from bench to bedside, laying a foundation for effective new treatment measures for I/R injury. Key questions addressed include:

> Why do promising preclinical findings not translate to clinical settings?
> Why did almost all clinical trials aiming to limit DGF (thus I/R injury) result in negative results?

Which pathophysiological mechanisms could underlie DGF after human kidney transplantation?
Which interventions could hypothetically decrease the chance of DGF after human kidney transplantation?
Which variables (donor / transplant procedure) affect the long-term outcome of kidney transplantation and does this have implications for the future donorpool?

In order to answer the questions above kidney transplantation was used to examine human I/R injury, as the I/R injured organ is accessible and the setting potentially offers consistency for the replication of experiments.

MODEL

During kidney transplantation, both initiation of ischemia and reperfusion are planned. The duration of ischemia is monitored and can be used as a variable. This unique situation allows kidney transplantation to be seen as "an experiment by nature". The studies summarized in this thesis are more difficult to perform in 'spontaneous' I/R like myocardial infarction or stroke. This is illustrated by the arteriovenous sampling method that was performed: the artery and renal vein were cannulated during the transplantation procedure and after initiation of reperfusion samples were taken on fixed moments.(Fig.1) Another benefit of transplantation compared to myocardial infarction and stroke is that transplantation always assures whole organ ischemia while infarct size differs between patients. Finally the transplantation setting advantageously provides a clearly quantifiable clinical readout of I/R injury: DGF.[1]

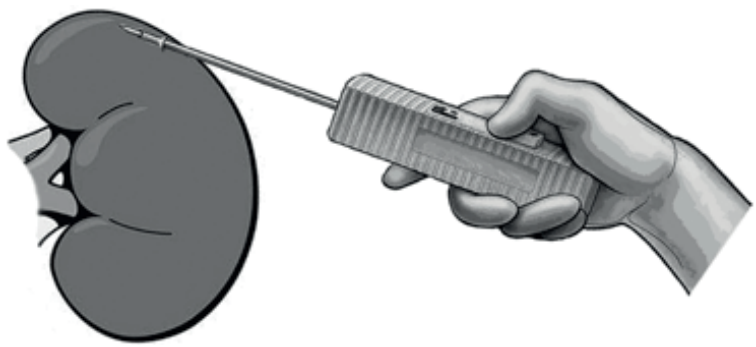

Figure 1A. Using kidney transplantation as a model of human ischemia/ reperfusion injury offers several benefits. During transplantation the kidney is accessible and biopsies can be taken during both ischemia and reperfusion.

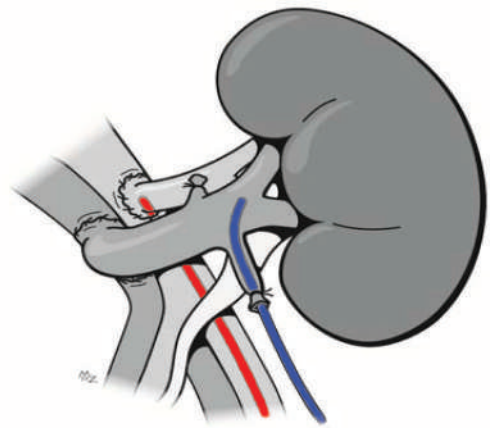

Figure 1B Kidney transplantation comprises a setting of whole organ ischemia, which enables arteriovenous measurements over the reperfused graft as a reproducible model.

OUTLINE OF THE THESIS

Following Chapter 1's general introduction to ischemia/reperfusion injury, the next 2 Chapters describe processes inconsistent with common I/R injury theories. Chapter 2 refutes the role of the hypoxanthine-XO (xanthine oxidase) axis in the development of DGF and discusses the role of ROS-mediated damage in I/R injury. Chapter 3 then examines whether succinate accumulation-induced ROS production drives I/R injury in human kidney transplantation. The Chapter pursues on interspecies differences in mitochondrial function and vulnerability against I/R, assessing the value of different animal models. Chapters 4 and 5 share new insights in human pathophysiology of I/R injury and identify targets that could limit DGF. Unbiased transcriptomic and metabolomic approaches describe processes and pathways underlying I/R injury. In both studies, grafts with a functional response upon ischemia and reperfusion (reference group of adequately recovering grafts) were compared with grafts that developed DGF after transplantation (i.e. I/R injury). Chapter 6 evaluates donor and transplantation procedure factors via a more epidemiological approach. Using the NOTR registry, long-term graft survival of grafts donated after brain death (DBD) and grafts donated after cardiac death (DCD) was compared. Analyses of the Leiden University Medical Center (LUMC) cohort were performed to evaluate functional graft recovery. Chapter 7 describes the role of mitochondrial pool recovery in advance of functional recovery after DGF. Chapter 8 elaborates on the role of mitochondrial (detoxifying) aldehyde dehydrogenase (ALDH) enzymes in I/R injury. Next the opportunity of simvastatine as protective pretreatment before I/R is described in

the exploratory trial in Chapter 9. Chapter 10 summarizes all the experimental and clinical studies performed on donor pretreatment in the context of kidney transplantation. To conclude, Chapter 11 summarizes the findings of this thesis and explains why unraveling the primordial triggers of I/R injury will provide focus in developing new strategies to mitigate clinical I/R injury.

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Part I

Pathophysiology of

Ischemia

Reperfusion

Injury

II

The hypoxanthine-xanthine oxidase axis is not involved in the initial phase of clinical transplantation-related ischemia/reperfusion injury

American Journal of Physiology – Renal Physiology

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ABSTRACT

The hypoxanthine-xanthine oxidase (XO) axis is considered to be a key driver of transplantation-related ischemia/reperfusion (I/R) injury. Whereas interference with this axis effectively quenches I/R injury in preclinical models, there is limited efficacy of XO inhibitors in clinical trials. In this context, we considered clinical evaluation of a role for the hypoxanthine-XO axis in human I/R to be relevant. Patients undergoing renal allograft transplantation were included (n=40) and classified based on duration of ischemia (short, intermediate, and prolonged). Purine metabolites excreted by the reperfused kidney (arteriovenous differences) were analyzed by the ultra performance liquid chromatography-tandem mass spectrometer (UPLCMS/MS) method and tissue XO activity was assessed by in situ enzymography. We confirmed progressive hypoxanthine accumulation ($P<0.006$) during ischemia, using kidney transplantation as a clinical model of I/R. Yet, arteriovenous concentration differences of uric acid and in situ enzymography of XO did not indicate significant XO activity in ischemic and reperfused kidney grafts. Furthermore, we tested a putative association between hypoxanthine accumulation and renal oxidative stress by assessing renal malondialdehyde and isoprostane levels and allantoin formation during the reperfusion period. Absent release of these markers is not consistent with an association between ischemic hypoxanthine accumulation and postreperfusion oxidative stress. On basis of these data for the human kidney we hypothesize that the role for the hypoxanthine-XO axis in clinical I/R injury is less than commonly thought, and as such the data provide an explanation for the apparent limited clinical efficacy of XO inhibitors.

INTRODUCTION

Graft ischemia and reperfusion is the inevitable consequence of kidney transplantation. During tissue ischemia, ATP catabolism along with changes in the redox state result in hypoxanthine accumulation and conversion of xanthine dehydrogenase into xanthine oxidase (XO). Upon tissue or organ reperfusion, the accumulated hypoxanthine is then oxidized by XO with a stoichiometric release of uric acid and superoxide. This hypoxanthine-XO axis is considered to be a major source of ischemia/reperfusion (I/R)-related reactive oxygen species (ROS), and as such a paramount, if not the prime driver, of I/R injury.[1–3]
A central role for the hypoxanthine-XO axis in experimental I/R is underscored by the clear benefit of interfering with the XO system in animal models of I/R.[4,5] Human studies on the other hand consistently fail to show a benefit of pharmaceutical XO inhibition on clinical endpoints.[6–9] This suggests that a role, if any, of the hypoxanthine-XO axis in clinical I/R injury may be less significant than generally assumed. Because of the apparent contrast between preclinical and clinical studies, we decided to evaluate the involvement of the hypoxanthine-XO axis in the I/R that occurs during kidney transplantation. This clinical study confirms ample hypoxanthine accumulation during ischemia, but postreperfusion XO activity and tissue ROS stress appeared to be minimal.

MATERIALS & METHODS

PATIENT POPULATION

Patients undergoing renal allograft transplantation were included (n=40). The study protocol was approved by the local ethics committee, and informed consent was obtained from each patient. Kidney transplantations were performed according to the local standardized protocol.[10] The immunosuppressive regimen was based on induction therapy with basiliximab on day 0 and 4 and tacrolimus or cyclosporine A in addition with mycophenolate mofetil and steroids. Postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. In living donors open nephrectomy was performed and HTK solution (Tramedico, Weesp, The Netherlands) was used for cold perfusion and storage of the kidney. Brain-dead and circulatory-dead donor kidneys were perfused and stored with either University of Wisconsin® (UW) solution or Custodiol® HTK solution.
For hypoxanthine analysis, patients were classified based on duration of ischemia (Flow chart in Fig. 1 and patient characteristics in Table 1).
Part of the deceased donor kidneys (intermediate/prolonged ischemia group) were preserved in UW solution. UW contains 1 mM of the XO inhibitor allopurinol (Table 2). Therefore separate strategies were used to assess post-reperfusion XO activity. XO activity in the HTK preserved grafts was assessed by in situ measurement of XO activity and uric acid release, and in grafts preserved in UW through quantifying oxypurinol production.

ARTERIOVENOUS SAMPLING

To analyze metabolic activity of the graft directly after reperfusion 1H NMR spectroscopy was performed on arteriovenous(AV) plasma samples. This arteriovenous sampling method was validated by blood gas analysis, as was illustrated earlier.[10] Prior to implantation of the graft, a 5 French umbilical vein catheter was placed in the renal vein through one of its side branches. At 30 s, 3, 5, 10, 20 and 30 min after reperfusion (i.e. t = 0), 10 mL blood aliquots were sampled. Paired arterial blood samples were obtained. Thirty minutes after reperfusion, the abdominal wall was closed and the endpoint of sampling was reached. Control AV samples over a physiologically-functioning kidney were taken from living donors undergoing open nephrectomy, prior to the induction of renal ischemia.
All samples were collected in pre-cooled EDTA tubes and immediately placed on melting ice. Blood samples were centrifuged (1550 g, 10 min 4°C) within one hour after collection and the derived plasma was re-centrifuged (10.000 g, 4 min, 4°C) to obtain leukocyte- and platelet-poor plasma. Aliquots were stored at -70°C until analysis.

MULTI-COMPONENT ANALYSIS OF PURINE METABOLITES

Hypoxanthine and allantoin in plasma were measured using an in-house developed UPLC-MS/MS method. Briefly, 30 µl plasma was mixed with 30 µl of a solution containing stable isotope-labelled internal standards. Samples were de-proteinized 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 formate (pH 4.0).

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 formate (pH 4.0)/acetonitrile gradient. Compounds were quantified using a Waters XEVO TQS tandem mass spectrometer (Waters), both in negative or positive electrospray ionization using specific MRM transitions.

MDA and total 15(S)-8-iso-PGF2a, that is, free and esterified 15(S)-8-iso-PGF2a, were assessed with an extensively validated GC-MS/MS method as described previously.[11]

HYPOXANTHINE LEVELS IN KIDNEY BIOPSIES

Renal cortical biopsies were obtained at the end of the ischemic period and at 45 min after reperfusion. Tissue was snap frozen in liquid nitrogen and stored at -80°C. Metabolic profiling of the tissue biopsies was performed by High-Resolution Magic-Angle-Spinning Nuclear Magnetic Resonance (HR MAS NMR) spectroscopy. Samples were prepared on ice and fitted in a leak-proof insert (30 µL; Kel-F, Bruker Biospin GmbH, Germany) used in a zirconium HR-MAS rotor (4 mm). The insert was filled with 3 µL cold (4°C) phosphate-buffered saline (PBS in D2O) containing 4.5 mM trimethylsilyl-tetradeuteropropionic sodium salt (TSP-d4) and 25 mM sodium formate (CHNaO4) 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 performed at 4°C while spinning the samples at a rate of 5 kHz. Relative concentrations of metabolites were calculated using the BATMAN[12] R package (https://cran.r-project.org). Spectra were normalized on basis of tissue weight.

IN SITU XO ACTIVITY

Renal cortical biopsies from HTK-preserved kidneys were obtained at the end of the ischemic period and 45 min after reperfusion. Tissue was snap frozen in liquid nitrogen and stored at -80°C. Frozen tissue sections (thickness 4 µm) were cut and XO activity was determined on the basis of the generation of superoxide anions from hypoxanthine, as described earlier.[13] Ischemic rat kidney and liver tissue were included as positive controls.

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.[10,14,15]

SPSS 22.0 (SPSSinc, Chicago, III) was used for statistical analysis.

In tissue hypoxanthine concentration and hypoxanthine release values were tested using ANOVA. For the uric acid, oxypurinol, allopurinol, MDA, 15(S)-8-iso-prostaglandin F2α and allantoin 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. Absence of release of metabolites means $P > 0.05$.

The level of significance was set at $P < 0.05$. All data represent mean ± SE.

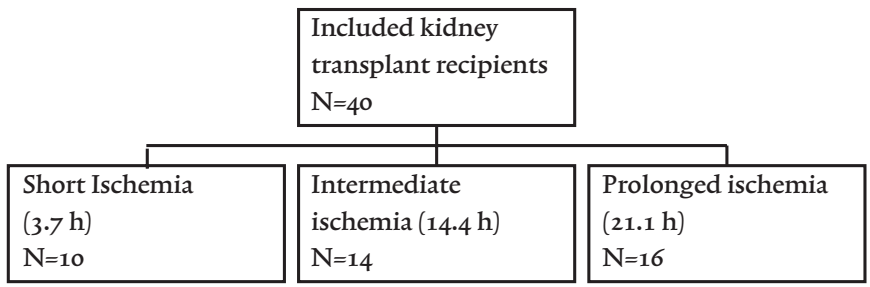

Figure 1. Flow chart of patient inclusion

Table 1. Patient characteristics

Duration of ischemia				Donor		Recipient			
Group	CIT (min)	WIT1 (min)	WIT2 (min)	D age	D sex	R age	R sex	Recipient disease	UW/ HTK
Short	215	0	28	63	F	62	M	Maligned hypertension	HTK
Short	223	0	26	69	M	65	F	Glomerulonephritis	HTK
Short	196	0	25	53	M	49	F	Sarcoidosis	HTK
Short	238	0	31	51	F	65	M	Nephrolithiasis	HTK
Short	205	0	44	33	M	32	F	Glomerulonephritis	HTK
Short	216	0	38	61	M	33	M	Polycystic kidney disease	HTK
Short	200	0	31	56	F	51	F	Glomerulonephritis	HTK
Short	246	0	40	52	F	48	M	DM type 1	HTK
Short	214	0	25	48	F	35	M	Renal failure e.c.i.	HTK
Short	265	0	49	69	F	54	F	Polycystic kidney disease	HTK
Intermediate	669	13	28	60	M	55	M	Polycystic kidney disease	UW
Intermediate	671	12	37	63	M	43	M	Renal failure e.c.i.	UW
Intermediate	675	33	30	67	M	70	F	Renal failure e.c.i.	UW
Intermediate	780	0	40	67	F	68	F	Polycystic kidney disease	UW
Intermediate	810	0	35	68	M	70	M	Polycystic kidney disease	UW
Intermediate	832	14	32	21	F	62	F	Maligned hypertension	UW
Intermediate	840	0	25	21	F	62	F	Maligned hypertension	UW
Intermediate	850	14	37	68	M	69	M	DM type 2	UW
Intermediate	960	24	30	58	F	46	M	Maligned hypertension	UW
Intermediate	965	23	30	56	M	58	F	Renal failure e.c.i.	UW
Intermediate	970	10	38	68	M	72	M	IgA nephropathy	HTK
Intermediate	1014	18	26	61	F	48	F	Renal failure e.c.i.	UW
Intermediate	1016	14	32	59	F	56	F	Glomerulonephritis	UW
Intermediate	1020	35	40	66	M	64	M	FSGS	HTK
Prolonged	1045	0	40	57	M	39	F	Basal membrane nephropathy	HTK
Prolonged	1065	0	31	46	F	57	F	Renal failure e.c.i.	HTK
Prolonged	1073	0	34	57	M	65	F	Renal failure e.c.i.	HTK
Prolonged	1127	0	30	57	M	46	M	Glomerulonephritis	UW
Prolonged	1140	24	30	48	F	64	F	Polycystic kidney disease	HTK
Prolonged	1146	14	22	63	M	53	M	Polycystic kidney disease	UW
Prolonged	1260	0	30	53	M	42	M	Renal failure e.c.i.	HTK
Prolonged	1270	11	41	56	M	30	M	Glomerulonephritis	UW
Prolonged	1280	24	42	41	F	39	M	Glomerulonephritis	HTK
Prolonged	1283	0	38	74	M	74	M	Glomerulonephritis	UW

Prolonged	1314	21	43	62	M	72	M	DM type 2	HTK
Prolonged	1410	0	32	62	F	56	M	DM type 2	UW
Prolonged	1418	0	32	63	M	47	F	Glomerulonephritis	HTK
Prolonged	1420	12	28	65	M	53	M	IgA nephropathy	UW
Prolonged	1440	0	25	63	F	40	F	Basal membrane nephropathy	UW
Prolonged	1520	0	40	10	F	64	F	Renal failure e.c.i.	UW

Table 1. Kidney transplantations (n=40) were categorized into 3 groups on the basis of duration of ischemia. Short ischemic kidneys (n=10) mean ischemia time: 3.7 h (s.e.m. 0.1 h). Intermediate ischemic kidneys (n=14): mean ischemia time 14.4 h (s.e.m. 0.6 h) and prolonged ischemic kidneys (n=16) mean ischemic time 21.1 h (s.e.m. 0.6 h).

CIT: cold ischemia time; WIT: warm ischemia time; D: donor; R: recipient; F: female; M: male; e.c.i.: e causa ignota; DM: diabetes mellitus; HTK: Histidine–tryptophan–ketoglutarate® solution; UW: University of Wisconsin® solution.

Table 2. Composition of kidney transplant preservation_uids: UW solution versus HTK solution.

UW®	HTK®
Potassium lactobionate: 100 mM	Sodium: 15 mM
KH_2PO_4: 25 mM	Potassium: 9 mM
$MgSO_4$: 5 mM	Magnesium: 4 mM
Raffinose: 30 mM	Calcium: 0.015 mM
Adenosine: 5 mM	Ketoglutarate/glutamic acid: 1 mM
Glutathione: 3 mM	Histidine: 198 mM
Allopurinol: 1 mM	Mannitol: 30 mM
Hydroxyethyl starch: 50 g/L	Tryptophan: 2 mM

RESULTS

Forty patients who received a kidney transplant were included in this study. Clinical characteristics are shown in Table 1. Kidney transplants were graded on basis of the overall duration of ischemia (i.e. period between circulatory arrest of the graft and subsequent reperfusion) into grafts with short (mean: 3.7 h), intermediate (mean 14.4 h) and prolonged (mean 21.1 h) ischemia periods (Fig. 1). Ten patients received a graft from a living donor and thirty patients from a deceased donor. Eighteen deceased donor grafts showed delayed graft function, a clinical manifestation of I/R injury.

A putative association between the period of ischemia and hypoxanthine accumulation was tested using high resolution magic angle spinning (HR MAS) NMR (tissue content) spectroscopy and by venous minus arterial (AV) concentration differences over the reperfused kidney (hypoxanthine washout from the graft). Figure 2A shows increasing graft hypoxanthine content with progressing periods of ischemia ($P<0.006$). Findings for the AV concentration measurements over the reperfused grafts (Fig. 2B–C) were in agreement with hypoxanthine tissue levels and indicate a clear association between duration of ischemia and hypoxanthine washout (mean(±SE) AV differences at reperfusion: short: 55.9(±12.7) μmol/L vs. intermediate 124.6(±16.6) μmol/L vs. prolonged 148.0(±24.0) μmol/L ($P<0.017$). These data confirm hypoxanthine accumulation during progressive renal ischemia.

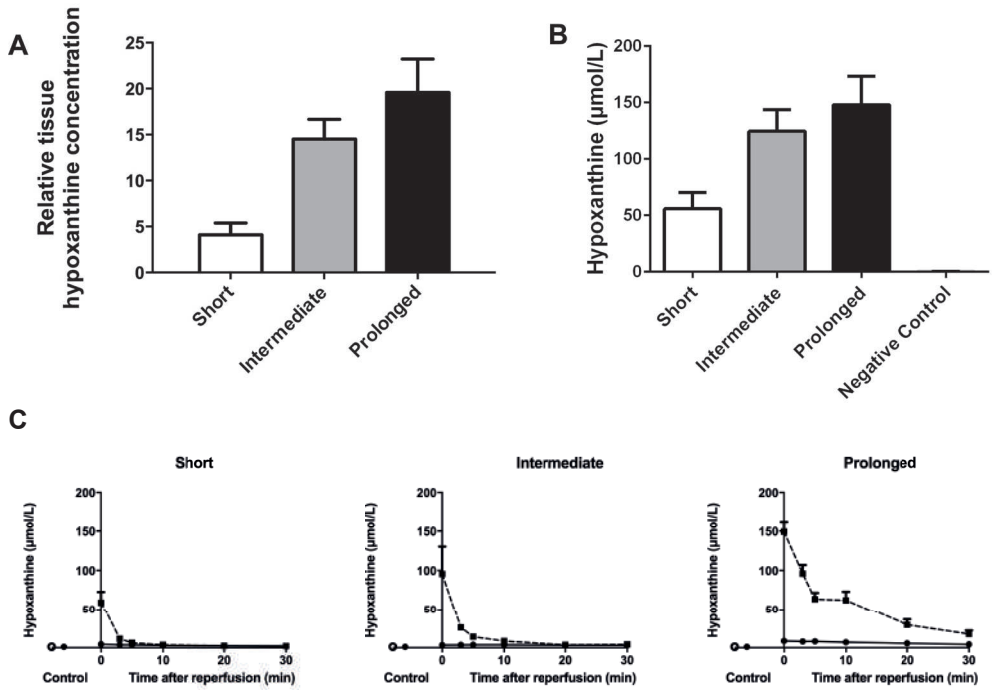

Figure 2. Hypoxanthine levels significantly increase with duration of ischemia. A) Hypoxanthine tissue accumulation (A) and tissue washout (B) following progressive kidney ischemia. (ANOVA, P<0.006 (A), respectively P<0.017 (B)). C) Arterio (solid line) venous (dashed line) concentration measurements over the transplanted kidney illustrating continued hypoxanthine washout in the prolonged ischemia group. Measurements shown left of the Y-axis (Control) represent reference values over the kidney of healthy controls. (Solid circle = arterial control. Open circle = venous control.)

We next tested postreperfusion hypoxanthine oxidation by quantifying graft uric acid production during the first half hour of reperfusion (AV differences). Figure 3 shows that uric acid release from reperfused donor grafts was absent under all conditions tested. These findings imply minimal XO activity in reperfused human kidney grafts. This notion was validated in a dual approach using enzymology and quantification of oxypurinol formation.
Results of the enzymology are shown in Figure 4. Abundant XO activity was observed in the positive control (ischemic rat kidney and liver) but minimal activity was observed in human kidney biopsies collected at the end of the ischemic period and after 30 minutes of reperfusion.

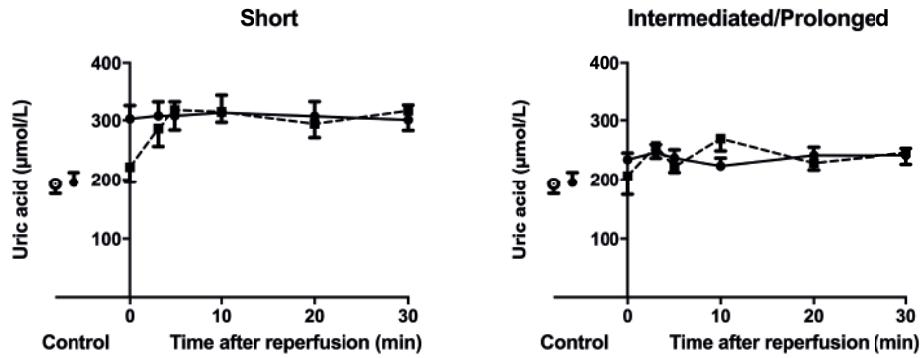

Figure 3. Arteriovenous concentration measurements of uric acid.
Absent AV differences for uric acid following reperfusion indicate minimal or absent uric acid production by the transplanted graft. (ANOVA: ns)
Solid line: arterial levels, dashed line: venous levels. Measurements shown left of the Y-axis (Control) represent reference values for healthy controls. (Solid circle = arterial control. Open circle = venous control.)

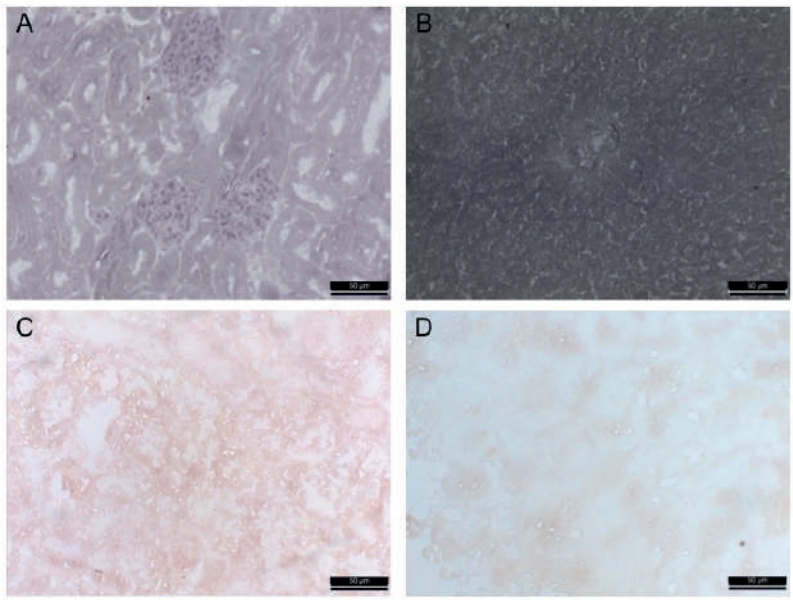

Figure 4. In situ assessment of xanthine oxidase activity. Bars represent 50 µm.
A) Positive control (ischemic rat kidney) and (B) ischemic rat liver[11] for xanthine oxidase in situ enzymography. The dense dark grey 3,3'-diaminobenzidine (DAB) precipitate reflects abundant XO activity.
C-D) Absent XO-activities formation in human renal biopsies taken before (C) and after (D) reperfusion (no DAB precipitate). Contrast in the images reflects the tissue contours (counter staining was omitted to enhance sensitivity).

As second functional validation, we quantified oxypurinol formation in grafts preserved in University of Wisconsin (UW) solution. UW solution, but not the HTK solution (Table 2) contains the pro-drug allopurinol. In the presence of active XO, allopurinol is rapidly converted into the competitive XO antagonist oxypurinol. Continued allopurinol release (Fig. 5A) but absent oxypurinol release from UW-preserved kidneys (Fig. 5B) further supports minimal XO activity in reperfused kidneys.

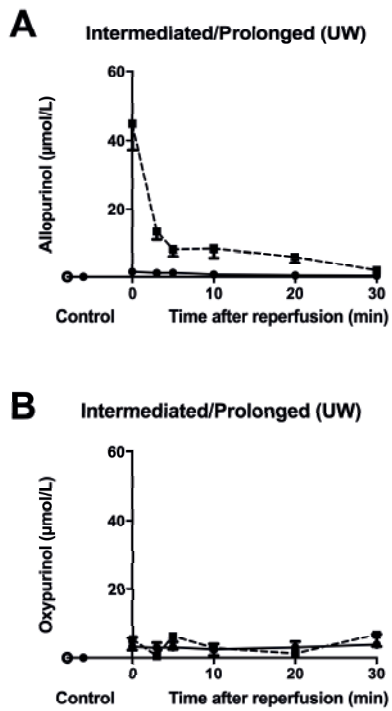

Figure 5. Arteriovenous concentration measurements of allopurinol and oxypurinol. A) Kidneys preserved in allopurinol containing UW solution (see Table 2) showed washout of allopurinol during the first minutes after reperfusion and sustained release of absorbed allopurinol up to 30 minutes after reperfusion. B) Absence of oxypurinol release from UW preserved grafts, indicating minimal or absent allopurinol oxidation. Solid line: arterial levels, dashed line: venous levels. Measurements shown left of the Y-axis (Control) represent reference values for healthy controls. (Solid circle = arterial control. Open circle = venous control.)

Altogether, although these observations confirm progressive hypoxanthine accumulation during ischemia, they challenge any prominent role of XO during I/R in human kidney transplantation.

A critical question is how this conclusion relates to a perceived excessive ROS formation during the reperfusion phase. To test the latter, we explored a putative relation between the period of ischemia (and thus hypoxanthine accumulation) and postreperfusion oxidative stress. In a first series of experiments, we assessed release of established biomarkers of oxidative damage (malondialdehyde (MDA);15(*S*)-8-iso-prostaglandin F2α) following graft reperfusion. Figure 6 shows similar and stable arterial and renal venous

concentrations of both factors, indicating that these markers were not released from the reperfused kidneys.

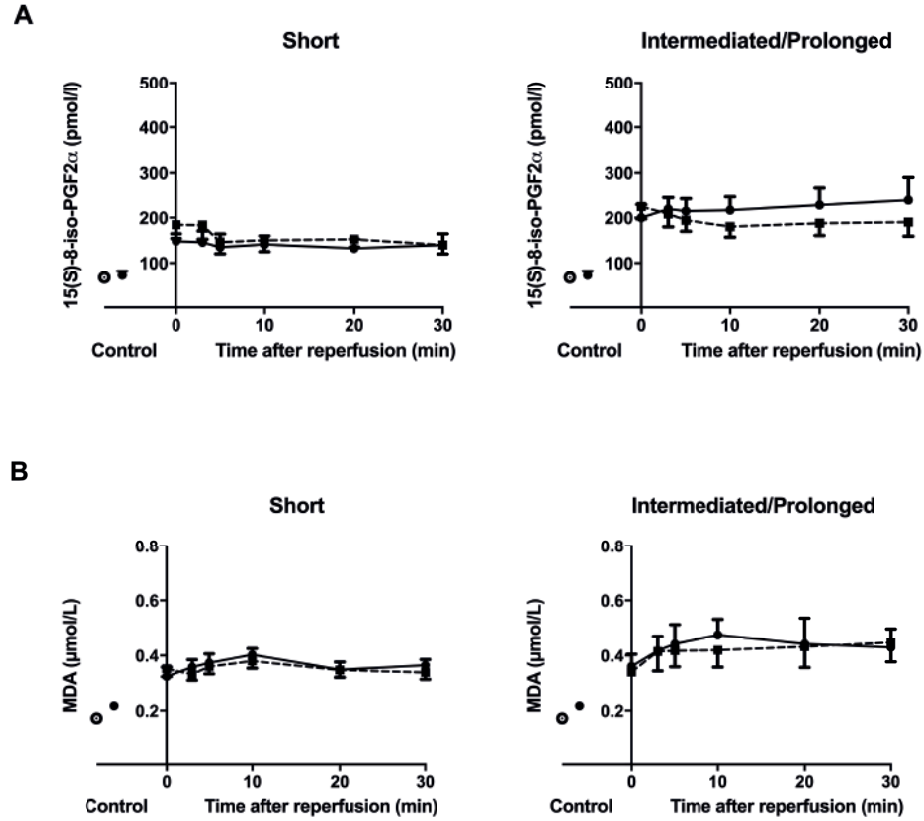

Figure 6. Arteriovenous concentration measurements of 15(S)-8-iso-PGF2a and malondialdehyde.
A) Absence of 15(S)-8-iso-PGF2a release during the first 30 min after reperfusion, independently of the duration of ischemia as indicated by similar arterial (solid line) and venous (dashed line) levels after short and intermediate/prolonged periods of ischemia.
B) Similar arterial and venous MDA levels indicating absent MDA release during the first 30 min after reperfusion.
Solid line: arterial levels, dashed line: venous levels. Measurements shown left of the Y-axis (Control) represent reference values for healthy controls. (Solid circle = arterial control. Open circle = venous control.)

This observation implies limited oxidative stress during renal reperfusion. Nevertheless, I/R-related oxidative stress may be concealed by a highly efficient endogenous antioxidant system that prevents formation of oxidative damage markers. As such negative findings for MDA and15(S)-8-iso-prostaglandin F2α do

not fully exclude oxidative stress. To that end, we quantified allantoin formation as a potentially more sensitive measure of oxidative stress. Findings for allantoin levels follow those of the biomarkers of oxidative stress with similar arterial and renal vein plasma concentrations (Fig. 7), showing that kidney graft reperfusion does not result in comprehensive oxidative stress.

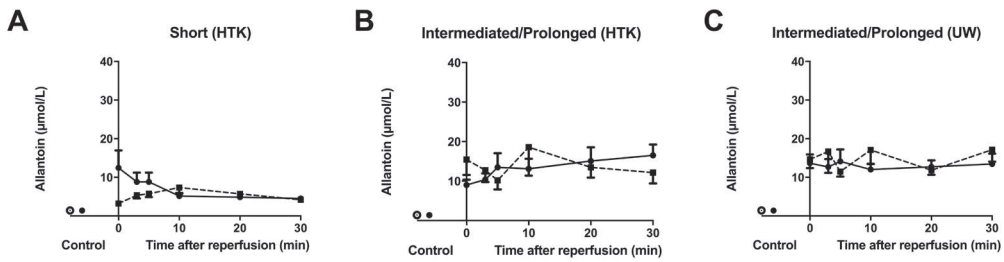

Figure 7. Absent allantoin release from the reperfused graft.
Similar arterial and venous allantoin levels indicate minimal or absent uric acid oxidation following reperfusion in HTK (A and B), and (C) allopurinol containing UW preserved grafts.
Solid line: arterial levels, dashed line: venous levels. Measurements shown left of the Y-axis (Control) represent reference values for healthy controls.

DISCUSSION

The hypoxanthine-XO axis is considered to be one of the primary drivers of I/R-related ROS formation, and as such a canonical factor in I/R injury. This notion is supported by more than one hundred experimental publications showing that interference with the hypoxanthine-XO axis alleviates I/R injury.[1–3,5] Clinical evidence on the other hand is limited and the few publications available indicate a limited effect, at the most. This suggests that a role, if any, for the hypoxanthine-XO axis in clinical I/R injury may be less than generally assumed.[16] In this context, we decided to re-evaluate the relevance of the hypoxanthine-XO axis in clinical I/R. The current study focuses on kidney transplantation-related ischemia reperfusion as a clinical and relevant example of full tissue I/R. This planned procedure has the advantage of scheduled reperfusion, and allows direct tissue access by assessment of AV differences, and by tissue biopsies. AV measurements have been proven efficient in exploring pathways of potential interest in human ischemia/reperfusion injury.[10,13,17,18] Moreover, procedure-related differences in ischemia time create "an experiment by nature" with considerable variation in ischemia time and short-term outcome (delayed graft function). In fact, incident-delayed graft function is considered to be a direct manifestation of I/R injury. Long-term graft survival in this study was 100%, showing that all ischemic periods times were within a clinically safe range.

We found progressive hypoxanthine accumulation during kidney ischemia. In fact, data on the cumulative hypoxanthine release from the prolonged ischemic grafts imply a more than 50% exhaustion of the graft's adenosine pool. Hence, these data show that hypoxanthine accumulation is a prominent feature of clinical ischemia. Yet, we were unable to detect appreciable XO activity in reperfused renal grafts despite a series of approaches that we applied. In the first approach, we tested for net release of uric acid, the end product of hypoxanthine oxidation. In the absence of urine production (as is the case in deceased donor grafts) and endogenous activity of uricase activity because the enzyme is absent in humans, local uric acid production results in a net AV difference for uric acid. Under these conditions, uric acid production can be used as a quantitative measure of XO activity. Hence, the absence of AV differences for uric acid for all conditions tested suggests minimal XO activity in the reperfused donor grafts. This conclusion is supported by in situ enzymography, and by the absence of allopurinol oxidation.

Allopurinol is the archetypical XO inhibitor, and as such included in the UW organ preservation fluid that was used in half of the donation procedures. Although these UW solution-preserved grafts were obviously excluded from the XO activity analyses, presence of allopurinol allows for a further functional validation of XO activity. Allopurinol is a pro-drug that, upon oxidation by XO, is converted into

the competitive XO inhibitor oxypurinol. As such, oxypurinol formation reflects XO activity. As a complementary strategy for the quantification of XO activity, we assessed allopurinol and oxypurinol release from UW preserved grafts. The results obtained for the first 30 minutes of reperfusion show a persistent, but gradual diminishing allopurinol release, without any oxypurinol release. Altogether, all approaches failed to identify significant XO activity in reperfused human kidney grafts.

XO-mediated oxidation of hypoxanthine is considered a significant, if not the most prominent source of ROS in I/R. In light of the non-confirmative findings for XO activity in post-ischemic renal grafts, we tested whether the escalating degrees of hypoxanthine accumulation during advancing ischemia translate into progressive oxidative stress. To that end, we quantified reperfusion-related release of MDA and 15(*S*)-8-iso-prostaglandin F2α, 2 established biomarkers of oxidative damage, and found no release of the biomarkers for all conditions tested. Although these results may be interpreted as signs of minimal oxidative stress, it is also possible that the results reflect a highly adequate antioxidant system, effectively quenching oxidants formed and thereby preventing oxidative damage, and thus formation of products such as MDA and isoprostanes. To validate this we assessed allantoin as a potentially more sensitive and non-redundant marker of oxidative stress. In humans, allantoin is the stable end product of the oxidation of uric acid, and as such it reflects ROS quenching by the antioxidant uric acid. Although allantoin would normally be cleared by the kidney, the deceased donor grafts in this study were all still nonfunctional at the end of the 30 minute study period. Absent allantoin release from the graft indicates a lack of uric acid oxidation during the first half hour of reperfusion for all conditions tested. Hence, even at the highest hypoxanthine levels encountered, oxidative stress does not result in uric acid oxidation, suggesting that even under extreme conditions the stress is effectively managed by other components of the antioxidant system. Separation of the deceased donors on basis of incident delayed graft function and manifestation of ischemia/reperfusion injury that ongoing hypoxanthine release upon reperfusion is not accompanied by allantoin production. Hence, even under conditions of manifest ischemia/reperfusion injury there is no evidence for global oxidative stress.[18]

The present study has several limitations. First, it is performed during clinical kidney transplantation and for this reason the window of measurement was limited to 30 minutes. Therefore, any ROS-related damage occurring afterwards will be missed. Yet, given the almost complete hypoxanthine washout from the graft after 30 minutes, we consider a delayed contribution of the hypoxanthine-XO axis unlikely. Another limitation is the extreme species and organ variation in XO

expression; as such these findings in the human kidney may not translate to other species[19,20], and potentially not or only partially to other human organs. Finally, as the studies were performed in a transplantation setting the organs were exposed to considerable periods of cold-ischemia, but variable periods of warm ischemia. Hence, findings may not directly translate to exclusive warm-ischemia such as during myocardial or brain infarction.

Nevertheless, this study indicates an insignificant role for the hypoxanthine-XO system in the first 30 minutes of clinical renal I/R injury, and therefore it provides a rationale for the apparent failure of XO inhibitors in the prevention of renal I/R injury. It remains to be determined whether these observations for the kidney translate to other forms of clinical I/R, and hallmark a major species differences for the involvement of the hypoxanthine-XO axis in I/R injury.[19]

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III

Succinate accumulation and ischemia/reperfusion injury: of mice but not men.

A study in renal ischemia/reperfusion

American Journal of Transplantation

Leonie G.M. Wijermars, Alexander F. Schaapherder, Sarantos Kostidis, Rob C.I. Wüst, Jan H.N. Lindeman

ABSTRACT

A recent seminal paper implicated ischemia-related succinate accumulation followed by succinate-driven reactive oxygen species formation as a key driver of ischemia/reperfusion injury. Although the data show that the mechanism is universal for all organs tested (kidney, liver, heart, and brain), a remaining question is to what extent these observations in mice translate to humans. We showed in this study that succinate accumulation is not a universal event during ischemia and does not occur during renal graft procurement; in fact, tissue succinate content progressively decreased with increasing graft ischemia time ($P<0.007$). Contrasting responses were also found with respect to mitochondrial susceptibility toward ischemia and reperfusion, with rodent mitochondria robustly resistant toward warm ischemia but human and pig mitochondria highly susceptible to warm ischemia ($P<0.05$). These observations suggest that succinate-driven reactive oxygen formation does not occur in the context of kidney transplantation. Moreover, absent allantoin release from the reperfused grafts suggests minimal oxidative stress during clinical reperfusion.

INTRODUCTION

Ischemia reperfusion (I/R) injury is the primary cause of organ dysfunction after events such as myocardial infarction, cerebrovascular accident and organ transplantation.[1] Despite decades of intensive research and an abundance of promising preclinical results, no interventions emerged that prevent or reduce clinical I/R injury.[2,3] A recent study in mice proposed a new mechanistic concept[4]: that progressive succinate accumulation during ischemia drives excessive post-reperfusion reactive oxygen species (ROS) formation as a result of reverse electron transport through mitochondrial complex I.[4] This excess ROS formation is suggested to drive I/R injury.

Despite a wealth of rodent studies in support of a role for ROS in I/R injury[5,6], human studies consistently fail to show a benefit with ROS scavenging (antioxidant) therapy.[7-9] Moreover, although clinical studies confirm increased redox stress during I/R, this is not followed by biomarkers of oxidative damage.[10] Consequently, a pivotal role for oxidative damage as driving force of I/R injury[4] and thus a role for succinate accumulation is unclear for clinical I/R injury. In this respect, it was noted that the proposed central role for succinate accumulation necessarily requires confirmation in the human setting.[11]

MATERIALS & METHODS

PATIENT GROUPS

Local ethics committees approved all experiments. Renal biopsies of 24 human kidney transplants were taken after written informed consent.[10] Patients were classified (n=6 per group) based on duration of cold ischemia: short (2-6 h), intermediate (6-12 h) and prolonged (12-24 h). Non-ischemic controls were biopsies taken from functioning living donor kidneys. Renal allograft transplantations were performed according to the local standardized protocol. All grafts were preserved by static cold storage. 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. Postoperative course was uneventful in all patients. One-year patient and graft survival was 100%. Patients in the short ischemia group did not develop clinical I/R injury (delayed graft function), whereas 14% of the intermediated ischemia group and 71% of the prolonged ischemia group developed delayed graft function.

The influence of warm and cold ischemia on murine kidney succinate content was tested in kidneys from healthy surplus male mice (C57bl background). Kidneys

were removed immediately upon sacrificing. Reference (control) kidneys (n=6) were directly snap-frozen. Two series of 6 kidneys were kept on melting ice or in a warm water bath (37°C) for 45 min in order to simulate cold and warm ischemia, respectively. Another 6 kidneys were kept on melting ice for 20 h before they were snap frozen, in order to simulate prolonged cold ischemia.

METABOLIC PROFILING

Metabolic profiling (hypoxanthine, succinate, and fumarate) of the tissue biopsies was performed by High-Resolution Magic-Angle-Spinning (HR MAS) Nuclear Magnetic Resonance (NMR) spectroscopy. Human tissue samples were snap frozen in liquid nitrogen and stored at -80°C. 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 D2O) containing 4.5 mM TSP-d4 (trimethylsilyl-tetradeuteropropionic acid) and 25 mM sodium formate ($CHNaO_2$) as internal standards. NMR-spectrometry was used for murine kidney succinate content, for which metabolites were extracted and dried. The dried material was reconstituted with 0.3 mL phosphate buffer solution (pH 7.4; 150 mM K_2HPO_4, Sigma-Aldrich, Germany) in deuterated water containing also 0.2 mM of bacteriostatic NaN_3 and 0.4 mM sodium TSP-d4 (Cambridge Isotope Laboratories Inc., Buchem, Apeldoorn, The Netherlands) as chemical shift reference and 0.19 mL of each sample was transferred to 3 mm NMR tubes (Bruker Biospin, GmbH, Germany) for NMR analysis. HR-MAS spectra were recorded on a 14.1 T (600MHz, 1H resonance frequency) 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.[12] Spectra were normalized based on the tissue weight used.

HIGH RESOLUTION RESPIROMETRY

Effect of ischemia-reoxygenation on mitochondrial efficacy was analysed by high-resolution respirometry (Oxygraph-2k; Oroboros Instruments, Innsbrück, Austria) and compared with different species: humans (N=7), pigs (N=10), rats (N=6) and mice (N=6). Kidney biopsies from a healthy kidney segment were taken immediately prior to clamping of the renal vasculature and stored on ice. Warm ischemia was simulated by maintaining biopsies in a warm-water bath (37°C) for 60 min.

On simulating 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), 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 bovine serum albumin (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). Adenosine diphosphate (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. Subsequently, complex I was blocked by rotenone (0.5 µM). Finally, antimycin A was added to inhibit complex III, and residual oxygen consumption measured (nonmitochondrial respiration), and subtracted from all values. All measurements were performed simultaneously and subsequently averaged. Values were mass normalized, and reported as pmol $O2s^{-1}.mg^{-1}$. The contribution of complex I function to the uncoupled maximal respiration was assessed by the relative decline of respiration after adding rotenone.

ALLANTOIN RELEASE

Post reperfusion allantoin formation was quantified by establishing arteriovenous concentration differences over the transplanted kidney. For maximum sensitivity we specifically selected patients that presented with manifest I/R injury (delayed graft function). These patients were all part from a previous study.[10]
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. The abdominal wall was closed about 45 min after reperfusion, and the endpoint of sampling was reached 30 min 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]
Allantoin in plasma was measured using an ultraperformance liquid chromatography (UPLC)–tandem mass spectrometry method developed in-house. 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, 10 000 g, 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), with both negative and positive electrospray ionization using specific MRM transitions.

STATISTICAL ANALYSIS

Maximal mitochondrial respiration values were tested using analysis of variance. Paired T-tests were used to compare the baseline respiratory rate with respiratory rate after experiencing warm ischemia (SPSS 22.0).

For the plasma allantoin 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 the following 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. The level of significance was set at $p<0.05$. All data represent mean ± SEM.

RESULTS

Similar to the report for the murine setting (which also specifically tested kidney I/R)[4], our data for the human kidney show progressive hypoxanthine accumulation during ischemia (Figure 1). Results for succinate (and fumarate) on the other hand contrast with those reported for the mouse.[4] Fumarate concentrations remained low and stable during ischemia, whereas our clinical data showed a paradoxical decline in tissue succinate content following progressive ischemia (Figure 1A), indicating that succinate accumulation[4] is not a universal metabolic signature of ischemia. This contrast between the reported murine data[4] and our human data may reflect species-specific differences and relate in part to contrasting effects of warm and cold ischemia. We tested the latter by exposing murine kidneys to cold and warm ischemia. Figure 1B shows that although cold ischemia results in progressive but moderate succinate exhaustion ($p<0.019$), tissue succinate levels remained stable during warm ischemia.

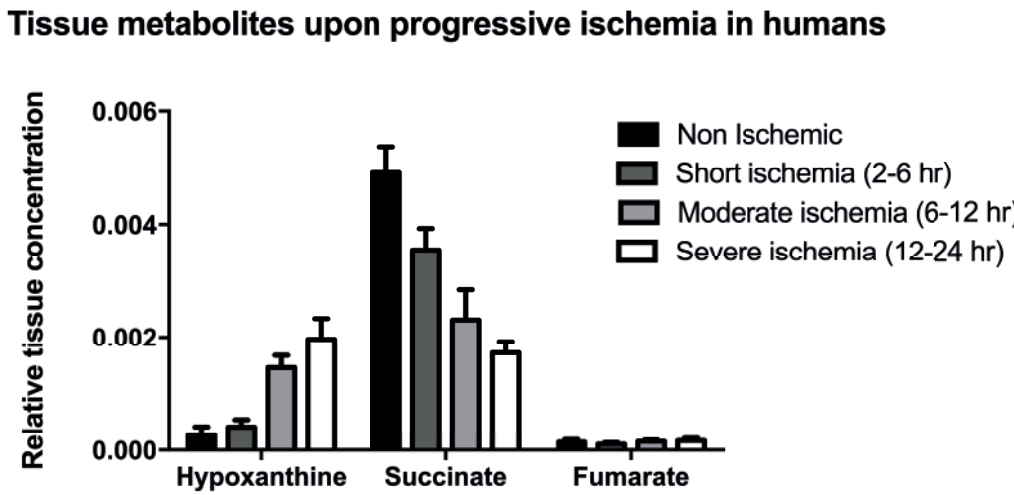

Figure 1A. Relative hypoxanthine, succinate and fumarate tissue content (HR MAS NMR) on incremental ischemia time (human kidney).
Increasing hypoxanthine (p‹0.031), but declining succinate levels (p‹0.007) during ischemia. Fumarate levels remained low and stable during ischemia. Non-ischemic control values are included as a reference.
All values were normalized on basis of tissue weight. (n = 6 per group, mean ± SEM).

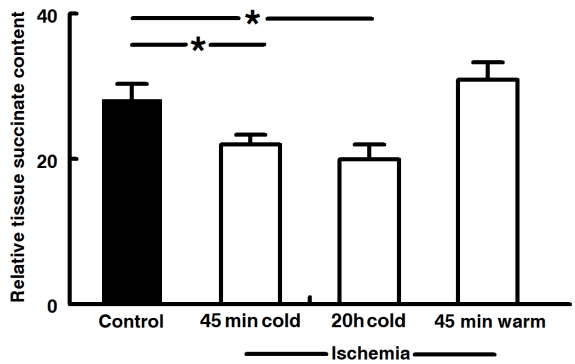

Figure 1B. Cold ischemia but not warm ischemia reduced murine kidney succinate content (ANOVA p‹0.019) (mean ± SEM).

Interspecies differences also exist with regard to mitochondrial respiratory efficiency and ROS production.[13] This is further illustrated by the clear reciprocal association between species body size and overall mitochondrial respiration (Fig.2A) and normalized activities of mitochondrial complex I and II activity (Figure 2B and C).

We tested for potential interspecies differences with regard to mitochondrial susceptibility to ischemia/reperfusion (Figure 2D-F), and in particular with respect to succinate metabolism. Figure 2D shows that while postischemic mouse mitochondria retained or even increase their ability to oxidize succinate, reperfused human mitochondria lose their ability to oxidize succinate. Results from validation experiments using rat and pig renal mitochondria parallel those for mice and humans, respectively (Figure 2D). In contrast to their rodent counterparts, postischemic human (and pig) mitochondria have a reduced capacity to oxidize succinate.

Respirometry revealed further interspecies differences in mitochondrial susceptibility. Not only are rodent mitochondria significantly more resistant to ischemia than human and pig mitochondria ($p<0.05$ and $P<0.03$, respectively) (Figure 2D and E), but it was observed that increases in complex II activity in rodent mitochondria compensate for loss of complex I function ($p<0,038$)[14] (Figure 2F).

These findings challenge a prominent role for succinate-driven ROS formation in the context of renal graft ischemia/reperfusion.[4] To that end, we tested reperfusion-related ROS formation in the context of clinical graft reperfusion by quantifying allantoin release from reperfused kidneys by means of ateriovenous concentration differences (Figure 3). Absent allantoin-release in the first 30 min following reperfusion implies negligible ROS quenching during reperfusion.

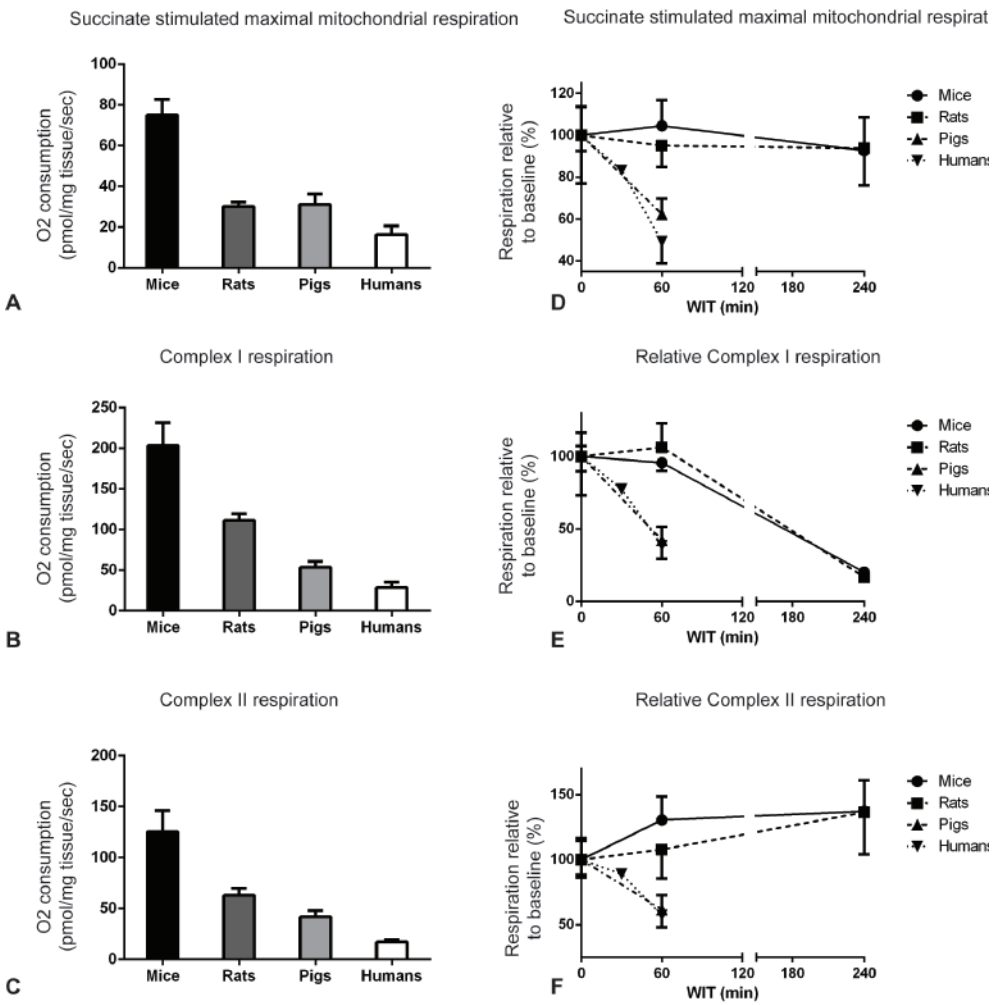

Figure 2. Interspecies differences in baseline mitochondrial respiration rates (A-C) and mitochondrial susceptibility towards ischemia and reperfusion (D-F).
A-C: Baseline aspects of mitochondrial oxidative phosphorylation (absolute values (oxygen consumption per mg tissue per second) for different species. Note the inverse associations ($p<0.000001$) between body weight and maximal oxidative phosphorylation capacity (A), complex I activity (B), and complex II activity (C). Bars represent mean ± SEM.
D-F: Effect of warm ischemia time and re-oxygenation on the aspects of mitochondrial function for different species (values relative to baseline values (baseline= 100%), Bars represent SEM). D) Sixty minutes of warm ischemia significantly ($p < 0.05$) reduced succinate-driven mitochondrial respiration (combined complex I and II activities) in human and pig mitochondria, whereas succinate-driven respiration in rat and mice mitochondria is not influenced by 4 h of warm ischemia.

E) Sixty minutes of warm ischemia significantly reduced complex I activity of human and pig mitochondria (p‹0.03). Complex I activity of mouse and rat mitochondria is not influenced by 60 minutes warm ischemia but significant drops are found after 4-hours of warm ischemia (p ‹ 0.001).
F) Sixty minutes warm of ischemia significantly reduced complex II (succinate dehydrogenase) activity of human and pig mitochondria (p‹ 0.008 resp. p‹ 0.04), but increased respectively stable Complex II activity in mouse and rat mitochondria (p‹0,038).

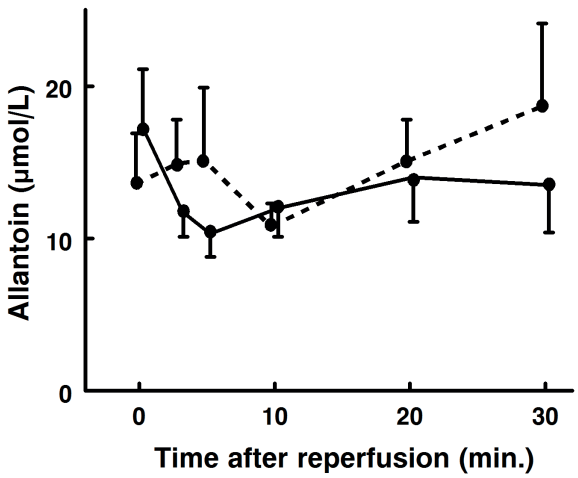

Figure 3. Absent postreperfusion uric acid ROS quenching (allantoin release from the reperfused grafts following prolonged ischemia). Dotted line represents arterial allantoin levels and solid line represents renal vein allantoin levels. (arteriovenous difference by area under the curve (AUC): p=0.368).

DISCUSSION

From these data, the picture emerges of divergent metabolic and mitochondrial responses to I/R in mice and in humans, and for succinate accumulation in murine kidneys between warm and cold ischemia. It is unclear how these observations translate to the human context. Pre-reperfusion succinate content of grafts from donation after cardiac death (DCD) was actually lower than that in from donation after brain death (Supplementary Figure 2), showing that the additional period of warm ischemia in DCD donors does not translate to increased graft succinate content.

In mice, progressive succinate accumulation during ischemia and reversed transport through mitochondrial complex-I drives reperfusion-related ROS formation.[4] In contrast, the human context is characterized by an inverse relationship between ischemia and succinate tissue levels, and ischemia-driven knockdown of mitochondrial respiration. As such, the question arises of whether clinical I/R in the context of kidney transplantation is accompanied by excess ROS formation.

As noted earlier, clinical studies consistently fail to show a beneficial effect of antioxidant therapy on I/R injury,[7-9] and markers for oxidative damage remain low following ischemia/reperfusion.[10] These observations would be consistent with a minor role for ROS in clinical I/R, but they may also indicate superior antioxidant responses, with effective ROS quenching, effectively preventing ROS-mediated damage. To test whether excess ROS is formed during clinical I/R, we quantified ROS quenching by establishing allantoin release from reperfused human kidneys. Allantoin is the stable end product of ROS quenching by the antioxidant uric acid and, as such, reflects the extent of oxidative stress. Absent allantoin release in the first 30 minutes following reperfusion implies that ROS quenching is negligible and that ROS stress during clinical I/R may be less than generally expected.

In summarizing the mechanism of succinate accumulation driving I/R injury, Heger et al.[11] pointed out that 'most research in I/R injury has been proven of limited translational value'. The findings noted for I/R in the clinical setting of kidney transplantation point to fundamental mechanistic differences between findings obtained in rodent models of I/R and the actual human context. It came to our attention that the above observations (partly) explain the apparent antioxidant paradox in the context of I/R with clear advantages in murine studies, but no apparent benefit in clinical studies.[7-9,15]

Supplementary Figure 1 and 2 are published online.

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IV

Defective postreperfusion metabolic recovery directly associates with incident delayed graft function

Kidney International

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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).

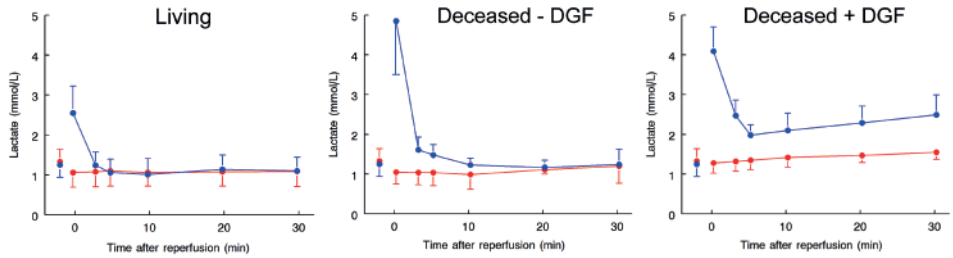


Figure 1. Persistent post reperfusion lactate release from +DGF grafts.
Arterial (red) and renal vein (blue) lactate levels (mean(±SEM)).
Left) Grafts from living donors (n=11) show absence of lactate release after a brief period of lactate washout upon reperfusion, which indicates an almost instantaneous 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.

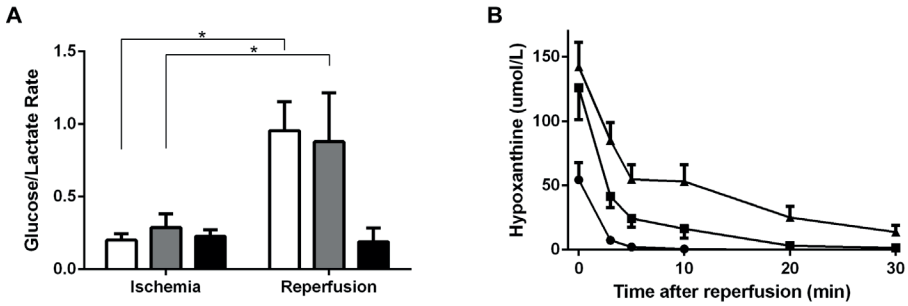


Figure 2. Readouts of tissue metabolic competence.
A) Recovery of tissue glucose/lactate ratio (mean(±SEM)) in living (n=6) and –DGF grafts (n=6) (Resp. living: glucose/lactate ratio before 0.19(±0.0) vs. after 0.90(±0.16) reperfusion (P‹ 0.0039) and –DGF grafts: before 0.28(±0.07) vs. after 0.87(±0.24) 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).

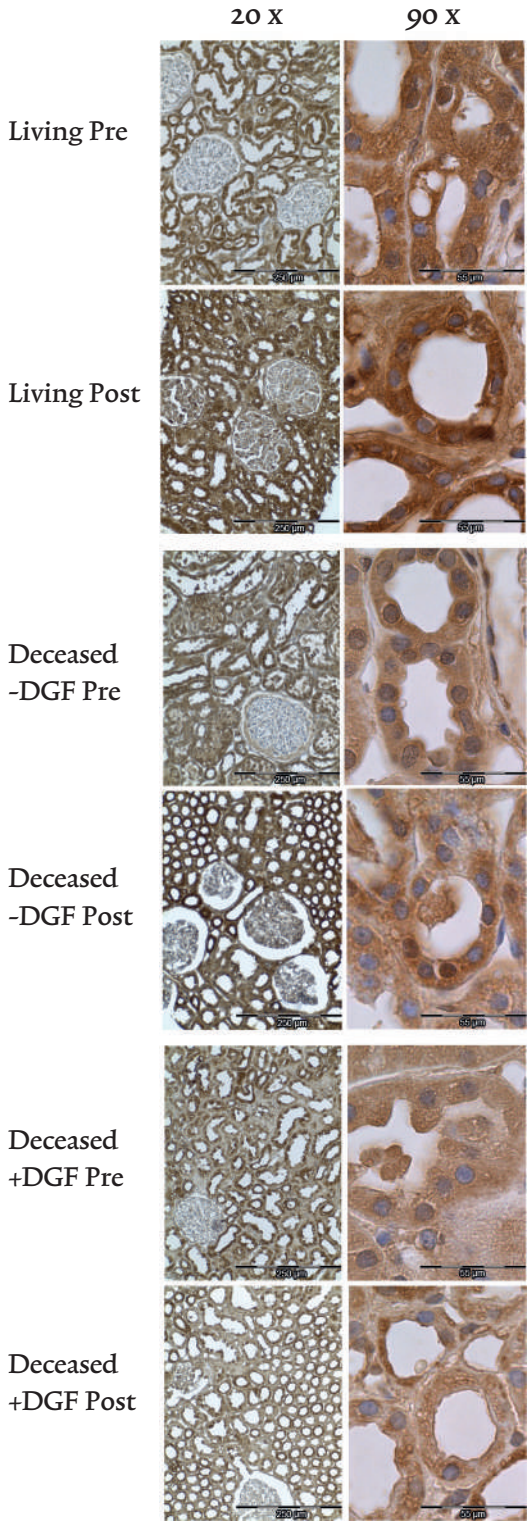


Figure 3. Overview of mitochondrial morphology in pre- and postreperfusion biopsies

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))

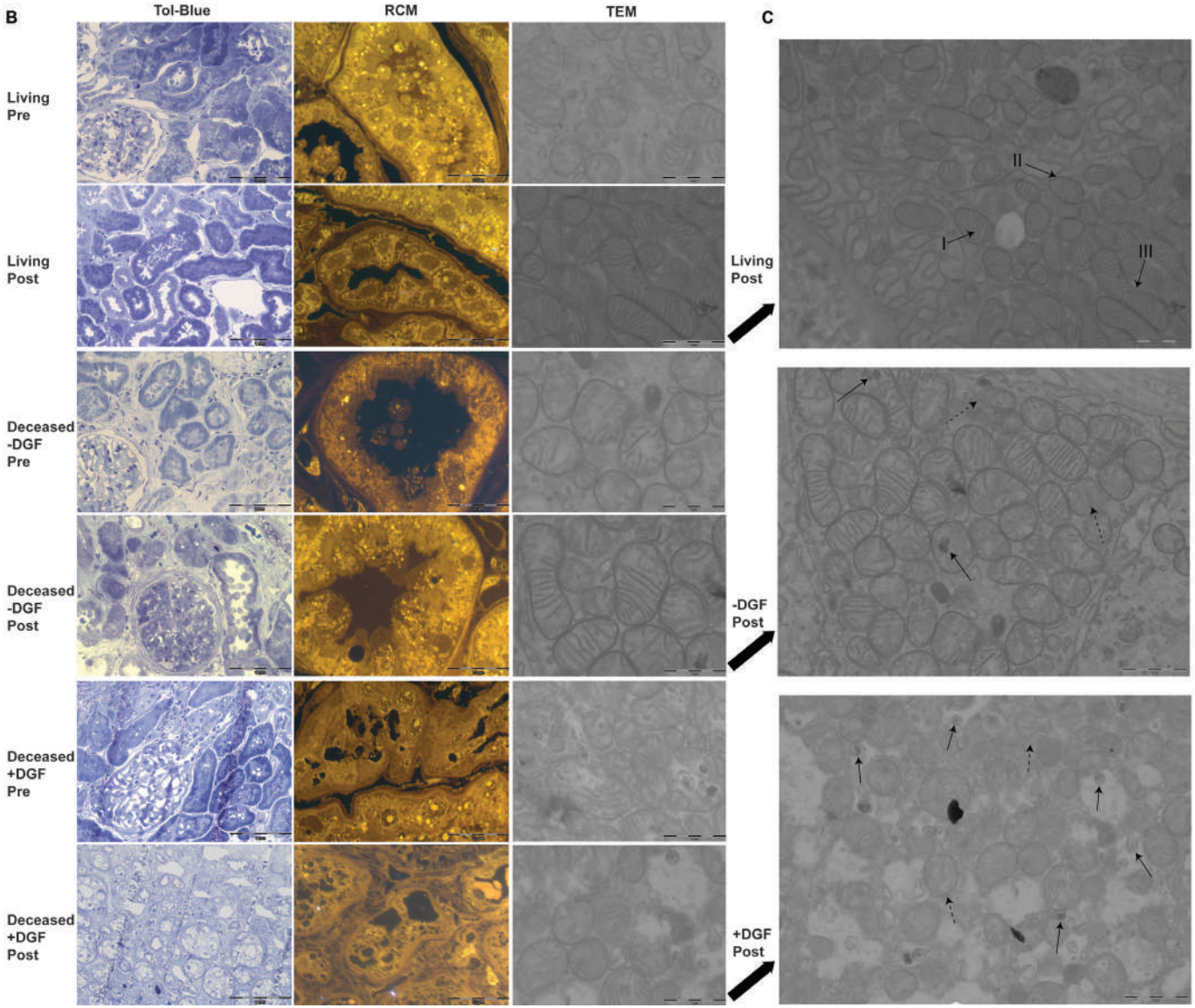

B
Tol-Blue
RCM
TEM
Living Pre
Living Post
Deceased -DGF Pre
Deceased -DGF Post
Deceased +DGF Pre
Deceased +DGF Post
C
Living Post
-DGF Post
+DGF Post
I
II
III

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]

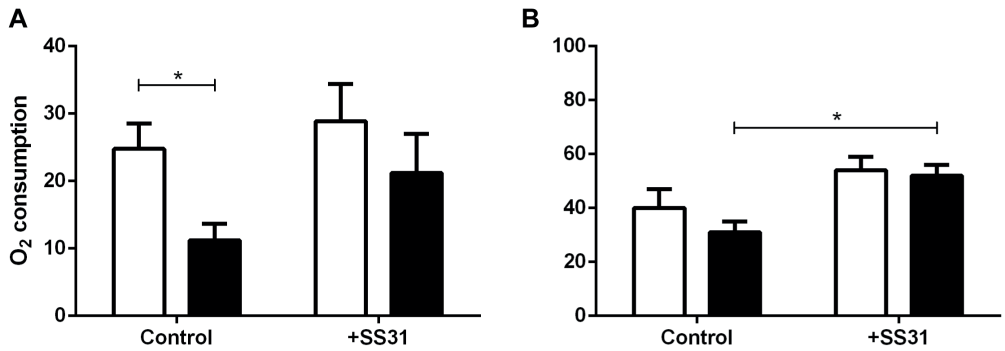


Figure 4. The mitochondrial stabilizing peptide SS-31 prevents ischemia-induced loss of mitochondrial function.
A) Maximal mitochondrial (uncoupled) respiration (pmol $O2^{s-1.mg-1}$). SS-31 preserves maximal oxidative capacity (mean(±SEM)) following 60 minutes of warm ischemia. With SS-31: 28.8(±5.53) 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 ($P<0.00061$).

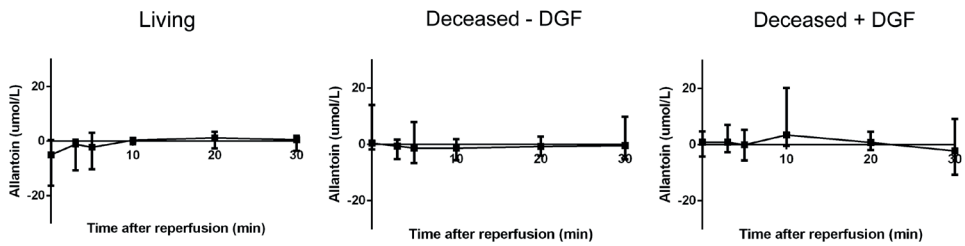


Figure 5. Absence of allantoin release from living, -DGF and +DGF grafts in the first 30 minutes after reperfusion.
Allantoin, the final end product when uric acid performs as reactive oxygen species (ROS) scavenger is not released following renal ischemia/reperfusion (mean(±SEM): +DGF (n=8), -DGF (n=5),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

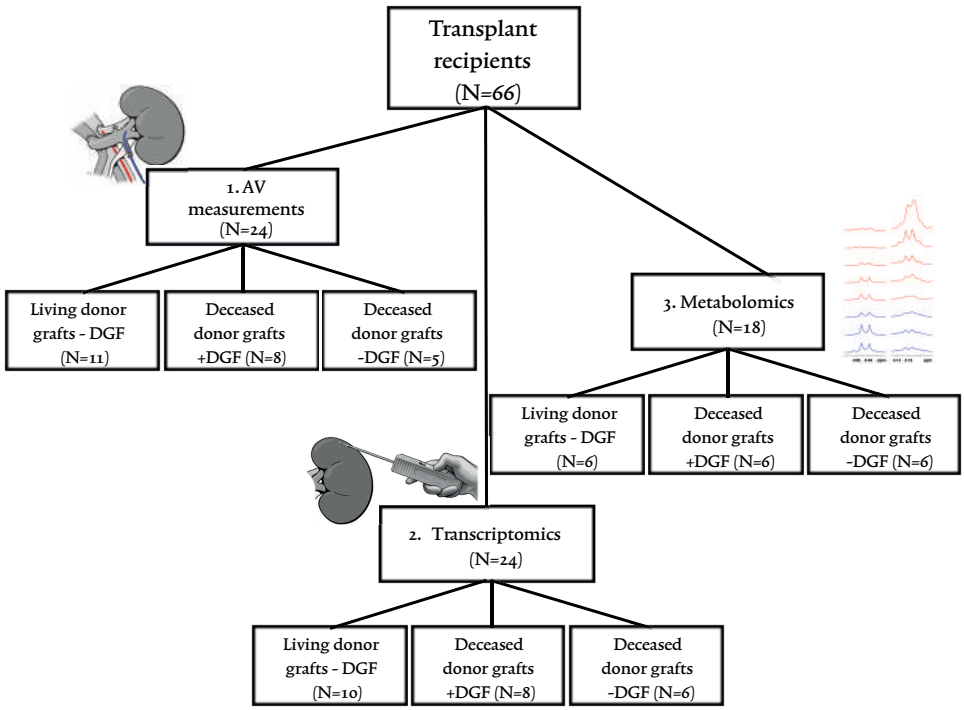
Limitations: This is a relative small study and as such we were unable to address specific points such as the influence of specific donor characteristics, types of anesthesia used during transplantation, or specific recipient characteristics. Moreover, no machine preservation was used in this cohort.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

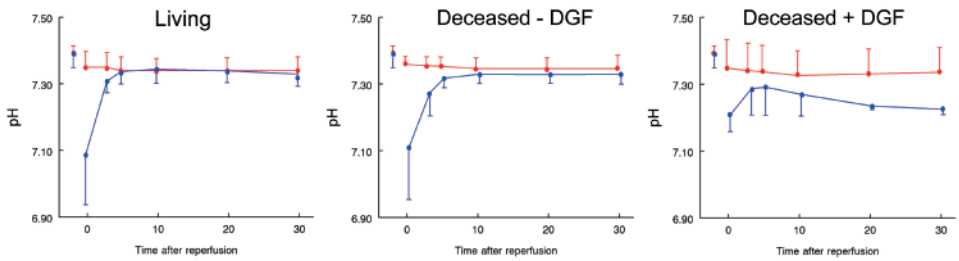
Although these studies have been performed in the context of kidney transplantation it came to our attention that observations from this study may also apply to other clinical situations of ischemia and reperfusion, such as myocardial infarction and cerebrovascular accidents. For the context of kidney 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

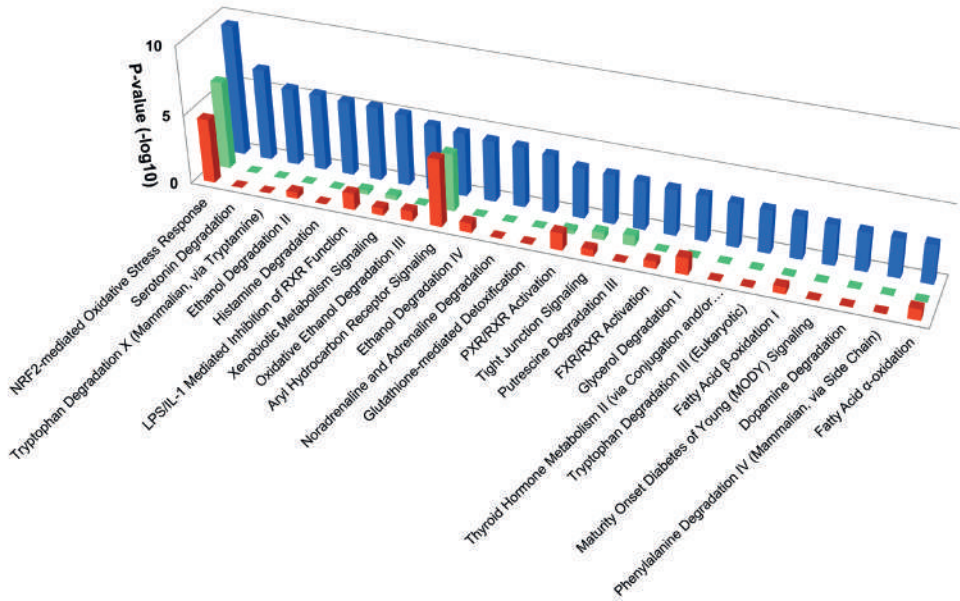
SUPPLEMENTARY FIGURES



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.

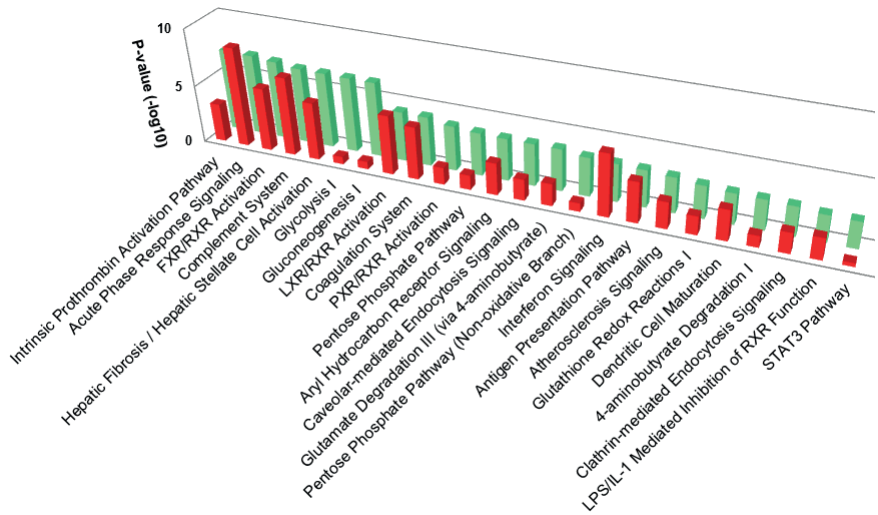


Supplementary Figure 2. Persistent acidosis upon reperfusion of +DGF grafts. Arterial (red) and renal vein (blue) pH (mean, SEM). Left + Middle) Rapid normalization of venous pH in living and –DGF grafts. Right) Persistent acidosis in +DGF grafts. (P‹ 0.004)



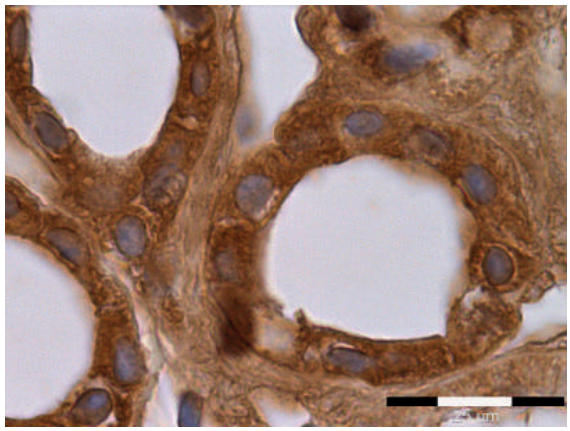
Supplementary Figure 3. Ingenuity Pathway Analysis–based analysis of gene expression.

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 o-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.



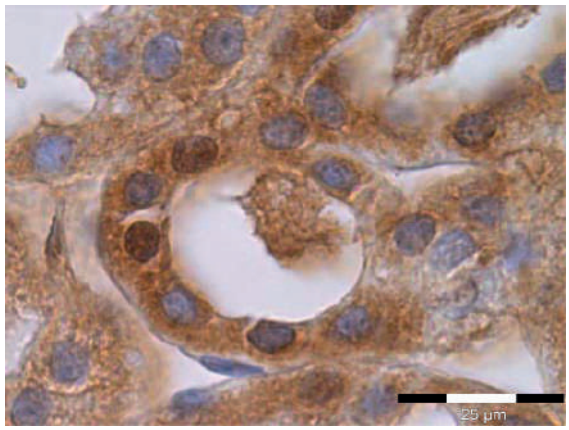
Supplementary Figure 3. Ingenuity Pathway Analysis–based analysis of gene expression.

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

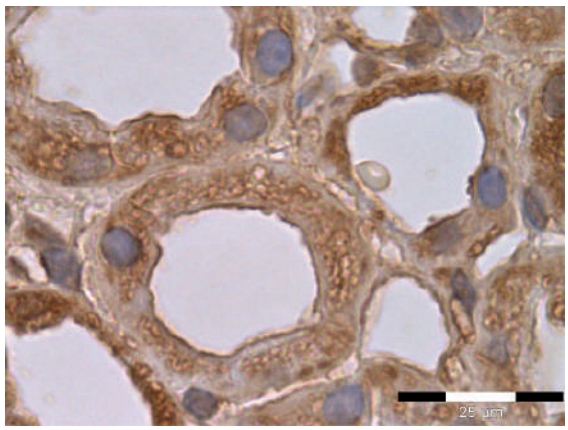


Supplementary Figure 4. Cytochrome C immunohistochemistry.

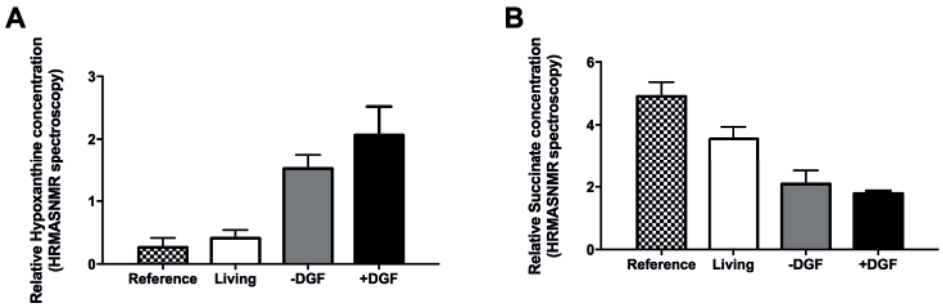
Supplementary Figure 4A. Cytochrome C staining (90 fold) of a living donor graft 45 minutes after reperfusion showing high density and punctuate stained pattern of Cytochrome C. (scale bar: 25 µm)



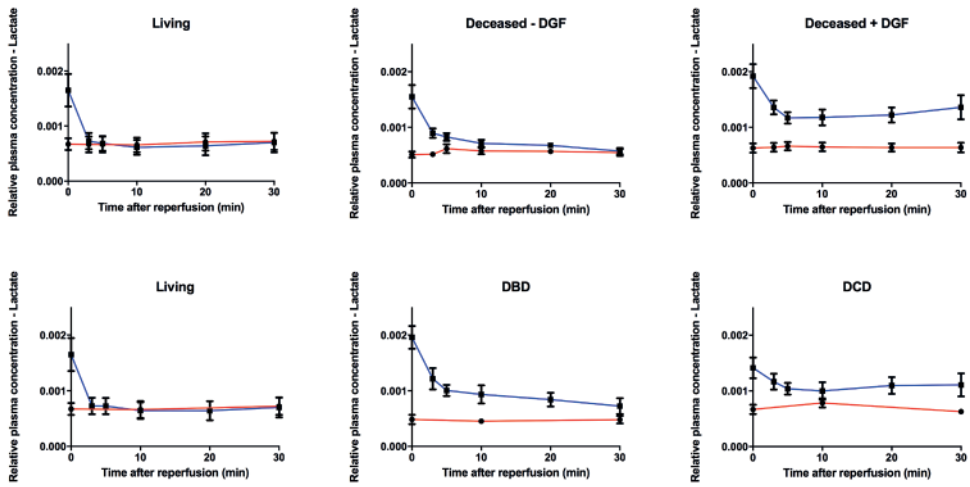
Supplementary Figure 4B. Cytochrome C staining (90 fold) of –DGF kidney 45 minutes after reperfusion showing sustained punctuate pattern of Cytochrome C. (scale bar: 25 µm)



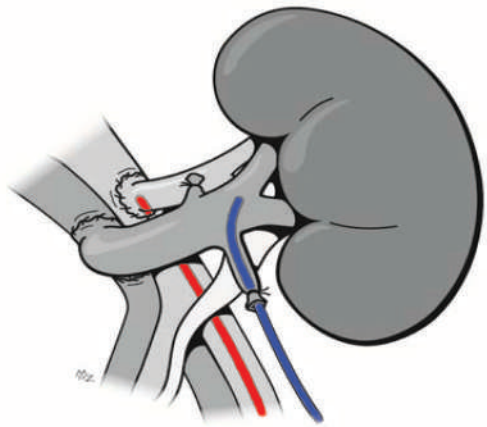
Supplementary Figure 4C. Cytochrome C staining (90 fold) of +DGF kidney 45 minutes after reperfusion showing loss of the punctate pattern of Cytochrome C. (scale bar: 25 µm)



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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V

An immediate post-reperfusion metabolic collapse associates with ischemia/reperfusion injury in the context of clinical kidney transplantation

Manuscript in preparation

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ABSTRACT

Background: kidney transplantation is the only curative option in end-stage renal disease. Due to everlasting organ shortages, more marginal grafts are used for transplantation. This leads to increased incidence of delayed graft function (DGF), the clinical readout of ischemia/reperfusion injury after transplantation. Previous studies indicated that DGF is driven by a metabolic incompetence in the (early) reperfusion period. To deepen knowledge on metabolic adaptation of DGF grafts in the acute phase of reperfusion a metabolome-broad approach was taken.
Materials & Methods: this report is based on sequential studies: a total of 52 kidney transplant recipients were included, of which 22 (16/34 and 6/18) developed DGF. Human kidney samples, taken from living donors prior to donation (n=10), served as non-ischemic controls. Renal artery and vein were cannulated during the transplantation procedure and paired plasma samples were taken immediately after reperfusion (at 0, 3, 5, 10, 20 and 30 minutes (n=34)). Biopsies were taken at the end of the ischemic period and 45 minutes after reperfusion (n=18). Metabolomics was performed on both plasma and tissue samples.
Results: DGF grafts show decreasing phosphocreatine levels (P=0,002) and ongoing ATP catabolism (i.e. persistent release of (hypo)xanthine (resp. P=0,040, P=0,007). Compared to adequately functioning control grafts, DGF grafts are hallmarked by the continuous release of lactate (P=0,001), acetylcarnitine (P=0,016), pyruvate (P=0,022), α-ketoglutarate (P=0,008), short-chain carnitines and phospholipids in the first 45 minutes after reperfusion. It was realized that the metabolomic-differences between DGF grafts and controls are collectively covered in 5 functional clusters: metabolic collapse (power outage), Krebs cycle (entry) defects, glycolysis/glutamine oxidation, lipid oxidation and phospholipolysis.
Conclusion: Incident DGF is preceded by a profound metabolic incompetence caused by mitochondrial dysfunction. Release of the Krebs cycle intermediate α-ketoglutarate, in combination with the absence of succinate recovery implies graded defects of oxoglutarate dehydrogenase activity in DGF grafts. This indicates that incident DGF not only associates with mitochondrial dysfunction, but that damage extends beyond the membrane bound respiratory complexes and also involves complexes located in the mitochondrial cytosol.

INTRODUCTION

Delayed Graft Function (DGF), clinically defined as the need for dialysis in the first week following transplantation, is the manifestation of ischemia/reperfusion injury in the context of kidney transplantation.[1,2] Incident DGF detrimentally impacts graft function and survival.[1,3] Despite major research efforts, there is currently no therapy available that prevents or alleviates clinical ischemia/reperfusion injury.[4] As such, the high incidence of DGF constitutes a major obstacle for a more liberal use of grafts donated after cardiac death and from so called marginal donors in an era of organ shortages.

In an effort to identify the factor(s) driving DGF, we systematically evaluated processes implicated in renal graft ischemia/reperfusion injury. More specifically, we sequentially assessed arterial-venous metabolite concentration differences over the reperfused renal graft to obtain organ specific information. Findings from these clinical studies challenge commonly implicated culprits of ischemia/reperfusion injury such as oxidative damage, neutrophil or complement activation, or inflammation as initiators of early ischemia/reperfusion injury.[5–9] In contrast, clear evidence was found for an association between a profound post-reperfusion metabolic deficit resulting from extensive mitochondrial damage, and incident DGF.[10]

These observations suggest that in the context of renal transplantation, ischemia/reperfusion injury is driven by a metabolic incompetence in the (early) reperfusion period. Consequently, attempts to reduce DGF should focus on preventing or overcoming the post-perfusion metabolic deficit. In this context, we considered a clinical exploration of post-reperfusion metabolic profiles of donor grafts with and without incident DGF relevant. Data from living-donor grafts; procedures with superior immediate and long-term functional outcome were included as reference profiles.

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.

In this prospective study, 68 successive transplant recipients were enrolled. Ten patients refused participation, and six patients were excluded after cancellation of the transplantation (because of positive crossmatch, poor recipient condition or moderate quality of the graft). In total 52 patients were included.

Renal allograft transplantations were performed according to the local protocol.[10] In living donors, open minimal access nephrectomy was performed, and Custodiol® HTK (histidine–tryptophan–ketoglutarate) preservation solution was used for cold perfusion and storage. Deceased donor grafts 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.

Patient allocation was defined by presence of delayed graft function (DGF) following transplantation. DGF was defined as the need dialysis in the first week after transplantation.[2]

ARTERIOVENOUS SAMPLING AND KIDNEY BIOPSIES

Sequential arteriovenous (AV) blood sampling over the graft was performed in 34 patients. Reference AV blood samples for a normal, non-ischemic kidney were obtained before nephrectomy by sampling over the donor kidney in living donors (n=10).

The AV sampling procedure was performed as follows: prior to implantation of the donor graft, a 5 French umbilical vein catheter was positioned in the lumen of the renal vein through one of its side branches. Renal vein blood samples were collected at 30 s, and 3, 5, 10, 20 and 30 min after reperfusion (i.e. moment of reperfusion t=0). Paired arterial blood samples (arterial line) were obtained at 0, 10 and 30 min after reperfusion. Blood samples were collected in precooled containers and immediately placed on melting ice. The AV sampling method was validated earlier measuring oxygen saturation.[7]

Renal cortical biopsies were taken immediately prior to and 45 minutes after reperfusion (n=18). Six patients received a kidney from a living donor and 12 patients received a kidney from a deceased donor. Tissue biopsies were snap frozen in liquid nitrogen and stored at –80°C.

METABOLIC ANALYSES, PLASMA

Targeted metabolomics analyses were done using standard operating procedures from previously published methods. Detailed procedures and target lists are provided in the Supplemental methods with a brief overview of the five platforms used given below.

1.Plasma amino acids

The amine platform is an UPLC-MS/MS based method that covers amino acids and biogenic amines employing an Accq-tag derivatization strategy adapted from the protocol supplied by Waters.

2.Plasma carnitines

The acylcarnitine platform is an UPLC-MS/MS based method that allows for the separation and quantification of several important isomers of acylcarnitine species as well as trimethylamine-N-oxide, choline, betaine, deoxycarnitine, and carnitine.

3.Plasma organic acids

The organic acid platform uses a full scan gas chromatography-mass spectrometry (GC-MS) method combined with a target list of well-characterized metabolites for the analysis of organic acids. The method uses a combination of an oximation reaction using methoxyamine hydrochloride and a silylation reaction using N-Methyl-N-(trimethylsilyl) trifluoroacetamide.

4.Plasma purines and pyrimidines

Purines and pyrimidines were quantified using an in-house developed UPLC-MS/MS method using a Waters Acquity HSS T3 column and a Waters XEVO TQS tandem mass spectrometer using negative or positive electrospray ionization with specific MRM transitions.

METABOLIC ANALYSES: TISSUE (KIDNEY BIOPSIES)

5.Magic Angle Spinning High-resolution NMR spectroscopy (MAS HR-NMR)

Metabolic profiling of the tissue biopsies was performed by MAS HR-NMR spectrometry on a 14.1 T Bruker Avance III spectrometer. All measurements were performed at the MR Core Facility, Norwegian University of Science and Technology (NTNU). The MR core facility is funded by the Faculty of Medicine at NTNU and Central Norway Regional Health Authority.

STATISTICAL ANALYSIS

SPSS 22.0 (SPSSinc, Chicago, III) was used for statistical analysis. For heatmaps of arteriovenous concentrations differences between the three groups, venous/arterial ratios were calculated and expressed as the mean Z-score for each metabolite. For the heatmaps of tissue levels, pre-reperfusion/post-reperfusion rates were calculated and expressed in mean Z-score of the quantified concentrations. AV differences (per timepoint) were compared by Students T-test within group and tested with ANOVA between groups. Pre- and post-reperfusion levels of tissue metabolites were compared by Students T-test within group and pre/post reperfusion rates were compared and tested with ANOVA between groups.

If indicated, the area under the curve (AUC) was estimated for some plasma metabolites 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 (Living vs. –DGF vs. +DGF), 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.

Since most metabolites in the study are part of theoretical pathways we refrained from correction for multiple testing.

RESULTS

This study is based on data from a total of 52 kidney transplantations, 38 of these procedures concerned deceased donor graft transplant procedures. The clinical follow up showed that 22 of the deceased donor grafts fulfilled the criteria for DGF. These grafts were allocated to the +DGF group. The other 16 deceased donor grafts all displayed spontaneous recovery without the need for post-transplant dialysis and were therefore allocated to the –DGF group. Fourteen procedures concerned living donor grafts. All living grafts showed immediate functional recovery following reperfusion (urine production and creatinine clearance) and served as a reference. All grafts in the study showed ultimate functional recovery. Clinical details for the different groups are shown in Table 1).

Table 1. Patient characteristics

Patient Characteristics for arteriovenous concentration differences			
	Living (n=8)	Deceased – DGF (n=10)	Deceased +DGF (n=16)
Age recipient (yrs)	58,5±5,0	49,0±12,6	59,1±11,9
Sex recipient (% males)	55,6%	50%	68,8%
Recipient – cause of renal failure			
Glomerulonefritis	44,4%	40,0%	31,3%
*Polycystic kidney disease	11,1%	20,0%	18,8%
*DM type 2	0%	0%	25,0%
*Obstructive uropathy	11,1%	10,0%	6,25%
*Maligne hypertension	11,1%	20,0%	12,5%
*Renal failure e.c.i.	22,2%	10,0%	6,25%
Age donor (yrs)	55,5±10,7	55,6±13,8	57,4±15,3
Sex donor (% males)	66,7%	60%	56,2%
Donor cause of death			
*Living donor	100%	0%	0,0%
*CVA		20,0%	25,0%
*SAB		30,0%	12,5%
*TRAUMA		30,0%	25,0%
*CA-OHCA-AMI		20,0%	31,3%
*Suicide		0%	6,25%
*Miscellaneous		0%	0,0%
Ischemia time (hrs)	3,6±0,4	18,2±4,4	17,9±5,7
Histocompatibility (HLA mismatches, %)			

0	0%	10,0%	0,0%
1	33,3%	0%	18,8%
2	0%	60,0%	18,8%
3	11,1%	20,0%	37,5%
4	11,1%	10,0%	12,5%
5	33,3%	0%	12,5%
6	11,1%	0%	0,0%
Hospital Stay (days)	7,14±3,5	10,2±4,9	19,8±7,8

Patient Characteristics for HR MAS NMR spectroscopy in kidney biopsies			
	Living (n= 6)	Deceased – DGF (n= 6)	Deceased +DGF (n= 6)
Age recipient (yrs)	48,5±18.0	52,8±16,9	59,8±11,5
Sex recipient (% males)	100%	50%	100%
Recipient – cause of renal failure			
Glomerulonefritis	16,7%	33,3%	0%
*Polycystic kidney disease	16,7%	16,7%	16,7%
*DM type 2	16,7%	33,3%	33,3%
*Obstructive uropathy	16,7%	0%	0%
*Maligne hypertension	16,7%	16,7%	0%
*Renal failure e.c.i.	16,7%	0%	50%
Age donor (yrs)	50±8.1	59,3±11,3	56,5±13,2
Sex donor (% males)	66,7%	50%	33,3%
Donor cause of death			
*Living donor	100%	0%	0%
*CVA		33,3%	33,3%
*SAB		33,3%	0%
*TRAUMA		0%	16,7%
*CA-OHCA-AMI		33,3%	50%
*Suicide		0%	0%
*Miscellaneous		0%	0%
Ischemia time (hrs)	3,7±0,3	10,4±2,9	16,5±3,9
Histocompatibility (HLA mismatches, %)			
0	0%	0%	0%
1	0%	16,7%	0%
2	16,7%	16,7%	16,7%
3	16,7%	16,7%	50%
4	16,7%	33,3%	16,7%
5	33,3%	16,7%	16,7%
6	16,7%	0%	0%
Hospital Stay (days)	6,7±1,2	9,4±4,6	18,0±8,9

Table 1. Patient Characteristics. Patients were included in two recruitery rounds. Recipients of living donor grafts were taken as a reference since DGF is rare in this control group. Deceased donor grafts were classified based on outcome after transplantation. Delayed graft function (DGF) is status in which the transplant recipient is in need of dialysis in the first week(s) after transplantation and is caused by I/R injury.
Abbreviations: DM = diabetes mellitus, e.c.i. = e causa ignota.

We first explored putative differences in the metabolic signatures of +DGF deceased donor grafts, –DGF deceased donor grafts and those of the living donor grafts (reference) of the T=30 min post-reperfusion AV differences and the post-reperfusion tissue biopsies. The 30 min time-point for the AV differences was chosen to avoid interference from washout of metabolites accumulated during ischemia.
The AV differences are summarized in the heatmap shown in Figure 1A, and the tissue profiles in Figure 1B. The heatmap for the AV differences (Fig. 1A) shows parallel signatures for the living donor grafts and –DGF grafts, and a distinctive signature for the +DGF grafts. Although, differences on the tissue level (Figure 1B) were less outspoken than those for the AV differences, the –DGF signature matched that of the living donor reference group.
Functional clustering of the individual metabolic differences between –DGF and +DGF grafts identified 5 functional clusters: (I) metabolic collapse (power outage); (II) beta-oxidation; (III) glycolysis/glutamine oxidation and autophagy; (IV) Krebs cycle (entry) defects, and (V) phospholipolysis/cell damage. Collectively, these clusters cover all observed metabolic differences (the metabolic signature of future DGF). For the sake of clarity, it was decided to report the metabolites according to this clustering.

Figure 1A. Clustered heatmap for the arterial-venous metabolite concentration differences over the donor graft at 30 minutes after reperfusion.

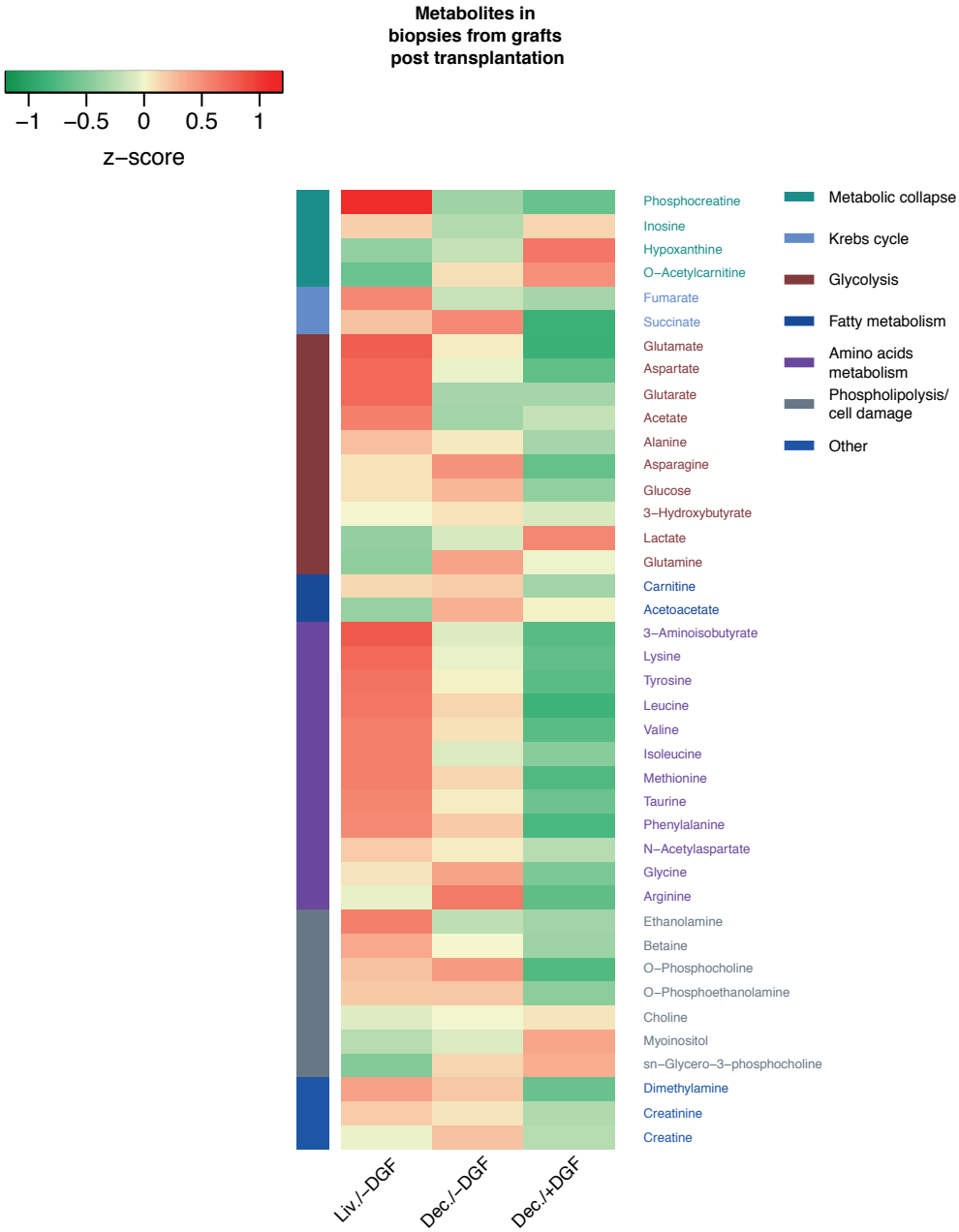


Figure 1B. Clustered heatmap for all identified tissue metabolites in the HR magic angle NMR analysis of graft biopsies taken 40 minutes after reperfusion.

Figure 1A. Clustered heatmap for the arterial-venous metabolite concentration differences over the donor graft at 30 minutes after reperfusion.
The three columns represent the three donor groups (living donor grafts (reference group); deceased donor grafts without later DGF (–DGF), and deceased donor grafts with later DGF (+DGF). Rows represent the arteriovenous concentrations differences between the three groups, expressed as the mean Z-score of the venous/ arterial ratios for each metabolite. Compounds are clustered according to the five metabolic clusters and, within each cluster ranked on basis of the Z-score of the living donors group. Green reflects net uptake by-, and red net release from the graft.

Figure 1B. Clustered heatmap for all identified tissue metabolites in the HR magic angle NMR analysis of graft biopsies taken 40 minutes after reperfusion. The three columns represent the three donor groups (living donor group (reference group), deceased donor grafts without later DGF (–DGF) and deceased donor grafts with later DGF (+DGF). Rows represent tissue content of each metabolite, expressed in mean Z-score of the quantified concentrations. Metabolites are clustered according to the five metabolic clusters and, within each cluster, ranked on basis of the Z-score in the living donor group. Red reflects a tissue content above, and green below the geometric mean of the three groups.

The first cluster of metabolites is consistent with a metabolic collapse ('power outage') in grafts with later DGF. This collapse is reflected by absent recovery of the high-energy phosphate buffer phosphocreatine in the tissue of +DGF grafts ($P<0.002$, Figure 2A), and by persistent hypoxanthine and xanthine release (AV-differences) from these grafts (Figure 2B). Although all 3 graft types showed an initial wash out of these ATP/GTP catabolites upon reperfusion, persistent release in grafts with later DGF implies continued ATP/GTP catabolism after reperfusion ($P<0.007$ and $P<0.04$ respectively for hypoxanthine and xanthine at T=30 min). Graded pre-reperfusion tissue accumulation of the ATP catabolites inosine and hypoxanthine in the three donor groups with the lowest tissue content in living, and the highest in +DGF donor grafts (Figure 2C) implies progressive degrees of high energy phosphate depletion during organ procurement and cold preservation. Low post-reperfusion contents in the +DGF group presumably relate to circulatory clearance (hypoxanthine), but possibly also to exhaustion of the ATP supply in these grafts.

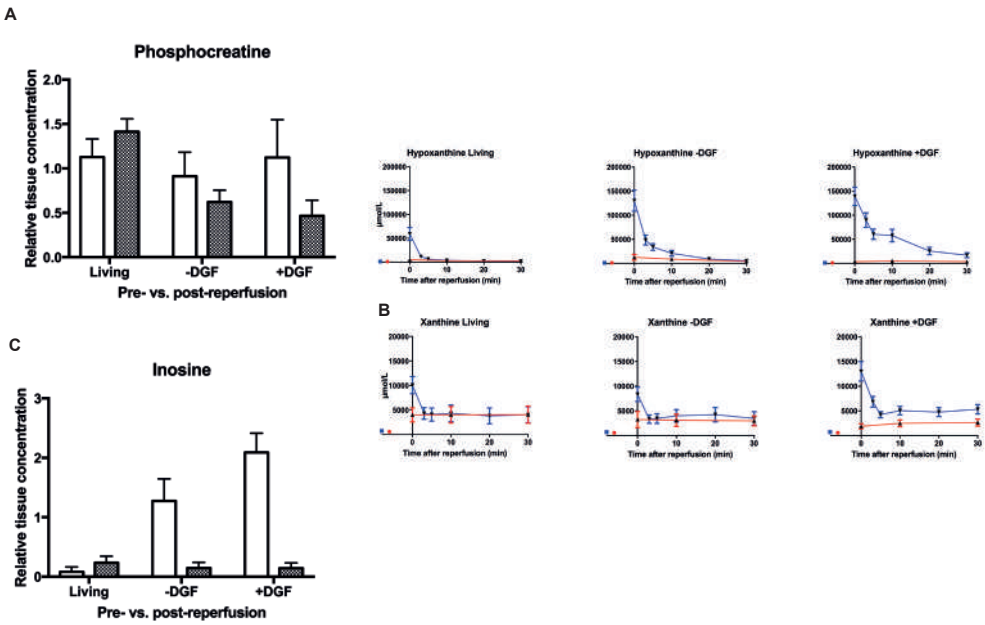


Figure 2. A post-reperfusion metabolic collapse associates with future DGF.
A) Pre and post-reperfusion(40 minutes post reperfusion) tissue phosphocreatine content in the three donor groups (living donor group (reference group), deceased donor grafts without later DGF (–DGF) and deceased donor grafts with later DGF (+DGF)). Stable post-reperfusion tissue phosphocreatine content in living donor grafts and –DGF grafts , but reduced post-reperfusion phosphocreatine tissue content (P‹0.002) in grafts with later DGF.
B) Post-reperfusion arterial (red) and venous (blue) concentrations for hypoxanthine and xanthine, the end products of nucleoside triphosphate catabolism. Stable arterial hypoxanthine and xanthine levels in all three study groups. Renal vein samples show an immediate wash out of accumulated hypoxanthine and xanthine following reperfusion in all three study groups. Persistent hypoxanthine and xanthine release from +DGF grafts (P‹0.007 and 0.04 respectively at T= 30 minutes after reperfusion) implies persistent nucleoside triphosphate catabolism in these grafts.
C) Progressive pre-reperfusion accumulation of inosine in the three graft types showing implying graded ATP catabolism during organ procurement.

Arterial (red) and venous (blue) data points left at the curve indicate plasma levels over normal kidneys.

The metabolic deficit in +DGF grafts occurred in spite of restoration of β-oxidation (Figure 3) and activation of normoxic glycolysis (Figure 4) and possibly autophagy (Figure 5). Uniform restoration of β-oxidation in all 3 graft types is reflected by emergence of hydroxybutyric acid in post-reperfusion tissue biopsies, as well as by clearance (uptake) of mid-chain fatty acids (C8-C12) from the circulation (Figure 3; Supplemental Figures). Tissue accumulation (-DGF and +DGF grafts) (Fig. 3C) and release of acetyl-carnitine in the circulation (Fig.3B) (+DGF grafts) (T=30 min: P‹0.016) implies various degrees of impaired disposal of this end-product of β-oxidation in the deceased donor grafts.

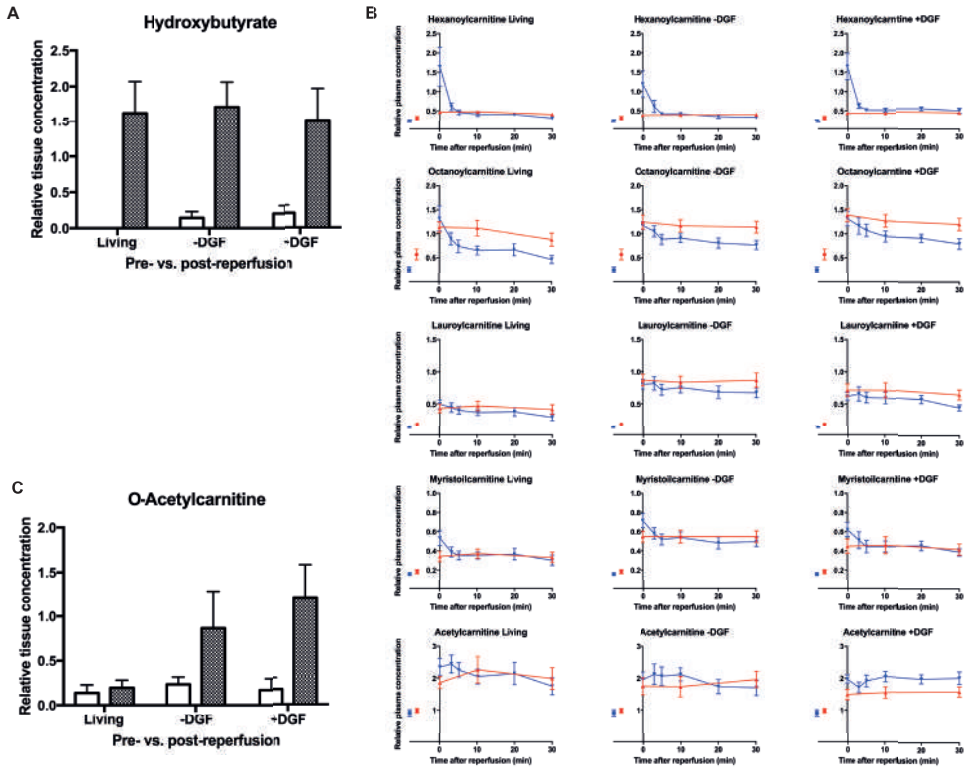


Figure 3. Reinstatement of β-oxidation (medium chain fatty acids) following reperfusion.

A) Post-reperfusion recovery hydroxybutyrate showing commencement of β-oxidation in all three donor groups

B) A-V differences for plasma carnitines show selective uptake of C8-C12 medium chain carnitines in all three donor groups. Transient (-DGF) and persistent acetylcarnitine release (AV differences, P‹0.007) and acetylcarnitine tissue accumulation C) indicate temporary respectively persistent inadequate acetyl disposal in – and +DGF grafts.

Arterial (red) and venous (blue) data points left at the curve indicate plasma levels over normal kidneys.

Mapping of glycolysis/glutaminolysis (Figure 4) indicated absent recovery of tissue glucose content following reperfusion, and persistent glycolysis in the +DGF donor grafts. Normoxic glycolysis in +DGF grafts is illustrated by the persistent release of lactate (T=30 min: $P<0.001$) and the transamination products alanine and aspartate (T=30 min: $P<0.006$ and $P<0.005$ respectively, Figure 4). Evident glutamine uptake and glutamic acid release (T=30 min: $P<0.006$) (AV differences) by the +DGF grafts points to a contribution of glutaminolysis in the energy supply. +DGF grafts showed net pyruvate release in the reperfusion phase (T=30 min: $P<0.022$), indicating that pyruvate production in these grafts exceeds pyruvate disposal (LDH, transaminases, Krebs cycle). Note that stable tissue glutamine, glutamate, alanine, and aspartate levels in +DGF grafts suggest an efficient blood-tissue exchange of these metabolites.

Some indications were found for autophagy (Figure 5) in +DGF grafts such as the net-release (AV differences) of selective amino-acids such as methionine and tyrosine (P for the area under the curves of methionine release in To-30 min after reperfusion: $P<0,000006$ and $P<2,3993E^{-7}$ respectively)) Figure 5). In this respect, progressive isovaleryl-carnitine release in +DGF indicates post-reperfusion leucine oxidation in these grafts.

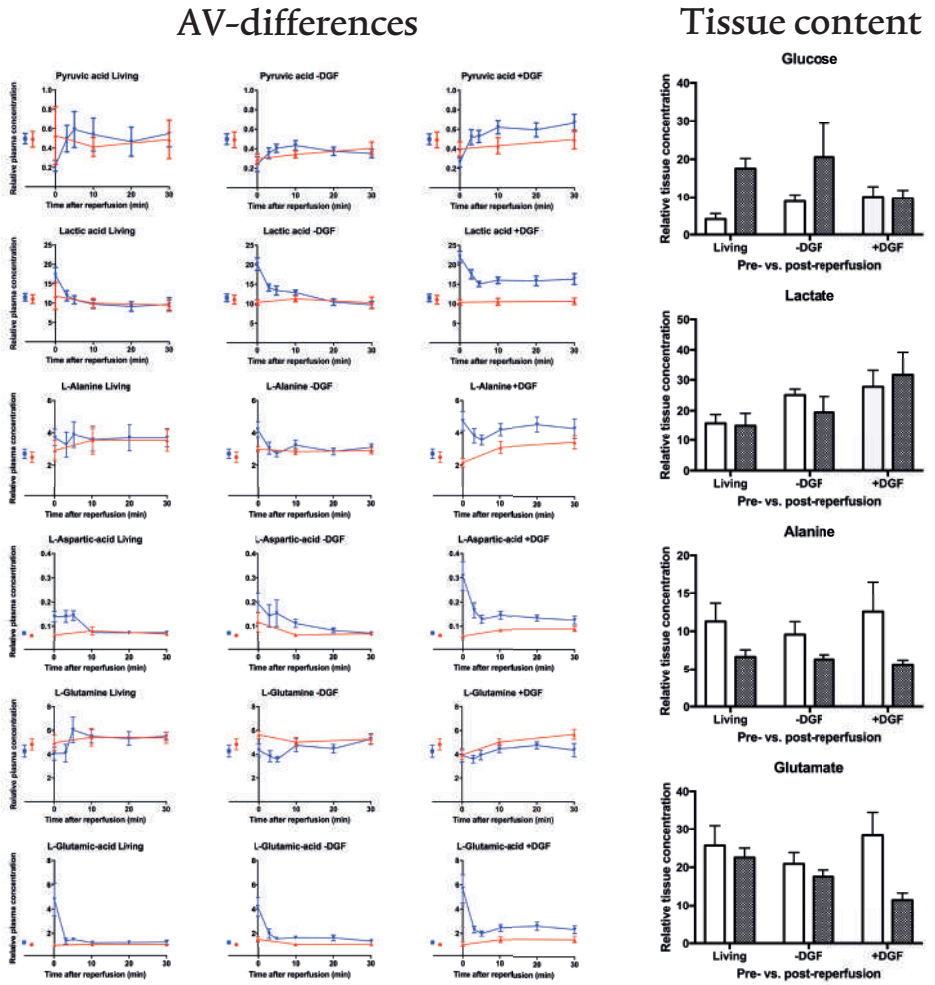


Figure 4. Post-reperfusion glycolysis and glutaminolysis.
Left column: A-V differences. Persistent post-reperfusion lactate, alanine (P‹0,006) and pyruvate (0.022) release from +DGF grafts indicate normoxic glycolysis in these grafts. Temporary resp. continued glutamine uptake and glutamate release (P‹0.006, (AV differences) from – and + DGF grafts show transient and persistent glutamine oxidation in these grafts.
Right column: tissue content. Pre and post reperfusion tissue contents of glucose, lactate, alanine and glutamine. Absent glucose recovery and persistently high lactate content in +DGF grafts. Stable alanine and glutamate contents presumably reflect wash out of these intermediates from the kidney.
Arterial (red) and venous (blue) data points left at the curve indicate plasma levels over normal kidneys.

AV-differences

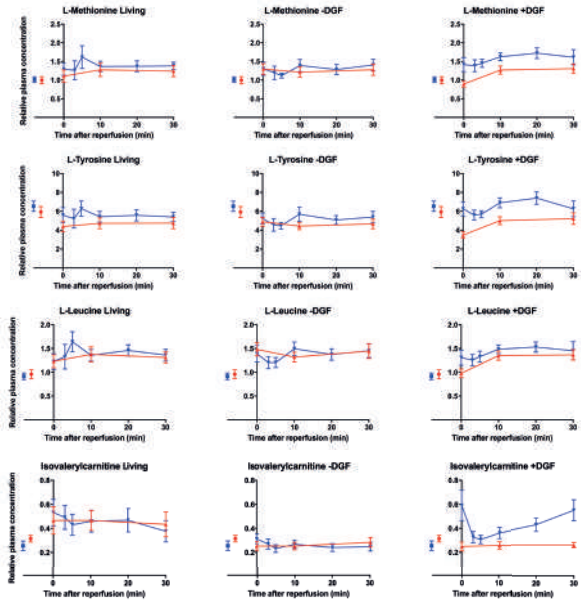


Figure 5. Post reperfusion amino acid metabolism.
A-V differences show post-reperfusion net release of the methionine, and the ketogenic amino acids tyrosine and leucine (P=0,000006; P=2,3993E-7 and P=0,017) from + DGF grafts. Selective and progressive isovalerylcarnitine release indicates post-reperfusion leucine oxidation in +DGF grafts.
Arterial (red) and venous (blue) data points left at the curve indicate plasma levels over normal kidneys.

The apparent metabolic deficit despite abundant activation of beta-oxidation, glycolysis and possibly autophagy, and the accumulation of the Krebs cycle entry-products acetyl-carnitine (Figure 3) and pyruvate (Figure 4) point to Krebs cycle defect(s) (Figure 6, Supplemental Figure 1) in +DGF grafts. Post-reperfusion release of the Krebs cycle intermediate α-ketoglutarate (T=30 min: P<0.008, Figure 6), but not its downstream intermediates succinate, fumarate and malate, and absent recovery of tissue succinate and fumarate in the +DGF grafts (Figure 6) indicate at least a defect at the level of oxoglutarate dehydrogenase in +DGF grafts.

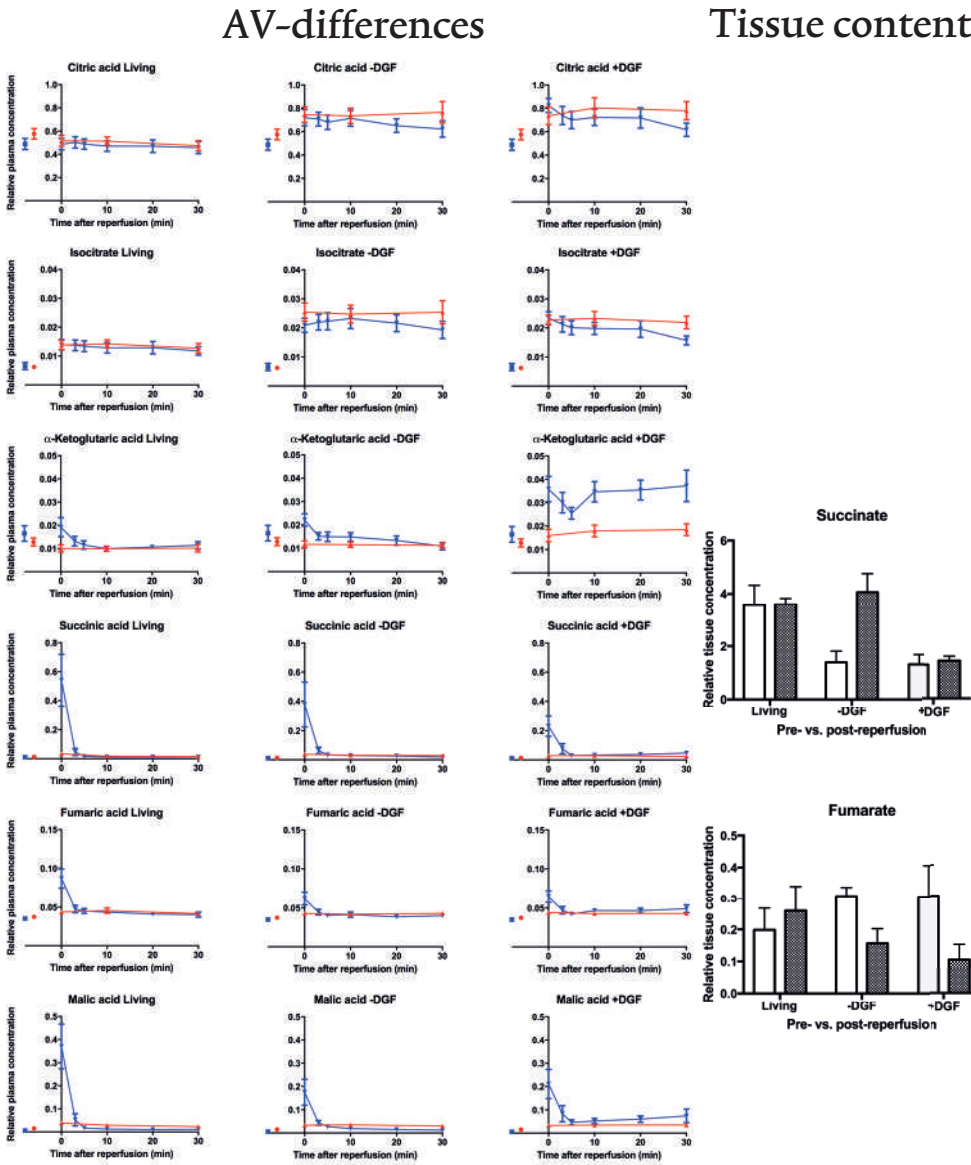


Figure 6. Post-reperfusion Krebs cycle defect in grafts with future DGF.
Left column: A-V differences. Release of the Krebs cycle intermediate α-ketoglutarate from +DGF grafts
Right column: tissue content. Absent succinate (+DGF grafts) and fumarate (- and +DGF grafts) recovery.
Arterial (red) and venous (blue) data points left at the curve indicate plasma levels over normal kidneys.

Results from the amino acid and purine analysis revealed a further discriminatory pattern that is consistent with post-reperfusion phospholipolysis/cell damage in +DGF grafts (Figure 7). This pattern includes release of uracil (T=30 min: P‹0.0002) and release of a cluster of phospholipid associated amino-acid constituents (viz. phospho-ethanolamine, ethanolamine, and phospho-serine (P for T=30 min resp.: ‹0.001, ‹0.004 and ‹0.003). Although choline was not released, its oxidation product betaine was progressively and selectively released from +DGF grafts (T=30 min: P‹0.0004).

Again, stable tissue content for these markers implies efficient tissue clearance and/or metabolism.

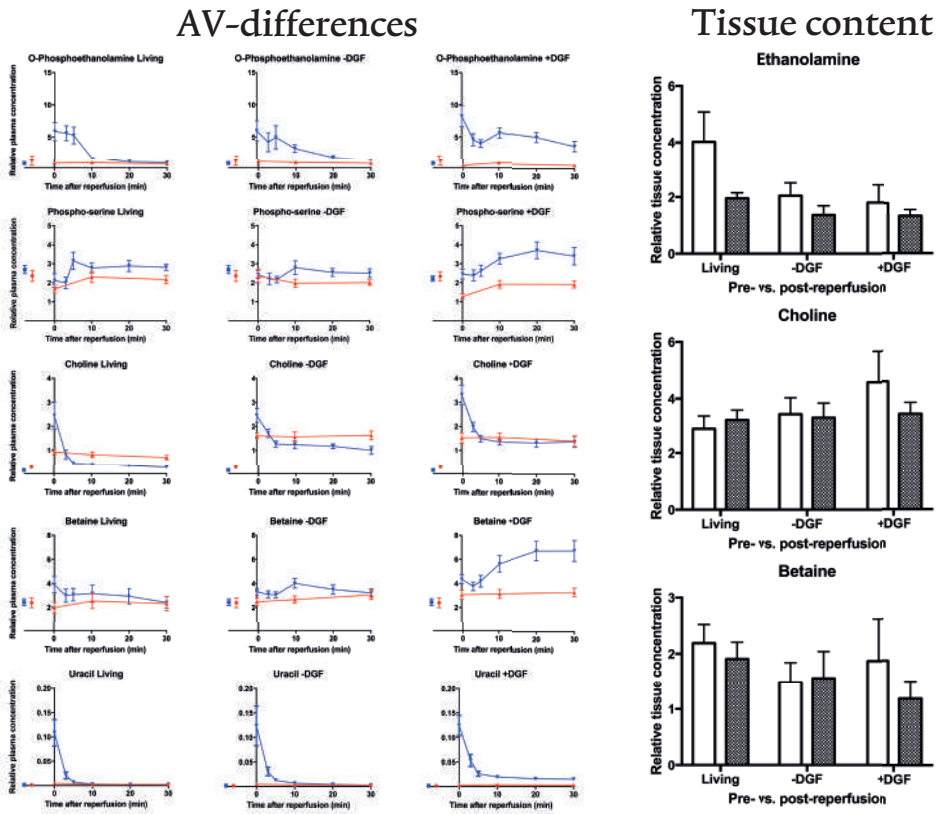


Figure 7. Selective and persistent post-reperfusion wash out of phospholipid-associated amino-acids and uracil from grafts with future DGF.
Initial and brief wash out of accumulated the phospholipid components (phospho) ethanolamine, and choline, and the pyrimidine uracil upon reperfusion for all three donor groups. A-V differences show persistent release of uracil (T=30 min: P‹0.00002), and phospholipid-associated amino acids (phosphor) ethanolamine, phosphoserine. Progressive post-reperfusion release of betaine, (T=30 min: P‹0.0004) the oxidation product of choline from +DGF grafts implies post-reperfusion choline oxidation. Tissue ethanolamine and betaine remain stable.
Arterial (red) and venous (blue) data points left at the curve indicate plasma levels over normal kidneys.

Above observations associate the incident ischemia/reperfusion injury with persistent post-reperfusion ATP catabolism and on-going cell damage in a context of mitochondrial failure and activation of glycolytic and lipolytic pathways.

DISCUSSION

From this study, performed during clinical kidney transplantation, the picture emerges of human renal ischemia/reperfusion injury being the consequence of an instantaneous, profound metabolic collapse in the early reperfusion phase. As a consequence, cells are unable to sustain adequate ATP levels, critically impairing their integrity, and perpetuating cellular injury. The data herein imply fundamentally different mechanisms for ischemia/reperfusion injury in humans compared to rodents.[11]

Conclusions from this study are based on integration of tissue data derived from paired pre- and post-reperfusion tissue biopsies and plasma-derived data based on sequential arteriovenous concentration differences over de graft during the first half hour of reperfusion. The plasma data allow for the appreciation of metabolite clearance into (elimination) or from (uptake) the circulation[12] and for directing the data from the paired tissue biopsies. In fact, we realized that sole reliance on tissue biopsies would have obscured most occurring events, as metabolite tissue levels/content remained stable due to effective clearance into the blood. Note that deceased donor grafts are anuric in the time frame of this study. As such, observations for the deceased donor grafts are not influenced by urinary metabolite clearance.
Mapping of the available metabolic data, shows that incident DGF (as a result of excessive tissue damage) is preceded by a metabolic crisis with persistent ATP catabolism in the reperfusion phase. This crisis is fully discriminatory for +DGF grafts, and appears to be primarily related to defects in the normoxic respiration (Krebs cycle defects), and an activated normoxic glycolysis that is unable to sustain the ATP supply. This latter conclusion is based on absent tissue phosphocreatine recovery,[13] and persistent post-reperfusion hypoxanthine and xanthine release in +DGF grafts. Hypoxanthine and xanthine represent end products of ATP (hypoxanthine and xanthine) and GTP (xanthine) degradation. Consequently, their continued release from +DGF grafts implies persistent ATP/GTP catabolism despite adequate graft perfusion (viz. oxygen and glucose supply). In fact, on basis of the reported post reperfusion renal blood flow it is estimated that the hypoxanthine lost in +DGF grafts during the first half hour of reperfusion represents more than 50% of the renal ATP pool/storage.

Impaired ATP regeneration in +DGF grafts appears to (partly) relate to impaired oxidative phosphorylation as result of Krebs cycle defect(s). This conclusion is based on release of pyruvate, and particular acetyl-carnitine, which indicates that these carbon flows exceed the capacity of the Krebs cycle; the release of the Krebs

cycle intermediate α-ketoglutarate, and absent tissue succinate recovery. Although it cannot be excluded that the α-ketoglutarate excess partly relates to glutamine oxidation, absent tissue succinate recovery in +DGF grafts, and to a lesser extend in –DGF grafts implies graded defects of oxoglutarate dehydrogenase activity.
These data extend conclusions from experimental studies and our earlier report that incident DGF associates with mitochondrial dysfunction[10,14] and that this damage not only extends beyond the membrane bound respiratory complexes, but also involves complexes located in the mitochondrial cytosol. Fully preserved β-oxidation in +DGF grafts shows that these effects are specific, and do not merely reflect gross mitochondrial damage.
An apparent persistent defect at the level of oxoglutarate dehydrogenase and absent succinate accumulation point to fundamental differences in the mechanisms involved in human and rodent (renal) ischemia and reperfusion. In fact, reported data for rat kidneys (and other organs) imply an oxidative burst within the first minutes of reperfusion, which is then followed by normalization of oxidative phosphorylation.[11] Human data herein imply a more protracted process with persistent mitochondrial failure, high-energy phosphate deficiencies, and absent gross oxidative damage.

Defects in oxidative phosphorylation in +DGF grafts are partially compensated by recruitment of normoxic glycolysis, as reflected by the continued lactate, alanine and asparagine release from these grafts, and possibly by the low post-reperfusion tissue glucose content. Although the latter could obviously relate to the increased glycolysis it cannot be excluded that a context of (relative) ATP deficiency interferes with insulin-mediated glucose uptake. Release of pyruvate in the circulation indicates that pyruvate flux in +DGF grafts exceeds the capacity of pyruvate dehydrogenase, lactate dehydrogenase, and aspartate transaminase and alanine transaminase.
Normoxic glycolysis in the +DGF grafts was paralleled by persistent glutaminolysis as shown by the glutamine uptake and glutamate release into the circulation. Early reperfusion glutamine uptake seen in living donor and –DGF grafts suggests transient glutamine oxidation as a metabolic adaptation in the first minutes following reperfusion.

The 3 graft groups showed similar post-reperfusion recruitment of lipid oxidation with recovery of tissue hydroxybutyrate and uptake of medium chain carnitines (C8-C12) and unsaturated C14 from the circulation. This latter observation implies that the kinetics of unsaturated C14 corresponds to that of a medium chain fatty acid. There appears to be a small but consistent release of short chain (C6 and smaller) carnitines from +DGF grafts. This phenomenon may indicate peroxisomal

β-oxidation since this reaction is limited to large and medium chain lipids and absent for smaller acyl chains. As peroxisomal lipid oxidations lack a valid electron acceptor, it is paralleled by a stoichiometric production of hydrogen peroxide. Consequently, putative peroxisomal β-oxidation in +DGF grafts merits attention as a route that may perpetuate ischemia/reperfusion injury through production of hydrogen peroxide.

The data for the amino acids identified 3 functional clusters. A cluster that relates to the process of glycolysis/glutaminolysis discussed earlier, a second cluster that is particularly enriched for amino acids incorporated in glycerophospholipids (see further), and a third cluster (methionine, tyrosine, serine and isovalerylcarnitine) that presumable reflects autophagy and failure to (completely) metabolize amino acids. The notion of autophagy and incomplete amino acid catabolism is supported by the isovalerylcarnitine release from +DGF grafts. Isovaleryl-CoA is an intermediate of leucine catabolism, that is normally further oxidized by isovaleryl-CoA dehydrogenase. In this context, selective release of isovalerylcarnitine may reflect excess substrate delivery (leucine from autophagy) and/or ischemia/reperfusion-related defects in the isovaleryl-CoA dehydrogenase complex.
We have no clear explanation for the asymmetrical amino acid release pattern (viz. release of abundant amino acids such as leucine and serine, but not of glycine). One possibility is that acetyl-CoA or NADPH accumulation in +DGF grafts interferes with leucine and serine catabolism. Albeit interesting and potentially relevant, we consider further exploration of observed asymmetrical amino acid release beyond the scope of this explorative paper.
Selective and sustained release of a class of amino acids is almost exclusively associated with glycerophospholipids in +DGF grafts, and implies that incident renal ischemia/reperfusion injury is associated with immediate cell membrane damage following reperfusion. In this context, the data for betaine, the oxidation product of choline is particularly revealing. While pre- and post-reperfusion tissue betaine levels remain stable, there is a selective and delayed post-reperfusion release of betaine from +DGF grafts. This observation implies post-reperfusion choline availability in +DGF grafts and that release of glycerophosolipid-associated amino acids is a reperfusion-related phenomenon, rather than a phenomenon related to ischemia-related damage. The pattern of sustained damage (phospholipolysis, uracil release) in +DGF grafts suggests clinical reperfusion injury is a protracted event. This notion is supported by release patterns of the Krebs cycle intermediates.

All in all, above conclusions imply that the mitochondrial defects and the associated metabolic collapse occur within the first minute(s) of reperfusion,

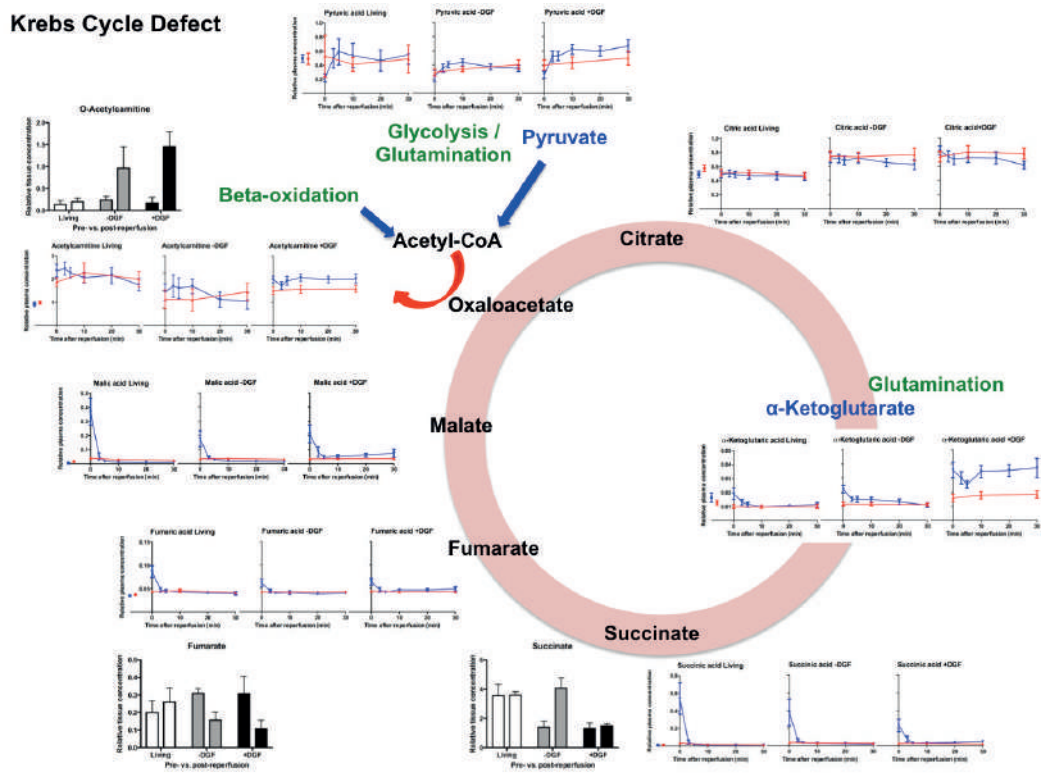
leaving a marginal window of opportunity for rescue therapies aimed at mitochondrial stabilization. Yet, unlike other forms of ischemia and reperfusion, organ transplantation does allow for preventive strategies aimed at mitochondrial stabilization. As such, in the context of organ transplantation (and other forms of planned ischemia and reperfusion), strategies aimed at mitochondrial stabilization, either as donor treatment or during organ storage (preservation) merit attention.[15,16]

Alternative, non-preventative strategies should focus on maximizing the available energy resources, in particular for normoxic glycolysis. Such an approach could include optimal substrate delivery and/or inhibition of futile or inhibitory pathways. In-depth analysis of the erythrocyte, a professional aerobic glycolytic cell that is devoid of mitochondria identified inosine as an energy dense alternative for glucose. Along this lines, inosine is superior to glucose in preserved cellular ATP content of eukaryotic cells during hypoxia[17] and ameliorates tissue damage in experimental ischemia/reperfusion models.[18,19]

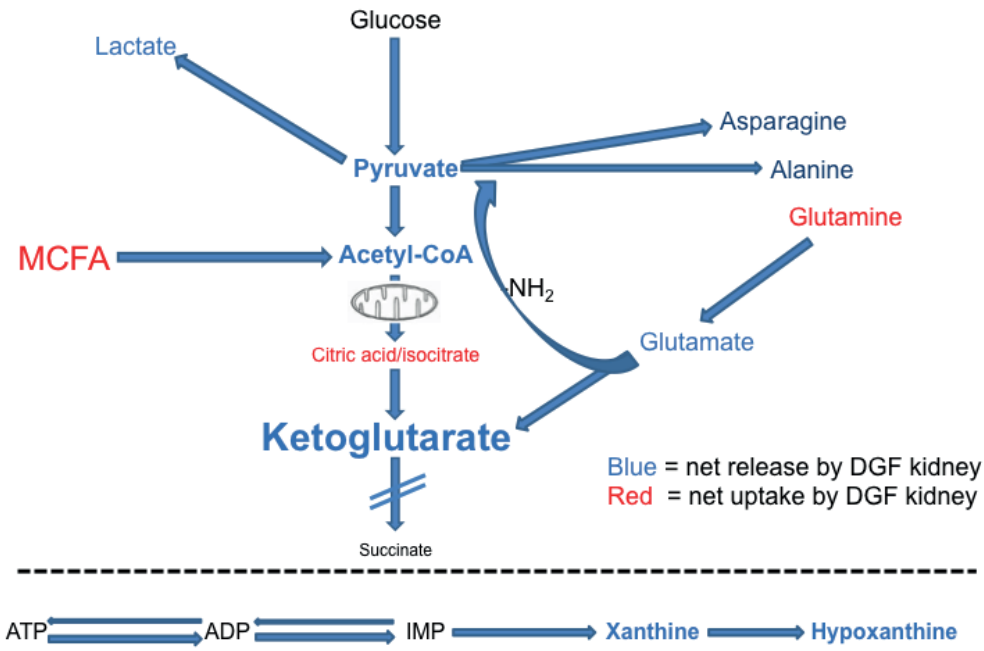
Strategies improving aerobic glycolysis could further include reduction of cellular acidosis via minimizing H+ formation in futile metabolic cycles. Reflexive pyruvate formation via activation of glutaminolysis is the natural response of somatic cells aimed at sustaining the Krebs cycle. Observed pyruvate release, and α-ketoglutarate from +DGF grafts show that, in the context of graft ischemia/reperfusion injury glutaminolysis is futile, yet does potentially contribute to cellular acidosis via release of H+ and consumption of NAD+. One could speculate that in such a context interference with glutaminolysis is beneficial.

In conclusion, results of this clinical evaluation show that incident graft ischemia/reperfusion injury is preceded by a profound metabolic incompetence caused by mitochondrial dysfunction, shedding light on the underlying mechanism of clinical ischemia/reperfusion injury. It came to our attention that although this difference may relate to fundamental differences in the pathophysiology of ischemia and reperfusion between rodent and humans, it may also imply that the injury inflicted in rodent models is insufficient to induce the metabolic deficit observed in human renal ischemia/reperfusion injury.

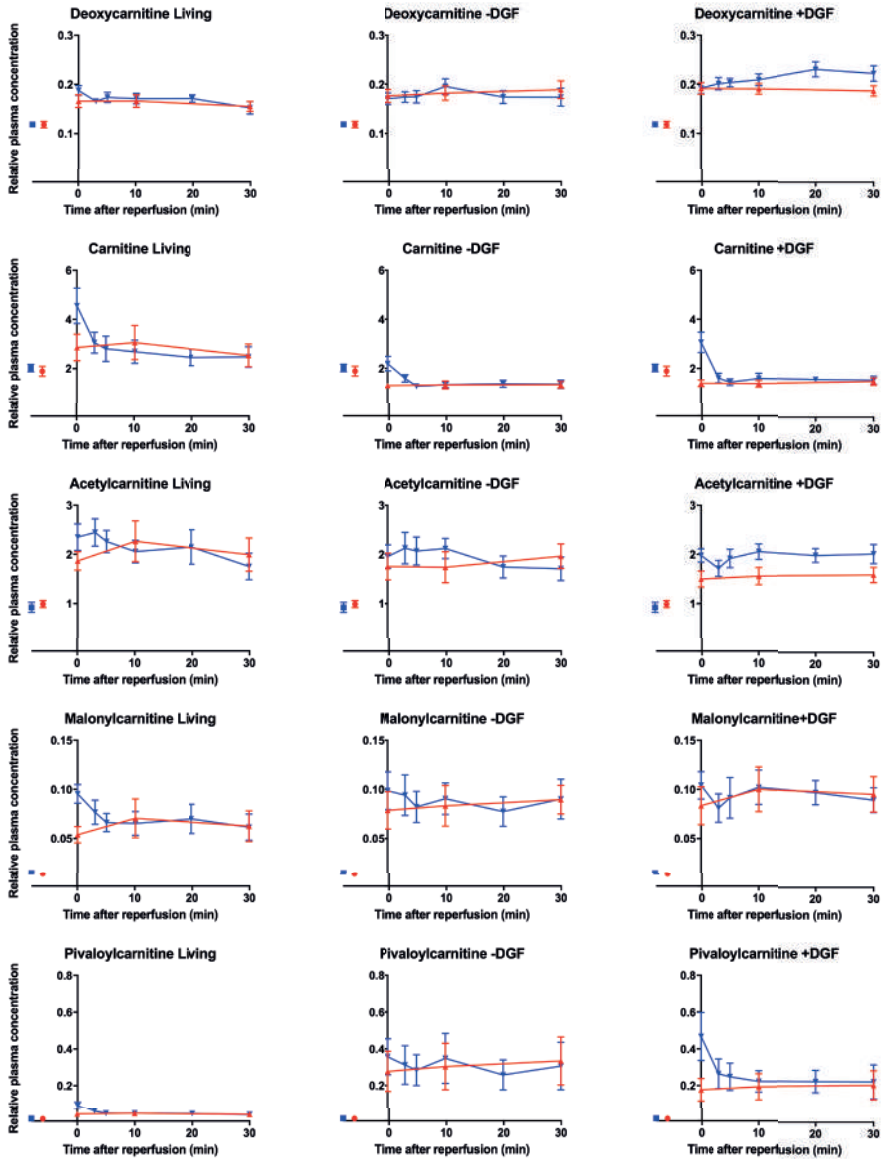
SUPPLEMENTARY FIGURES

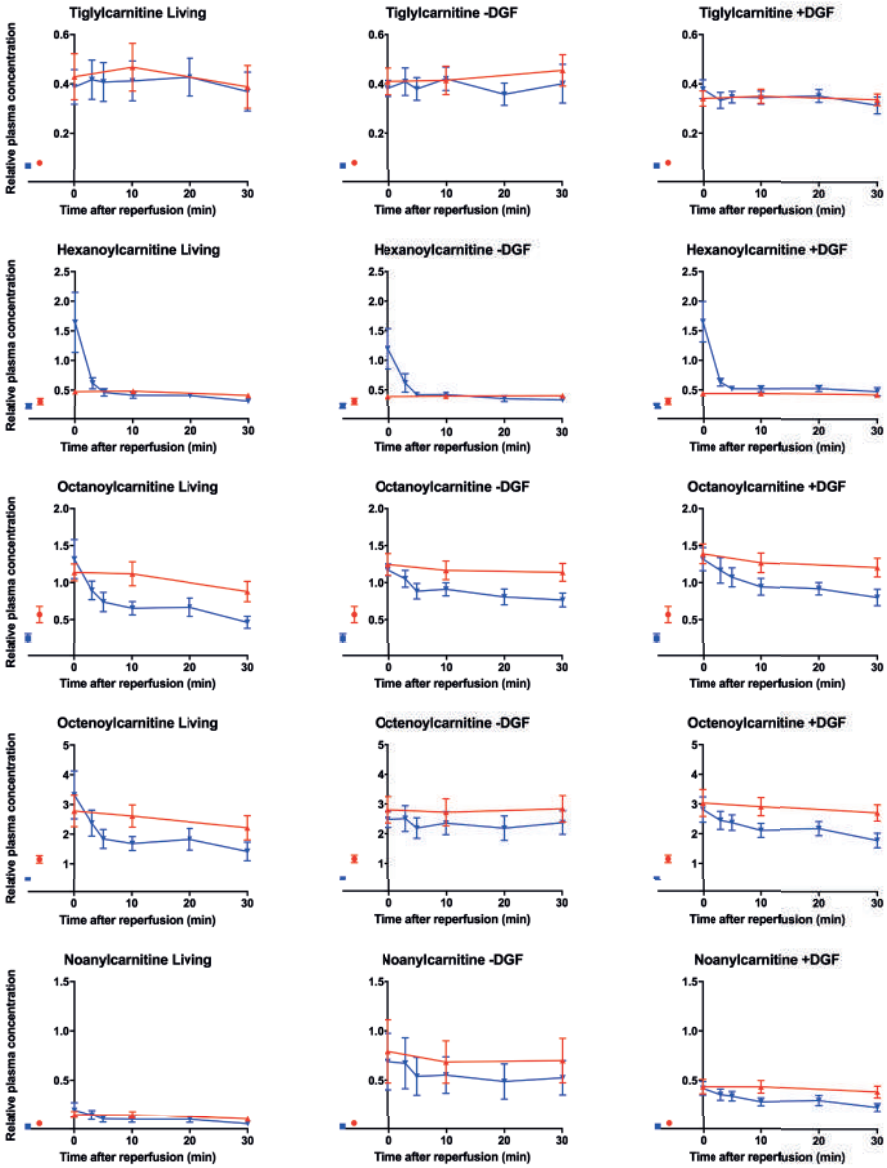


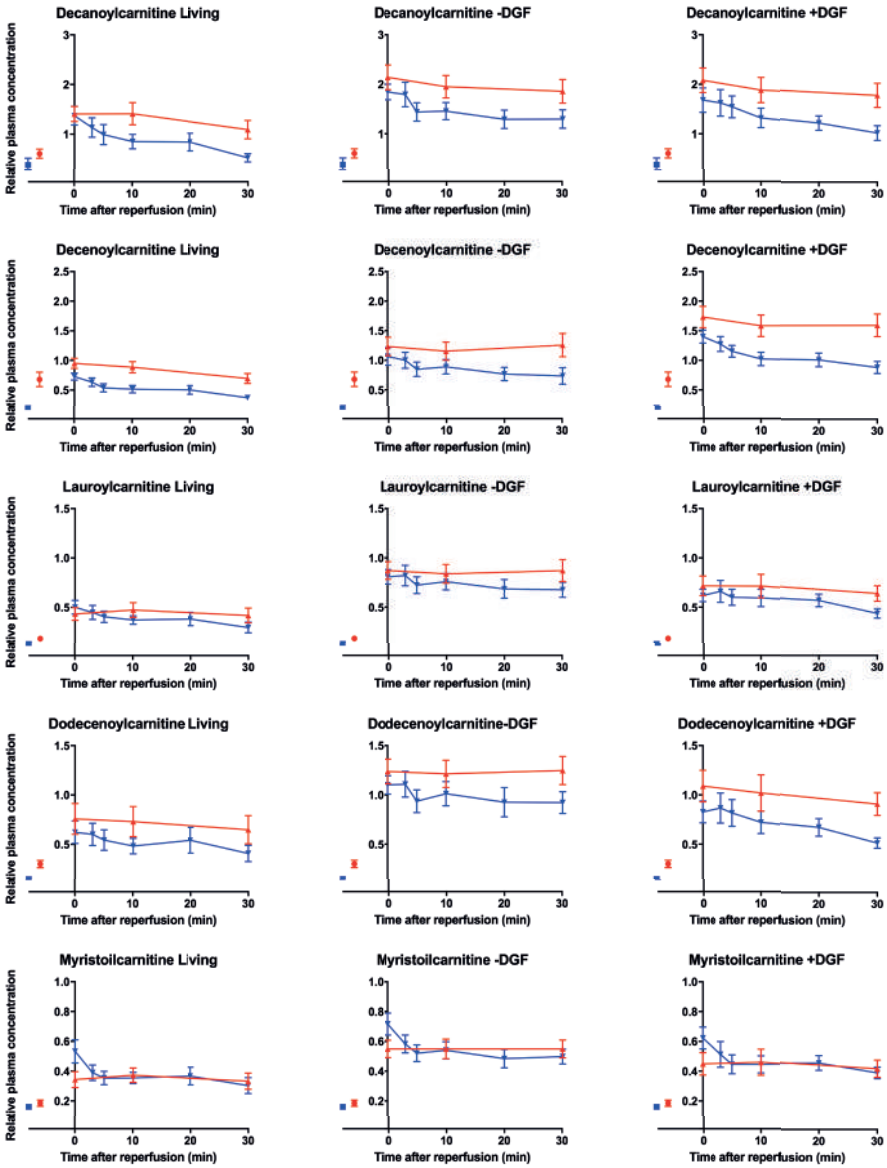
Supplemental Figure 1. Krebs Cycle (entry) Defect.
Accumulation of the Krebs cycle entry-products O-acetylcarnitine (P‹0,004), release of acetylcarnitine (T=30; P‹0,016), and pyruvate point to Krebs cycle defect(s) in +DGF grafts. Post-reperfusion release of the Krebs cycle intermediate α-ketoglutarate (T=30 min: P‹0.008), but not its downstream intermediates succinate, fumarate and malate, and absent recovery of tissue succinate and fumarate in the +DGF grafts indicate at least a defect at the level of oxoglutarate dehydrogenase in +DGF grafts.

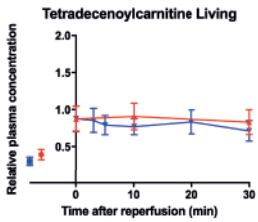


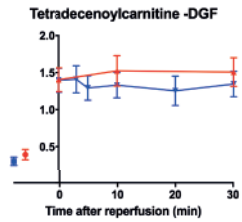
Supplemental Figure 2 summarizes the metabolic adaptation of +DGF grafts after reperfusion. The release of hypoxanthine, the end-product of ATP catabolism, as shown on the bottomline, indicates that ATP production is insufficient in +DGF grafts.
Medium chain fatty acids (MCFA) provide acetyl-CoA via beta-oxidation; as does pyruvate via normoxic glycolysis.
Due to defects in mitochondrial OXPHOS the Krebs cycle is spilled over and the Krebs cycle entry product acetyl-CoA is released.
The insufficient ATP production activates compensatory pathways producing Krebs cycle intermediates.For instance, ongoing glutaminolysis produces α-ketoglutarate.
Due to defects in the enzyme oxoglutarate dehydrogenase, α-ketoglutarate is not converted into succinate and it is released from +DGF grafts.
All in all, the only effective pathway of ATP production is the breakdown of pyruvate into lactate, asparagine and alanine via normoxic glycolysis.

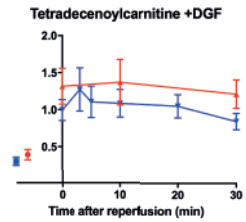

Deoxycarnitine Living
Deoxycarnitine -DGF
Deoxycarnitine +DGF
Carnitine Living
Carnitine -DGF
Carnitine +DGF
Acetylcarnitine Living
Acetylcarnitine -DGF
Acetylcarnitine +DGF
Malonylcarnitine Living
Malonylcarnitine -DGF
Malonylcarnitine+DGF
Pivaloylcarnitine Living
Pivaloylcarnitine -DGF
Pivaloylcarnitine +DGF
Relative plasma concentration
Time after reperfusion (min)
C2
C3
C4


Tiglylcarnitine Living
Tiglylcarnitine -DGF
Tiglylcarnitine +DGF
C5
Hexanoylcarnitine Living
Hexanoylcarnitine -DGF
Hexanoylcarnitine +DGF
C6
Octanoylcarnitine Living
Octanoylcarnitine -DGF
Octanoylcarnitine +DGF
C7
Octenoylcarnitine Living
Octenoylcarnitine -DGF
Octenoylcarnitine +DGF
C8
Noanylcarnitine Living
Noanylcarnitine -DGF
Noanylcarnitine +DGF
C9
Relative plasma concentration
Time after reperfusion (min)

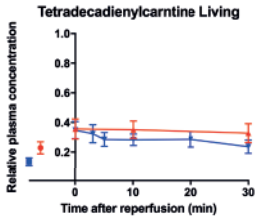

Decanoylcarnitine Living
Decanoylcarnitine -DGF
Decanoylcarnitine +DGF
C10
Decenoylcarnitine Living
Decenoylcarnitine -DGF
Decenoylcarnitine +DGF
C10
Lauroylcarnitine Living
Lauroylcarnitine -DGF
Lauroylcarnitine +DGF
C12
Dodecenoylcarnitine Living
Dodecenoylcarnitine-DGF
Dodecenoylcarnitine +DGF
C12
Myristoilcarnitine Living
Myristoilcarnitine -DGF
Myristoilcarnitine +DGF
C14
Relative plasma concentration
Time after reperfusion (min)

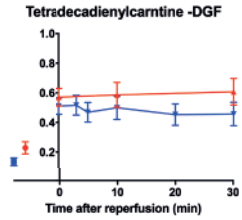


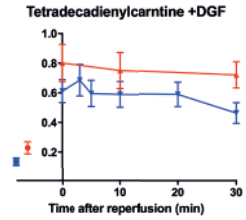




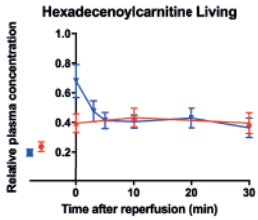
C14

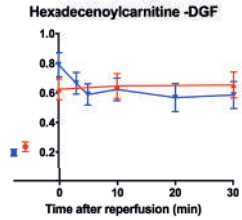


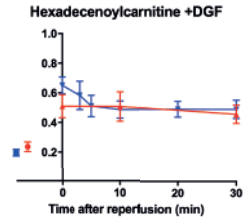




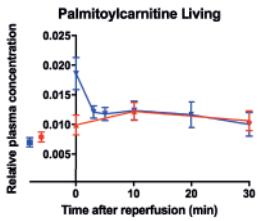
C14

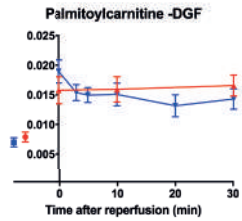


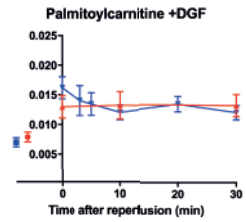




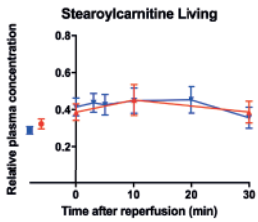
C16

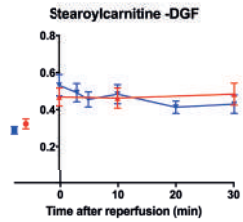


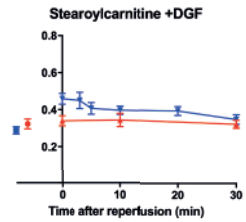




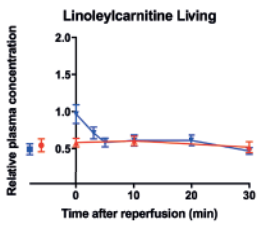
C16

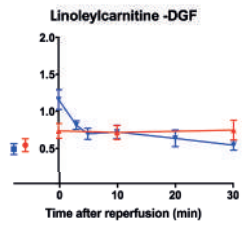


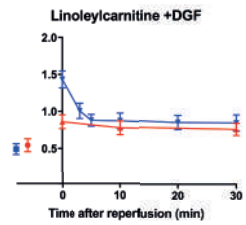




C18







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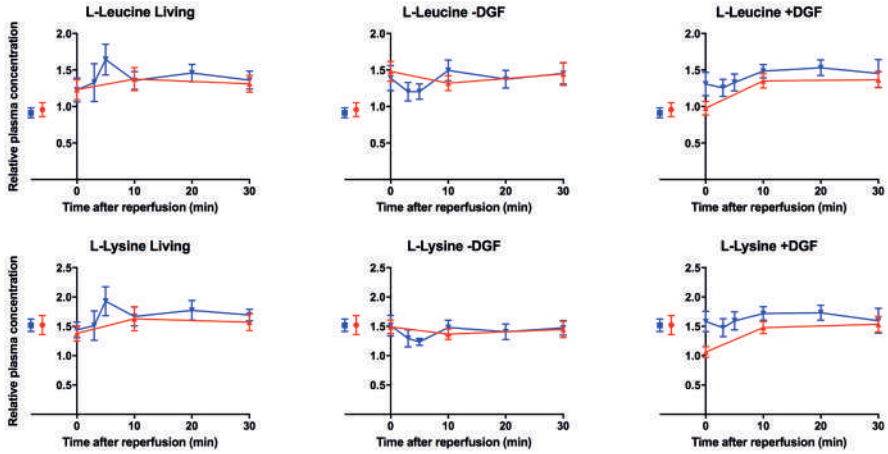
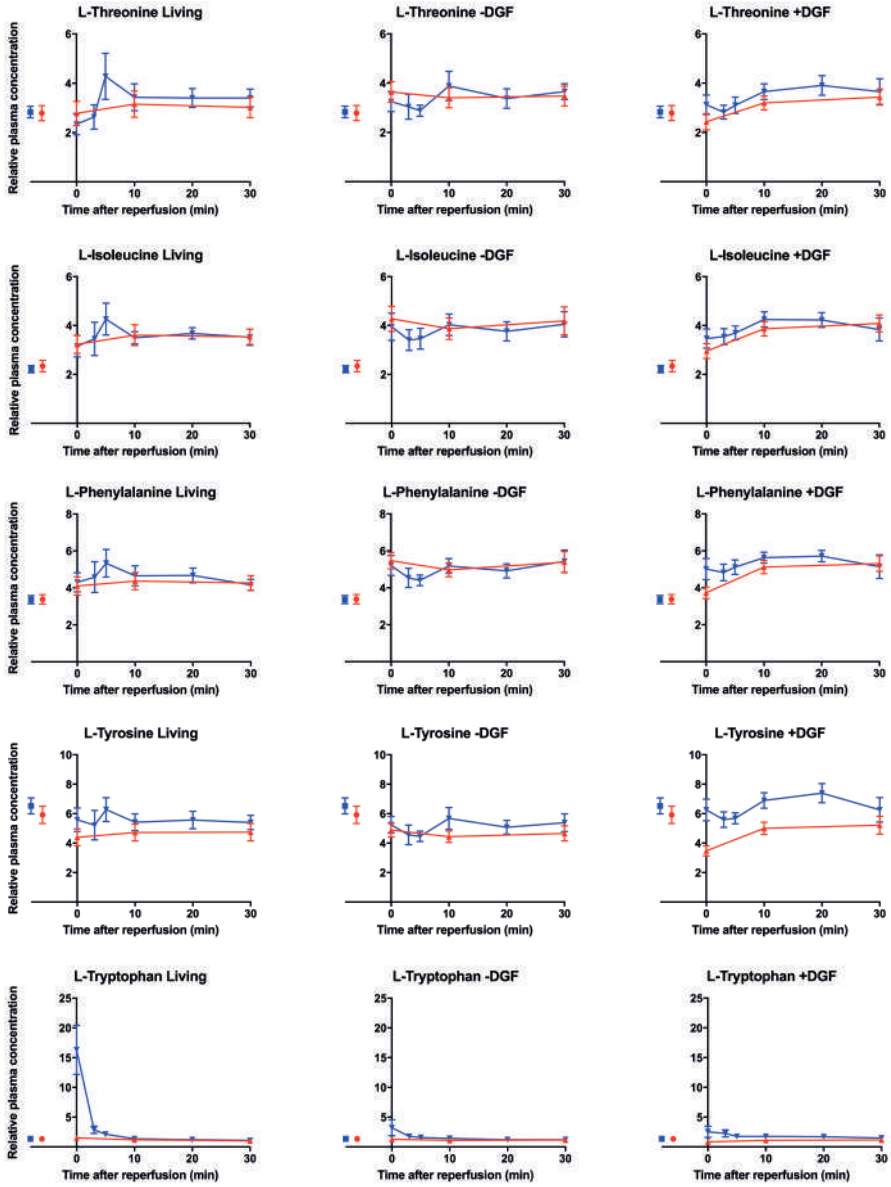
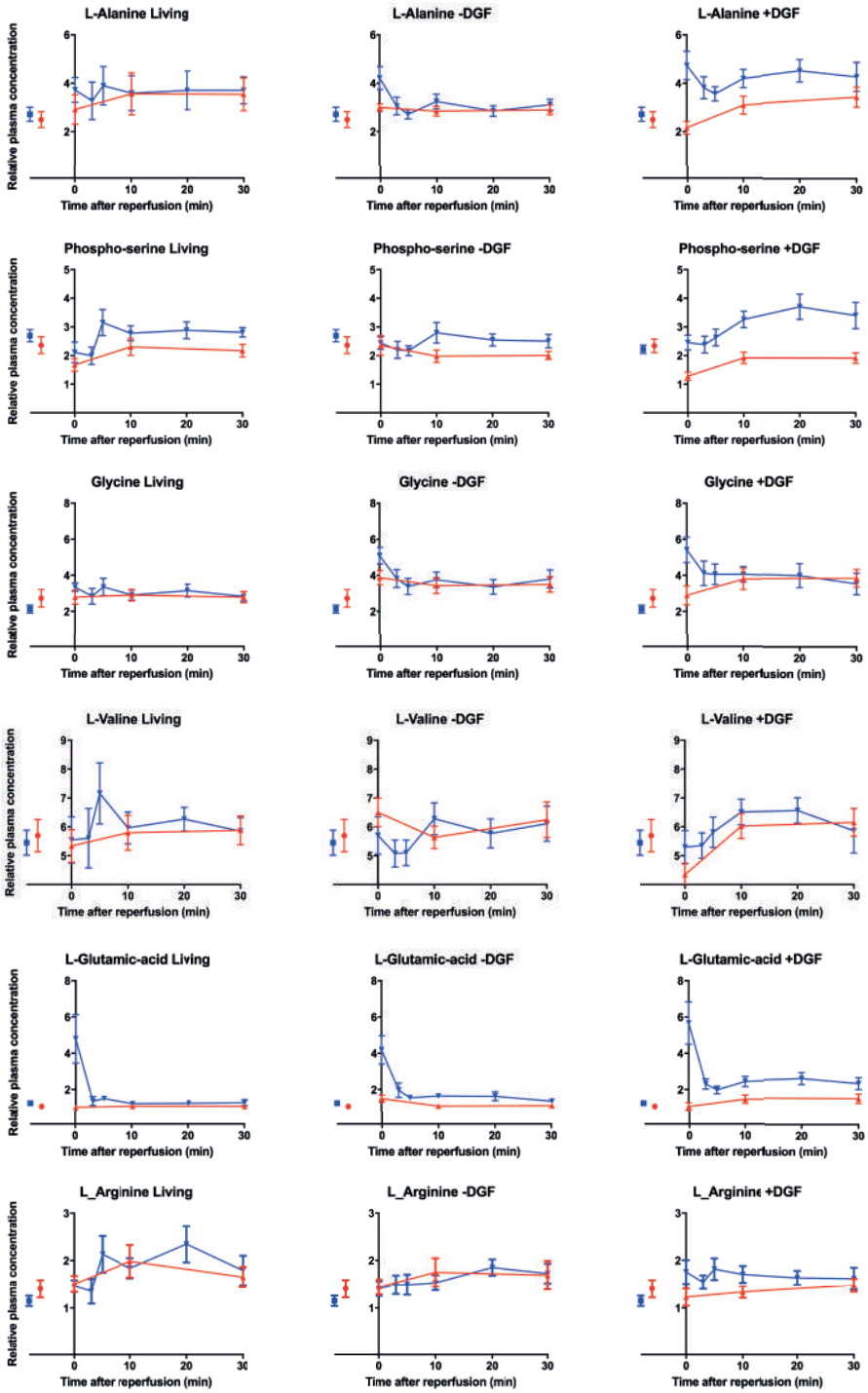

L-Leucine Living
L-Leucine -DGF
L-Leucine +DGF
L-Lysine Living
L-Lysine -DGF
L-Lysine +DGF
Relative plasma concentration
Time after reperfusion (min)

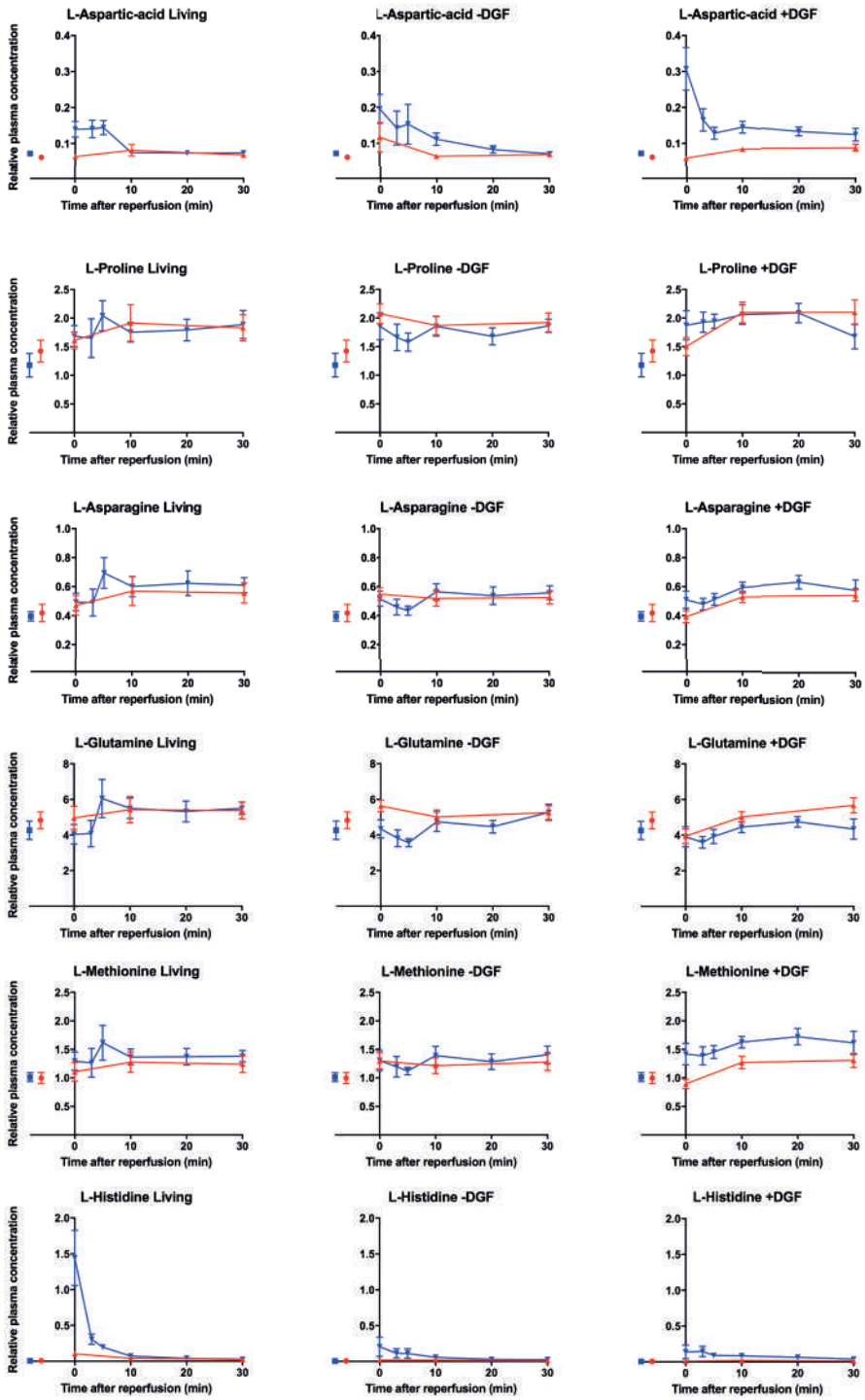
Ketogenic



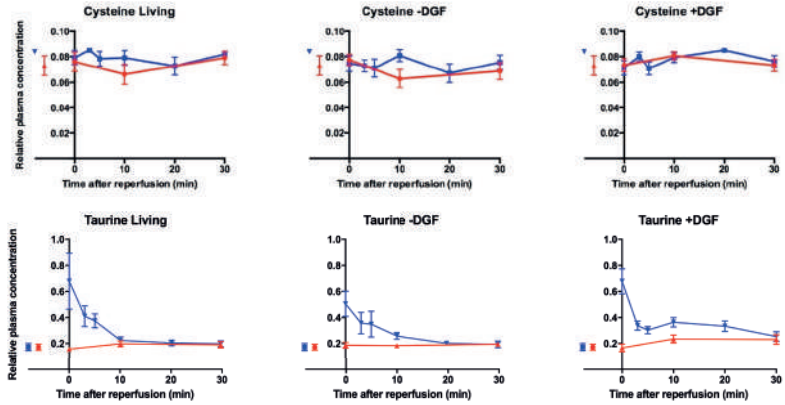
Ketogenic & glucogenic

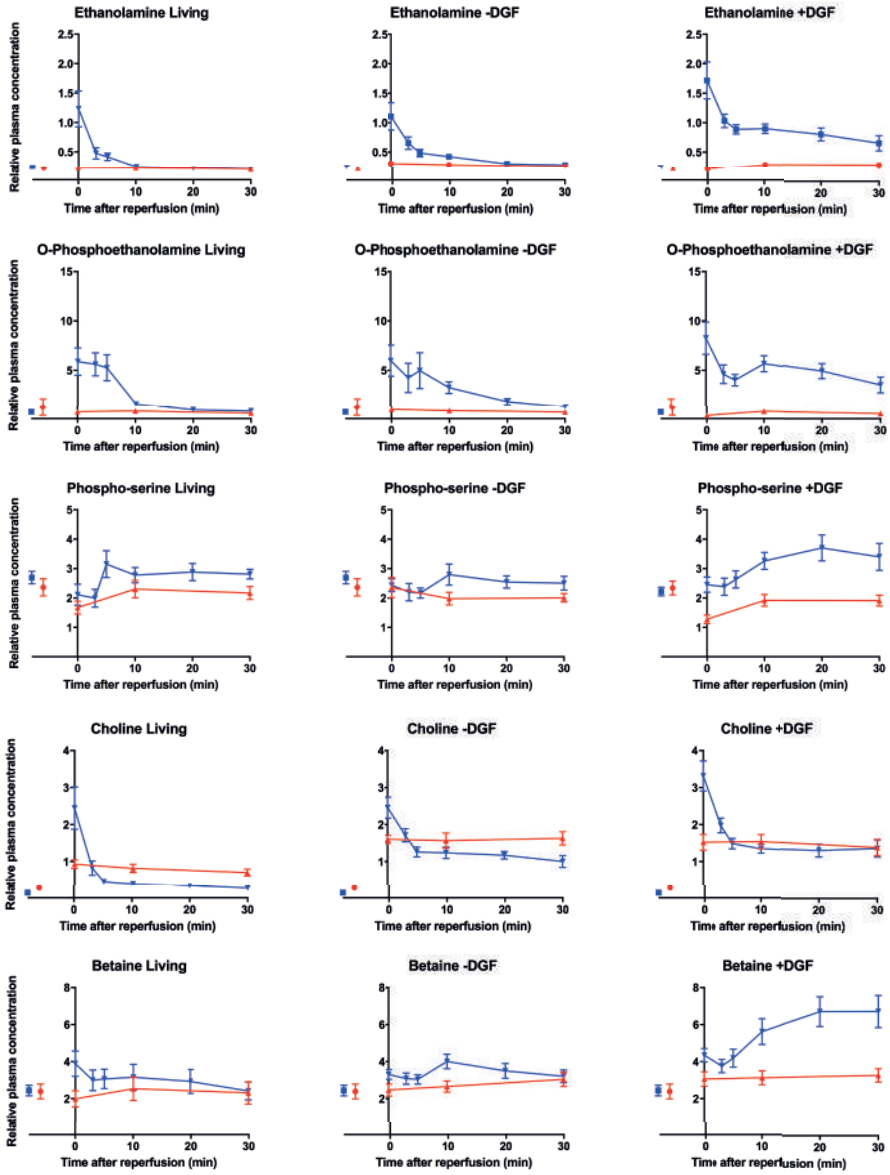

L-Alanine Living
L-Alanine -DGF
L-Alanine +DGF
Phospho-serine Living
Phospho-serine -DGF
Phospho-serine +DGF
Glycine Living
Glycine -DGF
Glycine +DGF
L-Valine Living
L-Valine -DGF
L-Valine +DGF
L-Glutamic-acid Living
L-Glutamic-acid -DGF
L-Glutamic-acid +DGF
L_Arginine Living
L_Arginine -DGF
L_Arginine +DGF
Relative plasma concentration
Time after reperfusion (min)

Glucogenic

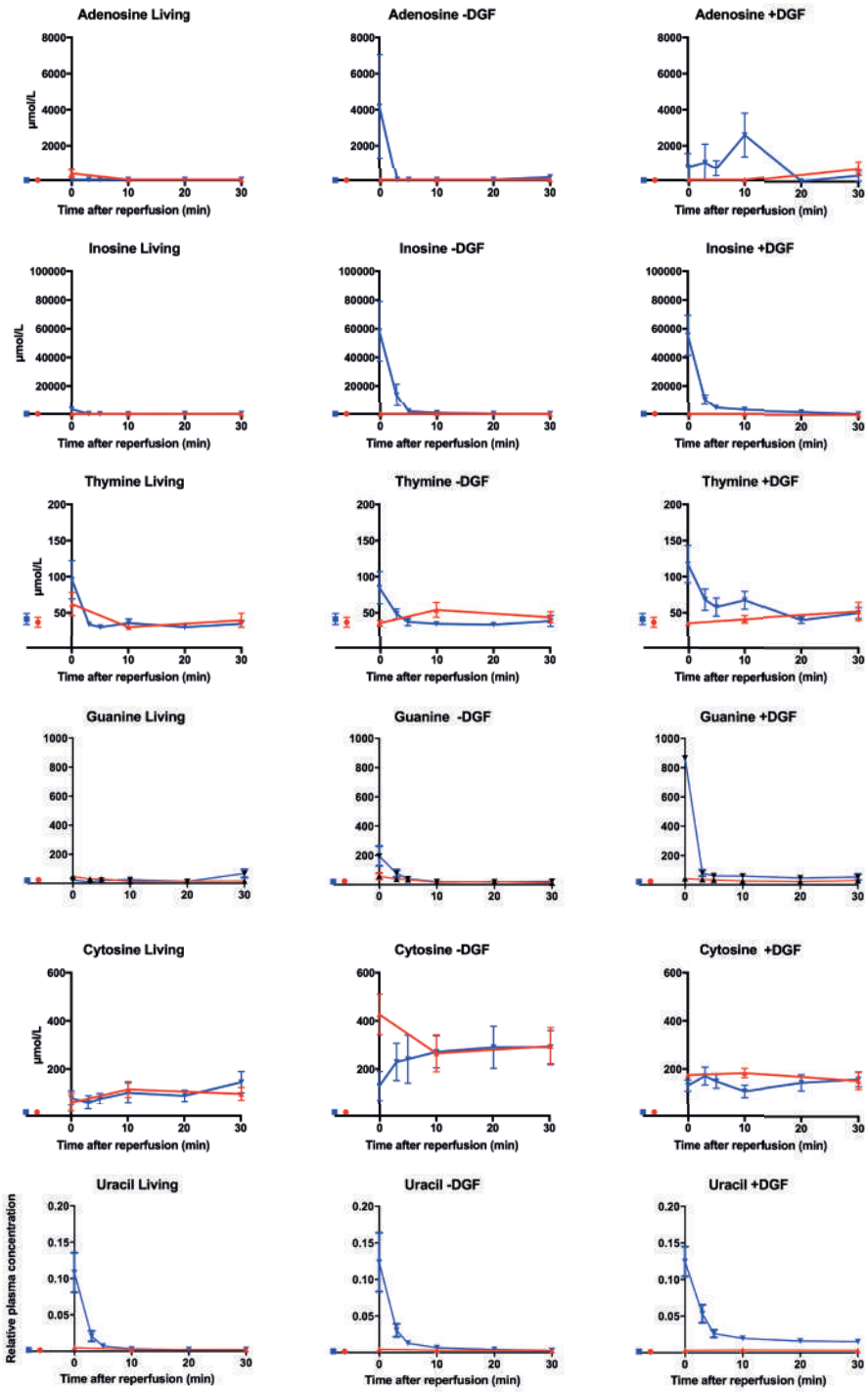


Glucogenic (cont.)

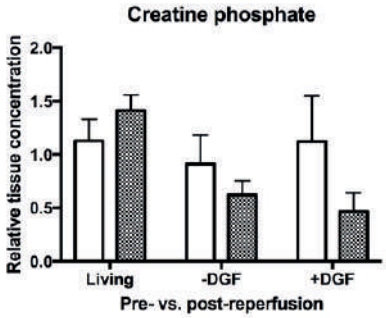

Cysteine Living
Cysteine -DGF
Cysteine +DGF
Taurine Living
Taurine -DGF
Taurine +DGF
Relative plasma concentration
Time after reperfusion (min)

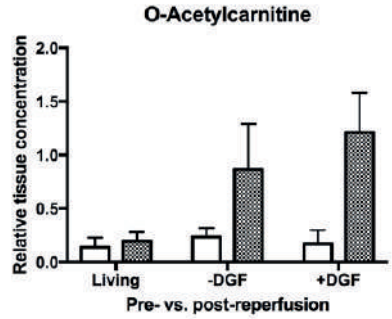


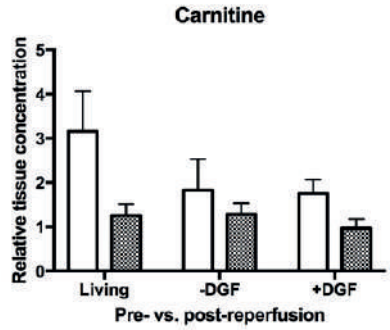
Phospholypolysis

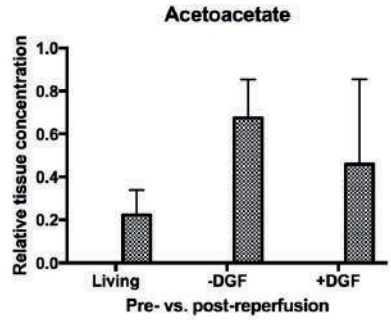


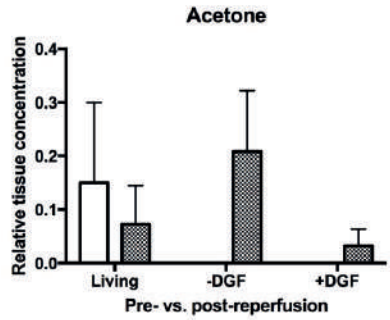
Nucleosides

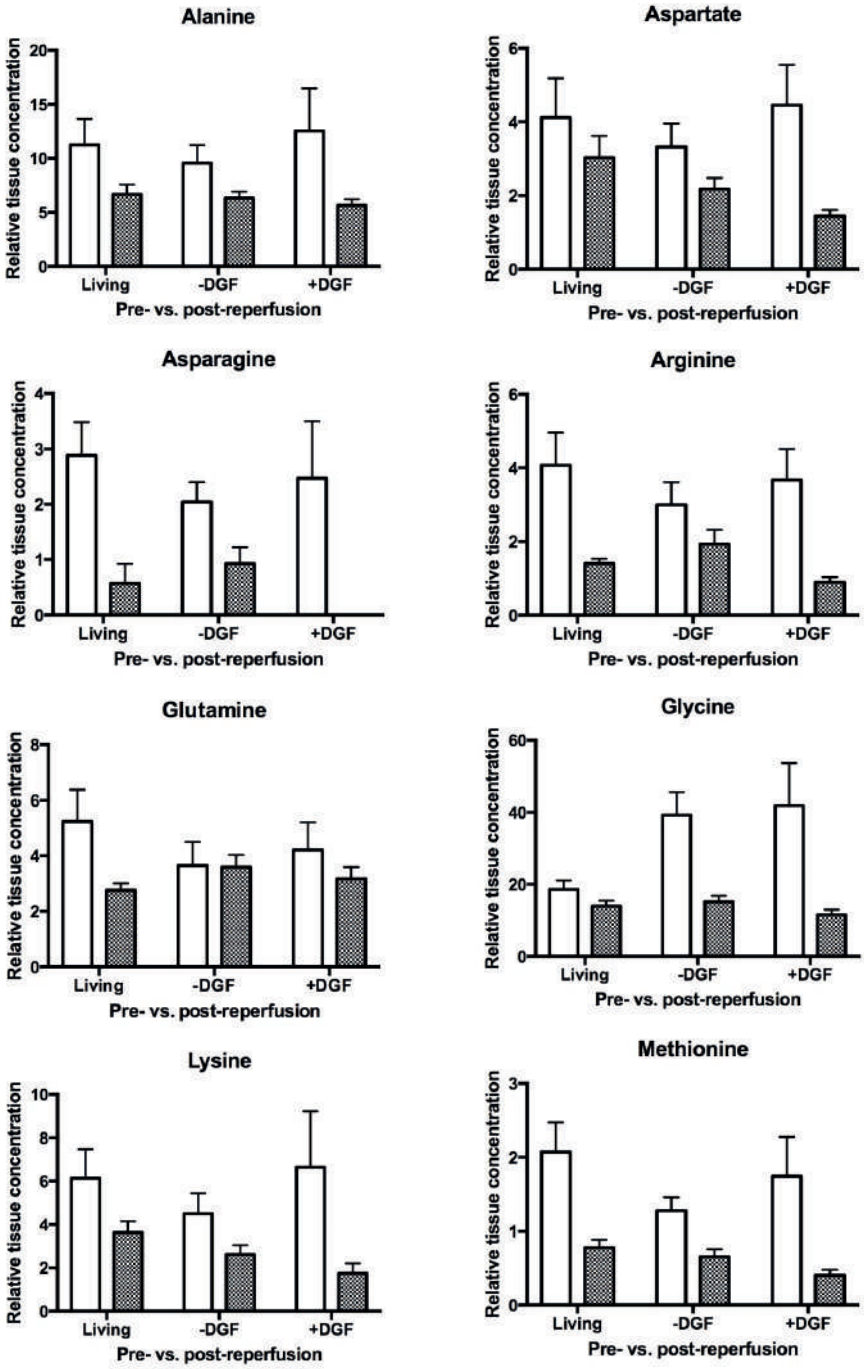

Creatine phosphate
Relative tissue concentration
2.0
1.5
1.0
0.5
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

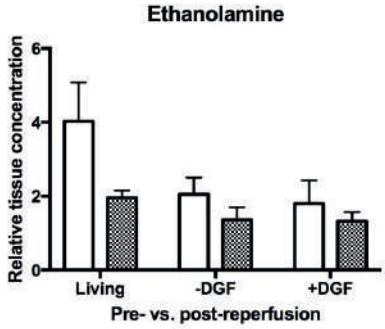

O-Acetylcarnitine
Relative tissue concentration
2.0
1.5
1.0
0.5
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

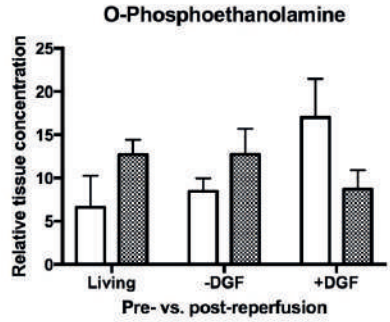

Carnitine
Relative tissue concentration
5
4
3
2
1
0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

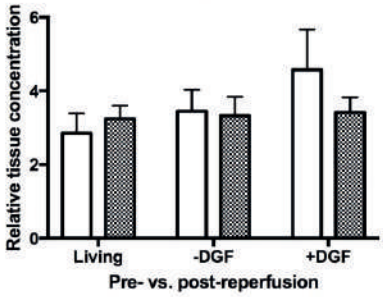

Acetoacetate
Relative tissue concentration
1.0
0.8
0.6
0.4
0.2
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

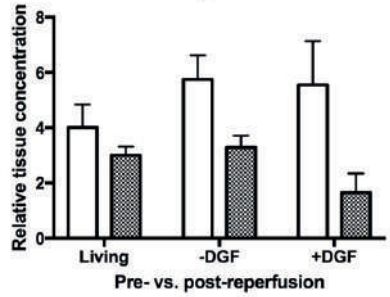

Acetone
Relative tissue concentration
0.4
0.3
0.2
0.1
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

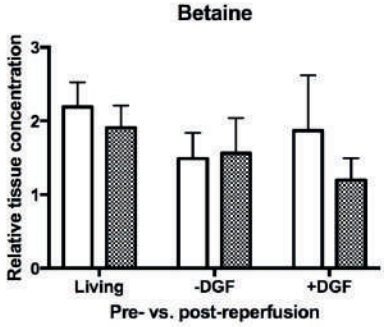

Alanine
Relative tissue concentration
0
5
10
15
20
Living
-DGF
+DGF
Pre- vs. post-reperfusion
Aspartate
Relative tissue concentration
0
2
4
6
Living
-DGF
+DGF
Pre- vs. post-reperfusion
Asparagine
Relative tissue concentration
0
1
2
3
4
Living
-DGF
+DGF
Pre- vs. post-reperfusion
Arginine
Relative tissue concentration
0
2
4
6
Living
-DGF
+DGF
Pre- vs. post-reperfusion
Glutamine
Relative tissue concentration
0
2
4
6
8
Living
-DGF
+DGF
Pre- vs. post-reperfusion
Glycine
Relative tissue concentration
0
20
40
60
Living
-DGF
+DGF
Pre- vs. post-reperfusion
Lysine
Relative tissue concentration
0
2
4
6
8
10
Living
-DGF
+DGF
Pre- vs. post-reperfusion
Methionine
Relative tissue concentration
0
1
2
3
Living
-DGF
+DGF
Pre- vs. post-reperfusion

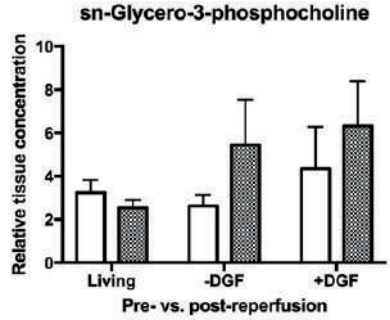

Ethanolamine
Relative tissue concentration
0
2
4
6
Living
-DGF
+DGF
Pre- vs. post-reperfusion

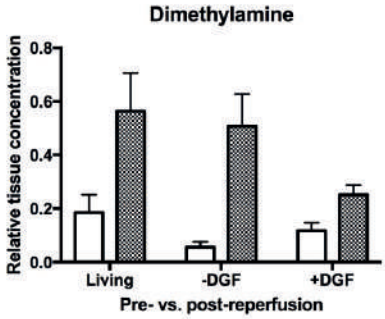

O-Phosphoethanolamine
Relative tissue concentration
0
5
10
15
20
25
Living
-DGF
+DGF
Pre- vs. post-reperfusion

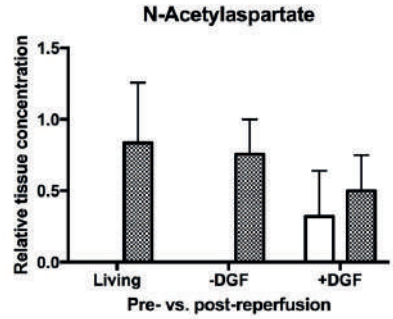

Choline
Relative tissue concentration
0
2
4
6
Living
-DGF
+DGF
Pre- vs. post-reperfusion

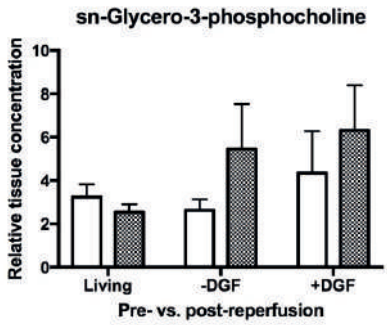

O-Phosphocholine
Relative tissue concentration
0
2
4
6
8
Living
-DGF
+DGF
Pre- vs. post-reperfusion

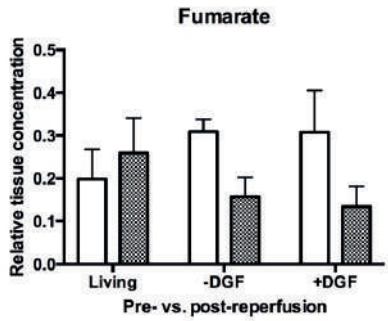

Betaine
Relative tissue concentration
0
1
2
3
Living
-DGF
+DGF
Pre- vs. post-reperfusion

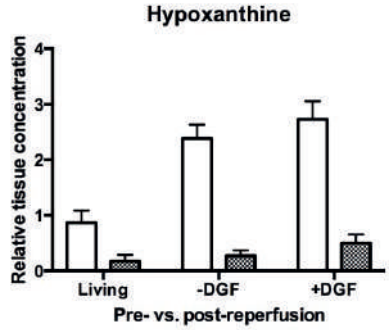

sn-Glycero-3-phosphocholine
Relative tissue concentration
0
2
4
6
8
10
Living
-DGF
+DGF
Pre- vs. post-reperfusion

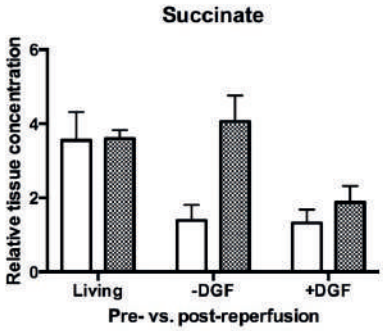

Dimethylamine
Relative tissue concentration
0.8
0.6
0.4
0.2
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

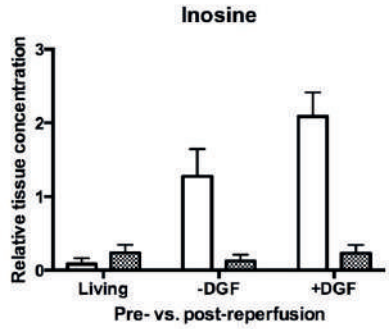

N-Acetylaspartate
Relative tissue concentration
1.5
1.0
0.5
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

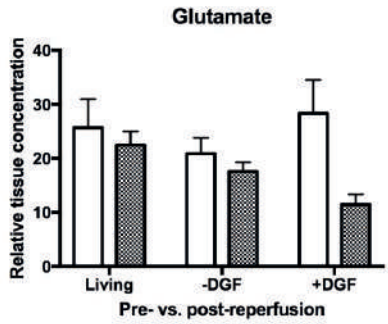

sn-Glycero-3-phosphocholine
Relative tissue concentration
10
8
6
4
2
0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

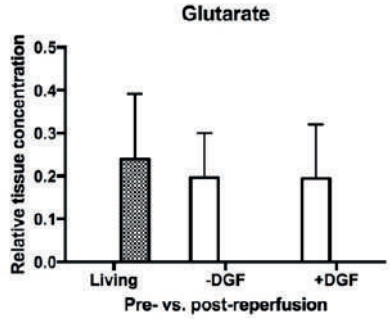

Fumarate
Relative tissue concentration
0.5
0.4
0.3
0.2
0.1
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

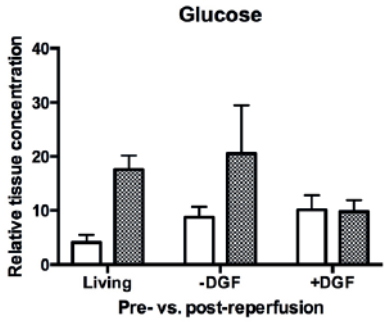

Hypoxanthine
Relative tissue concentration
4
3
2
1
0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

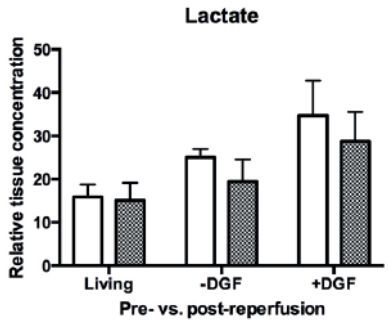

Succinate
Relative tissue concentration
6
4
2
0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

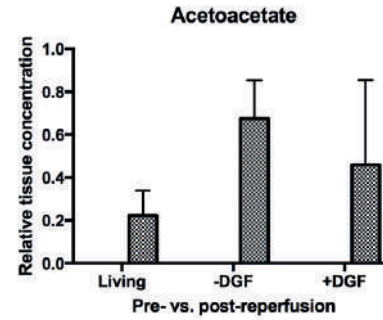

Inosine
Relative tissue concentration
3
2
1
0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

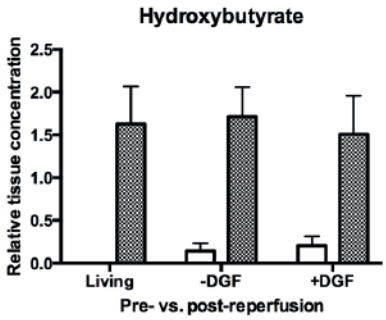

Glutamate
Relative tissue concentration
40
30
20
10
0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

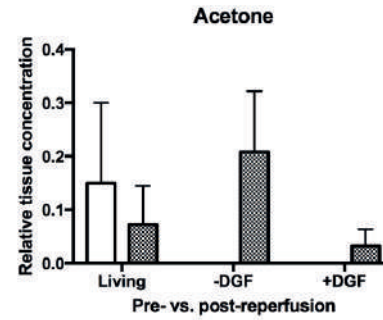

Glutarate
Relative tissue concentration
0.5
0.4
0.3
0.2
0.1
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion


Glucose
Relative tissue concentration
40
30
20
10
0
Living
-DGF
+DGF
Pre- vs. post-reperfusion


Lactate
Relative tissue concentration
50
40
30
20
10
0
Living
-DGF
+DGF
Pre- vs. post-reperfusion


Acetoacetate
Relative tissue concentration
1.0
0.8
0.6
0.4
0.2
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion


Hydroxybutyrate
Relative tissue concentration
2.5
2.0
1.5
1.0
0.5
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion


Acetone
Relative tissue concentration
0.4
0.3
0.2
0.1
0.0
Living
-DGF
+DGF
Pre- vs. post-reperfusion

SUPPLEMENTARY TABLES

Supplemental Table 1. Statistics metabolomics

Metabolite (pathway)	*Timepoint*	*Levels groups*	*P-value*
POWER OUTAGE			
Phosphocreatine	Post-reperfusion biopsy	+DGF: 0,39(0,19) mean(SEM) vs. control living: 1,41(0,35)	P=0,002
Hypoxanthine	Arteriovenous T=30	+DGF: arterial 3452,98(553,85) nmol/l mean(SEM) vs venous levels 17391,25(5042,63) nmol/l	P=0,007
Xanthine	Arteriovenous T=30	+DGF: arterial 2404,09(641,66) nmol/l mean(SEM) vs venous levels 4782,32(924,72) nmol/l	P=0,040
KREBS CYCLE DEFECT			
Acetylcarnitine	Post-reperfusion biopsy	controls 0,19(0,09) mean(SEM) vs. +DGF 1,45(0,34)	P=0,004
Acetylcarnitine	AV difference T=30	Living: 0.22(0.17) µmol/l mean(SEM) vs. +DGF: 0.45(0.16) µmol/l	P=0,016
Pyruvate	Area under the curve (venous vs. arterial curve) of To-T30 min	+DGF: 4,04(1,68) µmol/l mean(SEM)	P=0,022
Pyruvate	Area under the curve (venous vs. arterial curve) of To-T30 min	Living: 1,96(2,37)µmol/l mean(SEM)	P=0,41
Pyruvate	Area under the curve (venous vs. arterial curve) of To-T30 min	-DGF: 1,45(2,12) µmol/l mean(SEM)	P=0,49
a-Keto-glutarate	Arteriovenous T=30	+DGF: arterial 0,018(0,003) µmol/l mean(SEM) vs venous levels 0,037(0,007) µmol/l	P=0,008
Lactate	Arteriovenous T=30	+DGF: arterial 10,64(0,84) µmol/l mean(SEM) vs venous levels 16,34(1,40) µmol/l	P=0,001
Succinate	Pre-reperfusion biopsy vs. post-reperfusion biopsy	-DGF: pre 1,39(0,42) mean(SEM) vs. post 4,06(0,70)	P=0,007
Succinate	Post-reperfusion biopsies	Living vs. –DGF vs. +DGF. ANOVA	P=0,007
Succinic acid	Arteriovenous T=0	AV difference at T=0: 0,51(0.18) µmol/l mean(SEM) in living donor grafts vs. 0.21(0.07) µmol/l in +DGF grafts	P=0,095

Malic acid	Arteriovenous T=0	AV difference at T=0: 0.33(0.10) µmol/l mean(SEM) in living donor grafts vs. 0.18(0.06) µmol/l in +DGF grafts	P=0,182
Fumaric acid	Arteriovenous T=0	AV difference at T=0: 0,043(0.011) µmol/l mean(SEM) in living donor grafts vs. 0.023(0.006) µmol/l in +DGF grafts	P=0,114
ANAEROBIC GLYCOLYSIS			
Lactate	Arteriovenous T=30	+DGF: arterial 10,64(0,84) µmol/l mean(SEM) vs venous levels 16,34(1,40) µmol/l	P=0,001
Glucose	Post-reperfusion biopsies	Living: 17,54(2,64) mean(SEM) vs. +DGF 9,80(2,08)	P=0,044
Pyruvate	Area under the curve (venous vs. arterial curve) of To-T30 min	+DGF: 4,04(1,68) µmol/l mean(SEM)	P=0,022
GLUTAMINOLYSIS			
Glutamic acid	Arteriovenous T=30	+DGF: arterial 0,97(0,17) µmol/l mean(SEM) vs venous levels 1,82(0,25) µmol/l;	P=0,006
α-Ketoglutarate	Arteriovenous T=30	+DGF: arterial 0,018(0,003) µmol/l mean(SEM) vs venous levels 0,037(0,007) µmol/l	P=0,008
Alanine	Area under the curve (venous minus arterial curve) of To-T30 min	Living 7,09(8,85) mean(SEM) vs. –DGF 6,74(7,93) vs. +DGF 38,59(6,32); ANOVA	P=0,006
Aspartic acid	Arteriovenous T=30	+DGF: arterial 0,050(0,004) µmol/l mean(SEM) vs venous levels 0,106(0,021) µmol/l	P=0,005
Pyruvic acid	Area under the curve (venous minus arterial curve) of To-T30 min	+DGF: 4,04(1,68) µmol/l mean(SEM)	P=0,022
Glutamate	Pre-reperfusion biopsy vs. post-reperfusion biopsy	+DGF: pre 28,20(6,17) mean(SEM) vs. post 9,62(0,59)	P=0,017
Asparagine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	Living: pre 2,88(0,60) mean(SEM) vs. post 0,57(0,36)	P=0,008
Asparagine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	-DGF: pre 2,04(0,36) mean(SEM) vs. post 1,08(0,29)	P=0,067

Asparagine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	+DGF: pre 2,47(1,03) mean(SEM) vs. post 0,00(0,00)	P=0,026
Alanine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	Living: pre 11,26(2,39) mean(SEM) vs. post 6,65(0,95)	P=0,102
Alanine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	-DGF: pre 9,56(1,68) mean(SEM) vs. post 6,33(0,59)	P=0,094
Alanine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	+DGF: pre 12,54(3,94) mean(SEM) vs. post 5,65(1,44)	P=0,089
BETA-OXIDATION			
Valerylcarnitine	Arteriovenous T=30	+DGF: arterial 0,019(0,002) µmol/l mean(SEM) vs venous levels 0,079(0,023) µmol/l	P=0,006
Methylbutyroylcarnitine	AV difference T=30	Living: -0.13(0.06) µmol/l mean(SEM) vs. +DGF: 0.21(0.1) µmol/l	P=0,006
Linoleylcarnitine	AV difference T=30	Living: -0.04(0.06) µmol/l mean(SEM) vs. +DGF: 0.12(0.06) µmol/l	P=0,022
3-Hydroxybutyrate	Pre-reperfusion biopsy vs. post-reperfusion biopsy	+DGF: pre 0,20(0,11) mean(SEM) vs. post 1,51(0,45)	P=0,031
O-acetylcarnitine	Post-reperfusion biopsies	Living: 0,19(0,09) mean(SEM) vs. +DGF 1,45(0,34)	P=0,004
PHOSPHOLIPOLYSIS			
Phospho-serine	Arteriovenous T=30	+DGF: arterial 1,91(0,19) µmol/l mean(SEM) vs venous levels 3,40(0,46) µmol/l	P=0,003
Phospho-ethanolamine	Arteriovenous T=30	+DGF: arterial 0,53(0,06) µmol/l mean(SEM) vs venous levels 3,42(0,82) µmol/l	P=0,001
O-phosphoethanolamine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	+DGF: pre 17,0(4,48) mean(SEM) vs. post 6,59(0,64)	P=0,050
Ethanolamine	Arteriovenous T=30	+DGF: arterial 0,27(0,02) µmol/l mean(SEM) vs venous levels 0,65(0,13) µmol/l	P=0,004
O-phosphocholine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	+DGF: pre 5,55(1,58) mean(SEM) vs. post 0,98(0,19)	P=0,021

Betaine	Arteriovenous T=30	+DGF: arterial 3,25(0,36) µmol/l mean(SEM) vs venous levels 6,70(0,87) µmol/l	P=0,0004
Methionine	Area under the curve (venous minus arterial curve) of To-T30 min	Living: 5,92(3,49) mean(SEM)	P=0,100
Methionine	Area under the curve (venous minus arterial curve) of To-T30 min	–DGF: 1,15(3,12) mean(SEM)	P=0,370
Methionine	Area under the curve (venous minus arterial curve) of To-T30 min	+DGF: 13,50(2,49) mean(SEM	P=0,000006
Isovaleryl-carnitine	Arteriovenous T=30	+DGF: arterial 0,26(0,02) µmol/l mean(SEM) vs venous levels 0,55(0,09) µmol/l	P=0,001
Choline	Arteriovenous T=30	Living: arterial 0,71(0,09) µmol/l mean(SEM) vs venous levels 0,27(0,08) µmol/l	P=0,001
Choline	Arteriovenous T=30	+DGF: 1,63(0,18) µmol/l mean(SEM) vs venous levels 1,01(0,48) µmol/l;	P=0,018
Methionine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	Living pre 2,07(0,40) mean(SEM) vs. post 0,78(0,1)	P=0,011
Methionine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	-DGF pre 1,28(0,18) mean(SEM) vs. post 0,65(0,10)	P=0,012
Methionine	Pre-reperfusion biopsy vs. post-reperfusion biopsy	+DGF pre 1,74(0,53) mean(SEM) vs. post 0,40(0,08)	
	P=0,023		
Leucine	Area under the curve (venous minus arterial curve) of To-T30 min	Living = 3,94(3,19) mean(SEM)	P=0,227
Leucine	Area under the curve (venous minus arterial curve) of To-T30 min	–DGF -0,72(2,85) mean(SEM)	P=0,802
Leucine	Area under the curve (venous minus arterial curve) of To-T30 min	+DGF 5,74(2,28) mean(SEM)	P=0,017
Lysine	Area under the curve (venous minus arterial curve) of To-T30 min	Living = 5,56(4,03) mean(SEM)	P=0,178

Lysine	Area under the curve (venous minus arterial curve) of To-T30 min	–DGF -0,85(3,60) mean(SEM)	P=0,815
Lysine	Area under the curve (venous minus arterial curve) of To-T30 min	+DGF 8,42(2,87) mean(SEM)	P=0,006
Tyrosine	Area under the curve (venous minus arterial curve) of To-T30 min	Living = 34,09(14,12) mean(SEM)	P=0,022
Tyrosine	Area under the curve (venous minus arterial curve) of To-T30 min	–DGF 18,08 (12,61) mean(SEM)	P=0,162
Tyrosine	Area under the curve (venous minus arterial curve) of To-T30 min	+DGF 66,02(10,07) mean(SEM)	P=2,399 3E-7
CELL DAMAGE			
Uracil	Arteriovenous T=30	+DGF: arterial 0,003(0,0004) µmol/l mean(SEM) vs venous levels 0,015(0,0032) µmol/l	P=0,002

Supplementary Methods

METABOLITE ANALYSIS METHODS

General information

Samples were stored at -80°C until used further analysis. All samples were randomized and run in 5 batches which included a calibration line, QC samples and blanks. QC samples were analyzed every 10 samples (or every 15 samples in the oxidative stress platform). The acquired data were evaluated using MassHunter software (Agilent). An in-house written tool is applied using the QC samples to compensate for shifts in the sensitivity of the mass spectrometer throughout the batches.[21] Both internal standard correction and QC correction were applied to the data set before reporting results. All metabolites comply with the acceptance criteria of RSDQC <30%.

1. BIOGENIC AMINE PROFILING

The amine platform covers amino acids and biogenic amines employing an Accq-tag derivatization strategy adapted from the protocol supplied by Waters. 5 µL of each plasma sample was spiked with an internal standard solution, thiol amines are released from proteins and converted to reduced using TCEP. Then proteins are precipitated by the addition of methanol. The supernatant was transferred to a new eppendorf tube and taken to dryness in a speedvac. The residue was reconstituted in borate buffer (pH 8.5) with AQC reagent. After reaction, the vials were transferred to an autosampler tray and cooled to 10°C until the injection. 1.0 µL of the reaction mixture was injected into the UPLC-MS/MS system.

An Agilent 1290 Infinity UHPLC system with autosampler (Agilent, The Netherlands) was coupled online with a 6490 Triple quadrupole mass spectrometer (Agilent) operated using MassHunter data acquisition software (B.04.01; Agilent). The samples were analyzed by UPLC-MS/MS using an Accq-Tag Ultra column (Waters). The Triple quadrupole MS was used in the positive-ion electrospray mode and all analytes were monitored in dynamic Multiple Reaction Monitoring (dMRM) using nominal mass resolution.[22]

2. ORGANIC ACID PROFILING

This profiling platform, performed with GC-MS technology, covers 28 organic acids. Sample preparation was done by doing first protein precipitation of 50 uL of plasma with a crash solvent (MeOH/H2O) with ISTD added. After centrifugation and transferring the supernatant, the solvent was evaporated to complete dryness on the speedvac. Then, two-step derivatisation procedures with oximation using methoxyamine hydrochloride (MeOX, 15mg/mL in pyridine) as first reaction

and silylation using N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA) as second reaction were carried out. After this final step the samples were transferred to the auto sampler vials and 1 µL was injected on GC-MS20.

The metabolites were measured by gas chromatography on an Agilent Technologies 7890A equipped with an Agilent Technologies mass selective detector (MSD 5975C) and MultiPurpose Sampler (MPS, MXY016-02A, GERSTEL). Chromatographic separations were performed on a HP-5MS UI (5% Phenyl Methyl Silox), 30 m × 0.25 m ID column with a film thickness of 25 m, using helium as the carrier gas at a flow rate of 1,7 mL/min. A single-quadrupole mass spectrometer with electron impact ionization (EI, 70 eV) was used. The mass spectrometer was operated in SCAN mode mass range 50-500.[23]

3. ACYLCARNITINE PROFILING

The acylcarnitine platform covers acylcarnitines as well as Trimethylamine-N-oxide, Choline, Betaine, Deoxycarnitine and Carnitine. 10 µL of each sample was spiked with an internal standard solution and proteins were precipitated by the addition of MeOH. The supernatant was transferred to an autosampler vial. The vials were transferred to an autosampler tray and cooled to 4 until the injection. 1.0 µL of the reaction mixture was injected into the triple quadrupole mass spectrometer.

Chromatographic separation was achieved by UPLC (Agilent 1100, San Jose, CA, USA) on an Accq- Tag Ultra column (Waters) with a flow of 0.7 mL/min over a 11 min gradient. The UPLC was coupled to electrospray ionization on a triple quadrupole mass spectrometer (Agilent 6460, San Jose, CA, USA). Analytes were detected in the positive ion mode and monitored in Multiple Reaction Monitoring (MRM) using nominal mass resolution.

4.PURINES AND PYRIMIDINES

Purines and pyrimidines were identified by using an in-house developed UPLC-MS/MS 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 ammoniumformate buffer (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 formate (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 MRM transitions.

5. MAGIC ANGLE SPINNING HIGH-RESOLUTION NMR SPECTROSCOPY (MAS HR-NMR)

Metabolic profiling of the tissue biopsies was performed by MAS HR-NMR spectrometry on a 14.1 T Bruker Avance III spectrometer. All measurements were performed at the MR Core Facility, Norwegian University of Science and Technology (NTNU). MR core facility is funded by the Faculty of Medicine at NTNU and Central Norway Regional Health Authority. 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 MAS rotor (4 mm). The insert was filled with 3 µL cold (4°C) phosphate-buffered saline in D_2O containing 4.5 mM TSP-D_4 and 25 mM sodium formate ($CHNaO_2$) as internal standards. During NMR measurements the temperature was set to 4°C and the rotor spinning rate to 5 kHz. Per sample, a set of 3 NMR experiments were acquired including the 1D 1H NOESY, the 1D 1H CPMG and the 2D 1H J-resolved, with adjusted parameters as described previously.[24] An additional set of 2D NMR spectra for a subset of samples as well as the Bbio Refcode and ChenomX NMR suite 8.1 databases were used for identification of metabolites, while the latter was used for the quantification. In total, 41 compounds were quantified and their relative concentrations were normalized to tissue weight.

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Part II

Clinical

Translation

VI

Realistic restrictions in cold ischemia time result in similar outcomes for kidneys donated after brain and cardiac death.

Article under peer review

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ABSTRACT

Background: There are reservations with respect to the use of kidneys from cardiac death donors (DCD) in kidney transplantation. However, several outcome-based studies suggest that long-term graft survival of DCD grafts may be similar to survival of brain death donor (DBD) grafts. As such conclusions would have far reaching consequences, we considered an in-depth analysis of long-term outcomes after DBD and DCD kidney transplantations relevant.
Materials & Methods: Differences in 10-years graft survival were analysed in the Netherlands Organ Transplantation Registry (NOTR) database, for the 2000-2017 interval (DBD n=4084 (58.6%); DCD n=2891 (41.4%)), by means of calculating hazard ratios en plotting Kaplan Meier survival curves. Differences in graft survival for patients with and without DGF/PNF were calculated for the two graft types. Results were additionally stratified by intervals of cold ischemia times. For functional outcome (eGFR) we evaluated all transplantations performed in the Leiden University Medical Center (LUMC) (DBD n=370; DCD n=258) between 2007-2015.
Results: The NOTR data indicate a 50% higher incidence of primary non-function, and an almost tripled incidence of delayed graft function (DGF) in DCD grafts. After excluding the grafts with primary non-function (7,9% of all DCD and 4.5% of all DBD grafts) 10-year graft survival was similar for both donortypes (HR (DBD reference): 1.00 (95%CI 0.88-1.15); P=0.95). Further evaluation shows that duration of cold ischemia longer than 24 h disproportionally influences incident PNF and mitigates graft survival of DCD grafts (HR (DBD reference): 1.54 (1.21-1.96); P<0.001). Contrasting effects were seen for the impact of ischemia time on incident DGF. Thereby, it was shown that incident DGF negatively impacts graft survival in DBD grafts, while it does not so in DCD grafts (P=0.001). This differential impact of DGF on DBD grafts may reflect biological differences between the graft-types. Indeed, recovery of graft function is exponential in DCD grafts, resulting in long-term graft functions similar to those of DBD grafts, despite inferior initial function of DCD grafts.
Conclusion: Although DBD grafts have superior short-term outcomes, mid- and long-term graft survival is similar for DBD and DCD grafts. The increased susceptibility to longer ischemia times, in particular over 24 hours calls for stricter guidelines with respect to the logistics of DCD procedures.

INTRODUCTION

Kidney transplantation remains the only curative option for patients with end-stage renal disease. In an era of growing waiting lists for renal transplants, pressing donor shortages led to an increased use of so called "extended criteria grafts" and grafts donated following cardiac death (DCD). Transplantation procedures with DCD grafts associate with increased incidences of primary non-function (PNF) and delayed graft function (DGF). The latter phenomenon is considered to negatively influence graft function and long-term graft survival.[1-4]
Remarkably, a reticent attitude towards use of DCD grafts is not supported by data from some small cohort studies,[5-7] and follow-up data from the UK transplant registry.[8] In fact, the UK registry indicated equal 5-year graft survival rates for DCD grafts and brain-death donor (DBD) grafts. However, the registry data did signal potential concern with respect to differences in risk profiles; in particular a possible increased susceptibility of DCD grafts for (prolonged) cold-ischemia[9], thereby raising a cause of reservation on the generalizability of the observations. Nevertheless, reported similar survival outcome for DBD and DCD grafts is remarkable and lead some expert-opinions to call for a more liberal use of DCD grafts in the light of pressing donor shortages.[10] Still, use of DCD grafts is controversial[11], with many societies/countries refraining from these grafts.[12-16] In this light we considered an independent and adequately powered evaluation of outcomes of procedures with grafts donated after brain death (DCD) and DCD grafts relevant. From this perspective, the position of The Netherlands is unique as it has a long and relative liberal tradition with regard to use of DCD grafts. In fact, DCD procedures now account for 50% of the deceased donor procedures performed nationwide. The aim of this study was to compare long-term outcome (graft survival and functioning) of DBD and DCD grafts. This paper reports the outcomes for all 2891 DCD procedures performed in The Netherlands within the 2000 and 2017 interval and analyses functional outcome of 258 DCD procedures performed in our center.

MATERIALS & METHODS

The Netherlands Organ Transplant Registry (NOTR) registry is a nationwide registry of kidney transplant recipients from all eight kidney transplant centres in the Netherlands. The NOTR registry is managed by the Dutch Transplant Foundation and includes recipient and donor characteristics and a variety of outcome parameters (Table 1). In the first year after transplantation, registry follow-up is at 3 months, thereafter on a yearly basis. We retrieved data on recipient and donor characteristics, and transplantation outcomes for all transplants performed between January 1st 2000 and December 31st 2016.

DONOR CRITERIA AND PROCEEDINGS OF DONATION AFTER BRAIN AND CIRCULATORY DEATH

The NOTR collaborates with Eurotransplant, the organisation that facilitates organ allocation in Belgium, Germany, Luxembourg, Austria, Hungary, Slovenia, Croatia and the Netherlands. Donation takes places following the Eurotransplant quality standards and practices. For DBD grafts this means that a donor is diagnosed brain dead according to current national regulations and laws on transplantation. During the donation procedure the DBD donor is heparinized and after cannulation the organs are flushed with cold perfusion fluid (HTK/UW). After retrieval, organs are transported and stored by means of static cold storage preservation.
In DCD donation, the heart irreversibly stopped beating and the donor is diagnosed dead according to the respective national laws and regulations. All DCD kidneys were donated by a controlled DCD donor (category 3: awaiting cardiac arrest after withdrawal of life-supporting treatments in the ICU). After a no-touch period, organs were preserved via arterial cold perfusion generally with UW or HTK. Organs were retrieved and preserved by means of static cold storage.
All organs were matched and allocated following the Eurotransplant guidelines.

FUNCTIONAL RECOVERY RATE

Putative effects of donortype on functional (clearance) graft recovery were explored in a retrospective analysis of all deceased donor kidney transplantations (n= 628) performed between January 2007 and June 2015 in the Leiden University Medical Center (LUMC) (Sup.Table 1). All kidneys received from cardiac death donors were from the Maastricht category 3 (controlled DCD: awaiting cardiac arrest after withdrawal of life-supporting treatments in the ICU).[17] Data were retrieved from the (electronic) patient records.

STUDY END POINTS

Post transplantation outcome was classified in the following categories: primary function, delayed graft function (DGF) and primary non-function. DGF was defined by the need of dialysis because of initial non-function in the first week(s) after kidney transplantation that was followed by functional recovery. Primary non-function was defined as persistent non function of the kidney graft upon transplantation. The 'Kidney Donor Risk Index' (KDRI) was calculated using the coefficients provided[18] and creatinine clearance estimated by the Cockcroft-Gault Equation.

Weekly eGFR measurements in the first 12 weeks and at 3, 6, 9, 12, 18, 24, 36 and 48 months after transplantation were collected of all LUMC patients that showed functional graft recovery (i.e. PF and DGF). For grafts that developed DGF 'week 1', the first week of eGFR measurements, was defined as the first week following the last dialysis. Graft function recovery curves were generated up to 12 months after transplantation for the LUMC patients.

STATISTICAL ANALYSES

With respect to the NOTR database, differences between DBD and DCD donor grafts (i.e. recipient and donor characteristics and outcome parameters) were described (Table 1). A multivariate logistic regression analyses was performed to assess factors associated with primary non-function, stratified by graft type (*variables with a p-value ‹0.05 in the uni-variate analysis were entered in the multi-variate analysis*). Results are represented as OR with 95%CI. Differences in graft survival were calculated for DCD versus DBD as reference category and expressed as HR with corresponding 95%CI and plotted in Kaplan Meier survival figures. Graft survival was truncated at 10 years of follow-up. Results were additionally stratified by intervals of cold ischemia times. Differences in graft survival for patients with and without DGF were calculated for both DBD and DCD grafts. Analyses were performed using STATA/SE version 12.0 (StataCorp, Texas, USA).

RESULTS

The NOTR registry, for the 2000-2017 interval, include data for 4084 (58.6%) DBD and 2891 (41.4%) DCD kidney transplantations. With the exception of a higher proportion of male donors in the DCD group, and a different cause of death distribution, donor characteristics were similar for the 2 donor groups (Table 1). Equivalence of the two groups is also reflected in comparable mean KDRI's for the DBD and DCD grafts (1.29 and 1.21 respectively when excluding the DCD component from the equation).

Table 1. NOTR characteristics

	DBD	DCD
	N=4084 (58.6%)	N=2891 (41.4%)
Male	1950 (47.7%)	1689 (58.4%)
Age (mean (sd))	48.2 (25.0)	48.6 (15.5)
BMI (mean (sd))	25.0 (4.3)	25.2 (4.6)
Last Creatinine [IQR]	70 [54-89]	67 [53-84]
MDRD (mean (sd))	92 (37)	101 (38)
Cause of death Trauma Stroke Cardiac arrest Other	 906 (22.2%) 2404 (58.9%) 165 (4.0%) 609 (14.9%)	 901 (31.2%) 1090 (37.7%) 536 (18.5%) 364 (12.6%)
Hypertension No Yes Unknown	 2584 (63.3%) 1005 (24.6%) 495 (12.1%)	 2172 (75.1%) 543 (18.8%) 176 (6.1%)
Smoking No Yes Unknown	 1889 (46.3%) 1859 (45.5%) 336 (8.2%)	 1372 (47.5%) 1328 (45.9%) 191 (6.6%)
Cold Ischemia Time ‹12 hrs 12-18 hrs 18-24 hrs ›24 hrs Unknown	 787 (19.3%) 1398 (34.23%) 1001 (24.5%) 536 (13.1%) 362 (8.9%)	 497 (17.2%) 1110 (38.4%) 771 (26.7%) 304 (10.5%) 209 (7.2%)
Warm Ischemia Time (min) [IQR]	33 [26-41]	32 [26-40]
KDRI [IQR]	1.29 [1.04-1.62]	1.38 [1.12-1.71] 1.21 [0.98-1.50] (minus DCD component of KDRI equation)

Early Graft Loss			
Primary non function		183 (4.5%)	227 (7.9%)
Loss between day 8-90		122 (3.0%)	86 (3.0%)
Overall Graft Loss		1577 (38.6%)	1058 (36.6%)
Procedural		86 (5.5%)	86 (8.1%)
Permanent non function		55 (3.5%)	83 (7.8%)
Hyper acute rejection		21 (1.3%)	11 (1.0%)
Death recipient		785 (49.8%)	467 (44.1%)
Rejection		426 (27%)	255 (24.1%)
DGF			
No		2772 (73.4%)	918 (35.7%)
Yes		653 (17.3%)	1230 (47.8%)
Unknown		349 (9.2%)	427 (16.6%)
Sex Recipient (male)		2361 (57.8%)	1805 (62.4%)
Age Recipient		49.9 (16.1)	53.7 (13.4)
BMI recipient		25.0 (4.5)	28.8 (4.4)
Mismatch HLA-Dr	1	2011 (49.4%)	1713 (59.7%)
	2	466 (11.4%)	231 (8.1%)
HLA-A	1	2022 (49.6%)	1589 (55.1%)
	2	589 (14.5%)	428 (14.9%)
HLA-B	1	2005 (49.2%)	1725 (59.9%)
	2	1079 (26.5%)	685 (23.8%)
Panel reactive antibodies ›5%		14.8%	9%
3 months creatinine [IQR]		132 [105-167]	146 [116-188]
Year 1 creatinine [IQR]		127 [102-161]	136 [109-176]
-DGF		123 [100-154]	125 [102-159]
+DGF		145 [98-159]	145 [99-163]
Year 5 creatinine [IQR]		126 [99-166]	135 [106-180]
-DGF		124 [115-187]	126 [117-189]
+DGF		139 [108-188]	142 [112-191]

The registry data indicate an almost 50% higher incidence of primary non-function, and an almost tripled incidence of DGF in DCD grafts (Table 1). DCD procedures moderately impacted 10-year graft survival (HR for graft loss (DBD reference): 1.19 (95%CI: 1.06-1.32); P=0.002, Figure 1A). Recipient survival was similar for the two graft types (HR for recipient death after correcting for the higher recipient age in the DCD group: 0.95 (95%CI: 0.86-1.05), P=ns).
Further evaluation showed that the observed survival disadvantage of DCD grafts essentially relates to the higher incidence of primary non-function in these grafts, since exclusion of grafts with primary non-function resulted in similar 10-year graft survival (HR for graft loss (DBD is reference): 1.00 (95%CI 0.88-1.15); P=0.95), Sup.Fig.1).

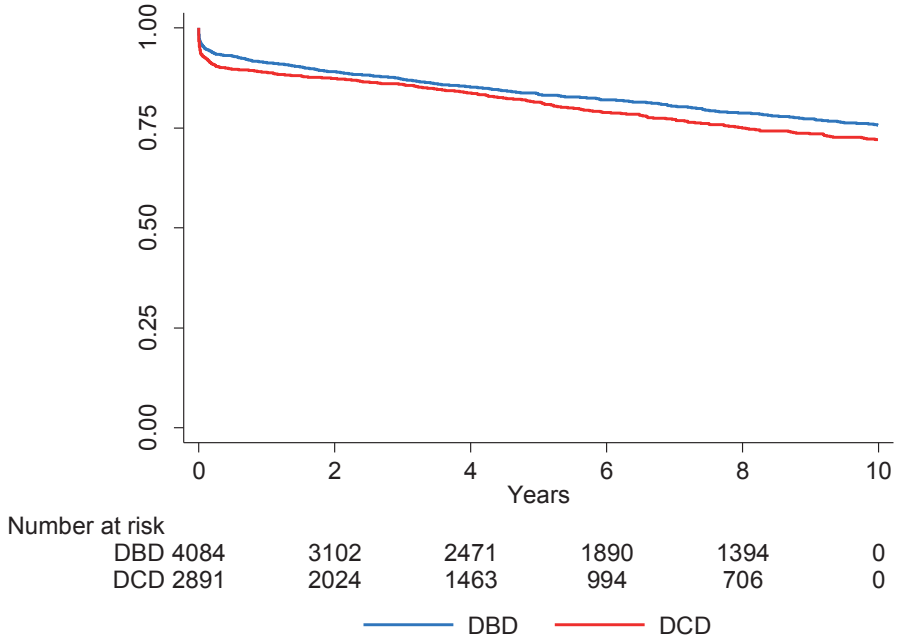


Figure 1A. 10-year graft survival of DBD(red) and DCD(blue) grafts transplanted in the Netherlands

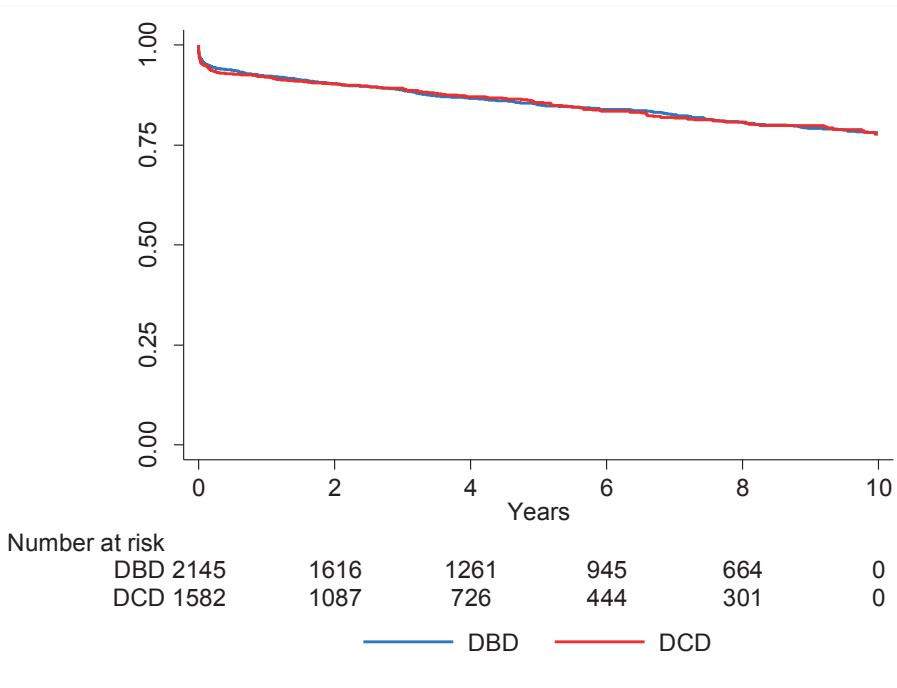


Figure 1B. 10-year graft survival of DBD(red) and DCD(blue) grafts with cold ischemia times restricted to 18 h or less

Incident primary non-function strongly associates with cold-ischemia time (P<1.10-11, Table 2). Along similar lines the UK registry data signalled concern with regard to an impact of longer ischemia times on DCD graft survival.[8,9] We therefore specifically addressed the impact of this potentially modifiable risk factor cold-ischemia time on outcome. Table 3 shows that longer ischemia times disproportionally influence incident PNF as well as long-term graft survival in DCD grafts as compared to DBD grafts. To test the impact of a policy with restricted ischemia times, we evaluated graft survival data for all procedures with a maximum ischemia times of 18 and 24 hours. This re-evaluation showed that a stricter policy with capped maximum cold ischemia times results in 10-year outcome equivalence for DBD and DCD grafts (HR for graft loss (DBD reference) for maximum cold-ischemia times of 18 and 24 hours respectively: 1.01 (95%CI 0.85-1.19); P=0.91) and 1.12 (95% CI: 0.99-1.27); P=0.08 (Figure 1B).

Table 2. Factors associated with primary non-function (multi-variate analysis)

	DBD	DCD
Date of transplant	1.002 (0.999-1.085)	1.007 (1.003-1.010) **
Donor age	1.023 (1.011-1.034)**	1.029 (1.018-1.041) **
Donor height	0.969 (0.957-0.98) **	0.985 (0.971-0.999) *
Cold Ischemia Time (hrs)	1.031 (1.007-1.055) *	1.043 (1.016-1.072) *
Warm Ischemia Time (min)	1.014 (1.004-1.024) *	1.022 (1.013-1.031) **

*P<0.04; **P<0.00015

Table 3. Discordant impact of longer cold ischemia times on DCD graft survival (DBD is reference)

DBD as reference	N	HR (95%CI)	p-value	Adjusted for donor age HR (95%CI)	p-value
< 12 h	1194	1.08 (0.76-1.54)	0.66	1.03 (0.72-1.46)	0.88
12 – 15 h	1690	0.93 (0.73-1.19)	0.56	0.91 (0.72-1.16)	0.47
16 – 17 h	843	1.06 (0.77-1.47)	0.70	1.08 (0.78-1.49)	0.63
18 – 19 h	657	1.12 (0.80-1.57)	0.51	1.16 (0.82-1.62)	0.40
20 – 21 h	581	1.32 (0.96-1.82)	0.09	1.35 (0.98-1.86)	0.07
22 – 23 h	487	1.33 (0.93-1.88)	0.11	1.38 (0.97-1.95)	0.07
≥24 h	969	1.48 (1.17-1.88)	0.001	1.54 (1.21-1.96)	<0.001

HR graft loss Cold ischemic time under 18h (DBD is reference) (n=3727): 1.01 (0.85-1.19); p=0.91

HR graft loss Cold ischemic time under 24h (DBD is reference) (n=5452): 1.12 (0.99-1.27); p=0.08

The almost tripled incidence of DGF in DCD grafts but comparable 10-year graft survival for DBD and DCD grafts implies a differential impact of DGF on outcome, with DBD grafts being more susceptible than DCD grafts. A discordant effect is supported by the differential impact of incident DGF on graft *survival* (HR for graft loss following DGF in the DCD group: 0.69 (95% CI: 0.59-0.86; P=0.001, Figure 2). The impact of DGF on graft *function* on the other hand, was similar for DBD and DCD grafts (Table 1: one and five year post-transplantation creatinine levels). In fact, reduced graft function in the DCD group was fully explained by the higher incidence of DGF in this group.

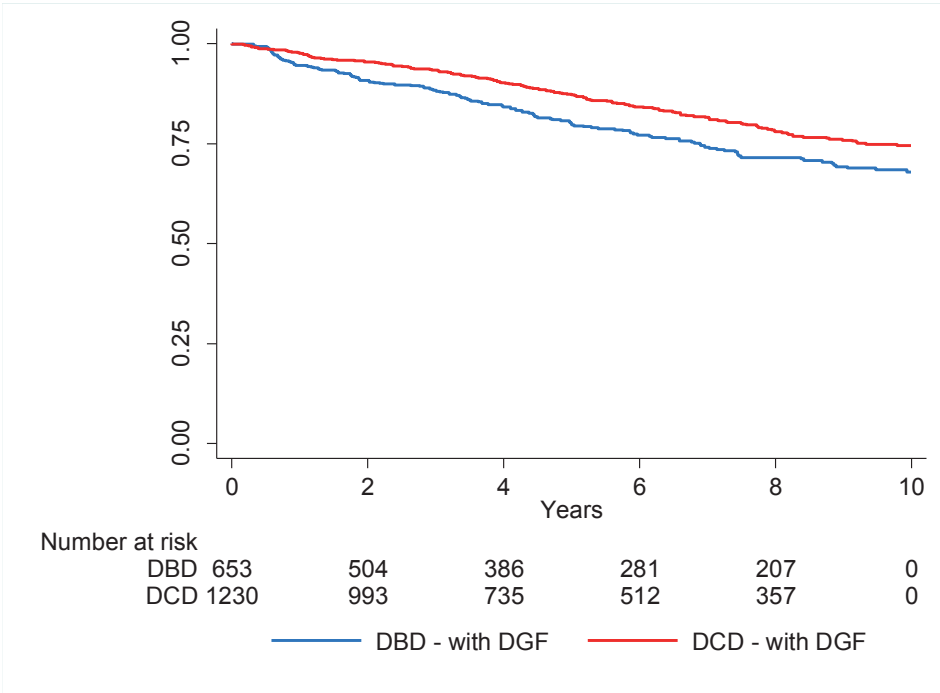


Figure 2 Differential impact of DGF on graft survival of DBD and DCD grafts (excluding grafts with primary non function) n=653 (DBD) and 1230 (DCD)). HR for graft loss (DBD reference): 0.69 (95%CI 0.55-0.86); P=0.001.

The differential impact of DGF on DBD and DCD grafts survival may imply biological differences in resilience of the two grafts types. To further explore such a difference we evaluated post-transplantation graft recovery dynamics in a subset of 628 grafts transplanted between 2010-2015 in our center (n=628, details of these grafts are provided in Supplemental Table 1) for which detailed recovery data is available. Data for DBD grafts without DGF show full functional reinstatement within the first week of transplantation (Figure 3), while DBD grafts with DGF

show a protracted recovery with an ultimate clearance that is approximate 12% less than that of grafts without DGF.
DCD grafts with and without DGF show parallel recovery dynamics that are clearly distinct from those of DBD grafts. Initial functions are profoundly inferior to those of DBD grafts, but the grafts show a catch up, with exponential recoveries in the first weeks of transplantation (Figure 3, and Table 4), ultimately resulting in long-term graft functions similar to those of DBD grafts (Table 1: similar 1 and 5-year post-transplantation creatinine levels in the full NOTR cohort)).

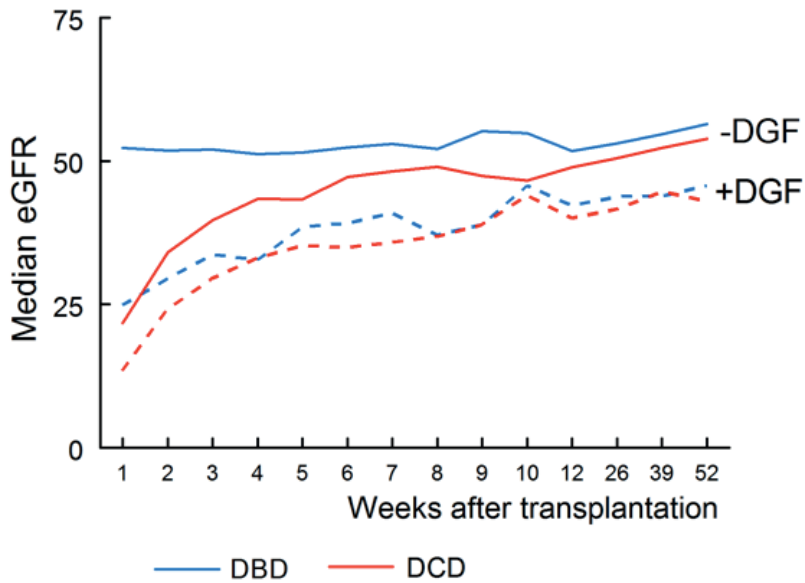


Figure 3. Graft recovery following transplantation. Solid lines: grafts without DGF, dashed lines grafts with DGF. Week 1 in the +DGF groups is defined as the first week without a need for dialysis.

Table 4. Post transplantation functional recovery of DBD and DCD grafts

	Relative eGFR recovery (week 26) vs baseline (week 1)	Mean absolute eGFR (ml/min/1,73m2) recovery at week 26 from baseline (week 1)	Number of dialysis performed because of DGF (mean(SD))
DBD -DGF	+ 1.55%	0.84 ml/min/1.73m2	
DBD +DGF	+ 55.9%	16.83 ml/min/1.73m2	4.78(3.1)
DCD -DGF	+ 95.4%	26.05 ml/min/1.73m2	
DCD+DGF	+ 155.4%	27.31 ml/min/1.73m2	5.02(2.9)

DISCUSSION

Results of this nation-wide evaluation of data from a society with an almost equal allocation of DCD and DBD renal grafts confirm and extend earlier indications of similar mid- and long-term outcomes for DCD and DBD grafts, after excluding patients with primary non-function.[6-9,19]

Pressing donor shortages in an era of progressive demand for donor kidneys have led to a progressive use of grafts from so-called marginal donors, and from DCD donors. Yet, high incidences of DGF and primary non-function in DCD grafts fuels concern on a more liberal use of this latter type of grafts.[13] In fact, in most societies including the USA, use of DCD procedures has stabilized at approximately 10% of the deceased donor procedures,[20] although some opinion leaders are now calling to amend the restrictive policy with regard to DCD grafts.
A less reticent attitude towards DCD grafts is supported by preliminary reports from small observational studies, and particularly by data from the UK registry.[5-9] Although the number of DCD procedures included in these studies remains limited, all reports indicate similar outcomes for DCD and DBD grafts. However, concern was raised with regard to a higher susceptibility of DCD grafts towards (prolonged) ischemia.[1,13] Although promising, these reports, and the reported more pronounced impact of prolonged ischemia on DCD outcomes require confirmation.

With an almost equal share of DBD and DCD grafts, the situation in the Netherlands is uniquely positioned to evaluate outcomes after DBD and DCD procedures. The context in the Netherlands not only allows for the evaluation of a large number of DCD procedures, but it also reduces selection biases that may result from a reticent attitude with regard to the use of DCD grafts (i.e. preference for DCD grafts with superior donor characteristics (i.e. young donor age; short ischemia time).[5-7]

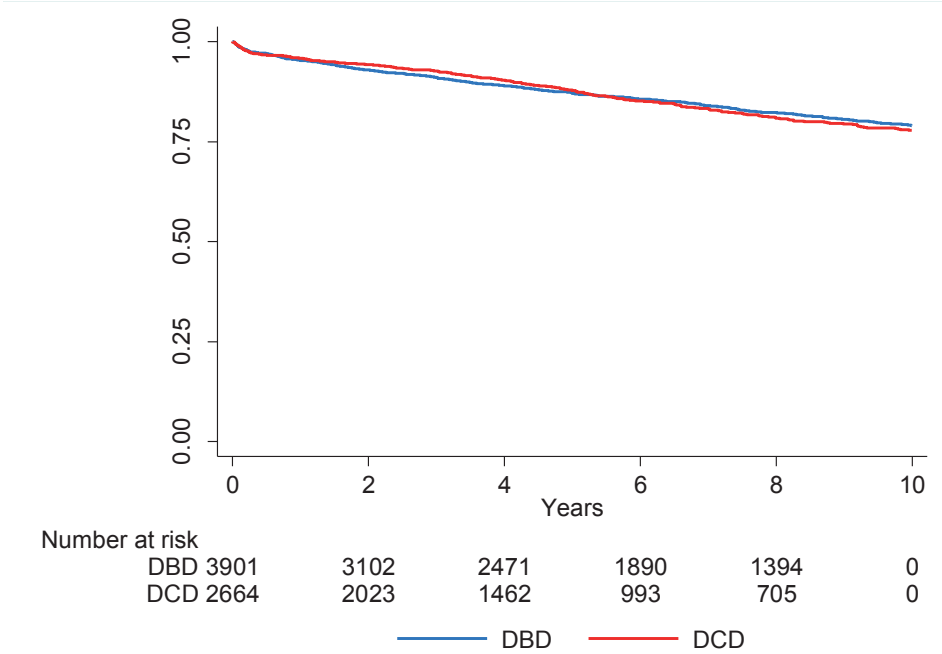
The liberal attitude towards DCD grafts for the Dutch context is reflected in the high proportion of DCD grafts (41% for the 17 year observation period in this study, 50% in 2016) and equal donor characteristics for DBD and DCD grafts. Although the cohort data confirm the higher incidence of primary non-function and DGF in-, and an (modestly) impaired 10-year survival of DCD grafts, results show that this disadvantage essentially relates to the use of DCD grafts with cold ischemia times over 24 hours. In fact we observed similar mid- and long-term outcomes for DBD and DCD grafts with cold-ischemia times less than 24 hours. Consequently, the data confirm observations from the UK registry and showed

discordant impacts of longer ischemia times on DCD graft survival for cold ischemia times in excess of 24 hours.

Discordant impact of longer ischemia times, and different impact of DGF on graft survival may imply differences in graft biology. Such a concept is supported by functional recovery analysis (eGFR) performed on all deceased donor procedures performed in our hospital. While this data extends the superior short-term outcomes for DBD grafts without DGF, it signals a remarkable recovery potential of DCD graft with an exponential catch up of kidney function in the first weeks following transplantation. This ultimately results in a functional and survival equivalence of DBD and DCD grafts, as outcomes for kidney transplantation should be based on mid- and long-term parameters
The notable recovery potential of DCD grafts, and superior survival of these grafts following DGF point to differences in graft biology. One could speculate that these differences relate to the negative impact of donor brain death on DBD grafts[21,22] and/or activation of tissue protective responses such as ischemic preconditioning,[23] and/or activation of the innate repair receptor[24] during the initial warm ischemia episode following cardiac death in DCD grafts.

In conclusion, this study that includes almost 2900 DCD procedures shows that mid- and long-term outcome after DBD and DCD kidney graft procedures is similar. A focus on short-term outcomes neglects the superior recovery potential of DCD grafts.
The increased susceptibility to longer ischemia times, in particular over 24 hours calls for stricter guidelines with respect to the logistics of DCD procedures.

SUPPLEMENTARY FIGURES



Supplemental Figure 1. Similar 10-year graft survival after excluding all grafts with primary non-function (n=410), HR (DBD reference): 1.00 (95%CI 0.88-1.15); P=0.95

SUPPLEMENTARY TABLES

Supplemental Table 1. Patient characteristics (donor, recipient and procedure) of the LUMC cohort.

Factors		*DBD*	*DCD*	*P-value*
DONOR				
Cause of death donor	CVA / SAB Trauma CA-OHCA-AMI Tumour Suicide – respiratory Miscellaneous	232 (63.6) 85 (23.3) 15 (4.1) 2 (0.6) 14 (3.8) 17 (4.7)	96 (38.1) 59 (23.4) 51 (20.2) 2 (0.8) 20 (7.9) 24 (9.5)	<0.001
Cardiac arrest donor	No Yes	259 (76.4) 80 (23.6)	147 (59.0) 102 (41.0)	<0.001
Age donor	≤40 years 41-59 years 60 or older	109 (29.9) 161 (44.1) 95 (26.0)	61 (24.2) 107 (42.5) 84 (33.3)	0.1
Sex donor	Male Female	187 (51.2) 178 (48.8)	149 (59.1) 103 (40.9)	0.05
BMI donor	Under 23 23-25 26 and higher	94 (25.9) 140 (38.6) 129 (35.5)	68 (27.0) 78 (30.9) 106 (42.1)	0.1
Diabetes donor	No Yes	320 (93.8) 21 (6.2)	231 (92.8) 18 (7.2)	0.6
Smoking status donor (Pack Years)	None Less than 25 25 or more	175 (50.7) 113 (32.8) 57 (16.5)	112 (46.7) 79 (32.9) 49 (20.4)	0.4
Duration hospital admission donor	2 or less 3-4 More than 4 days	237 (65.1) 55 (15.1) 72 (19.8)	73 (29.1) 75 (29.9) 103 (41.0)	<0.001
Hypotensive period donor	No Yes	242 (71.8) 95 (28.2)	189 (77.1) 56 (22.9)	0.15
Hypotensive minutes	0 1 or more	263 (72.1) 102 (27.9)	187 (74.2) 65 (25.8)	0.6
Terminal Creatinine Donor	<55 55-74 75 and higher	130 (35.8) 116 (32.0) 117 (32.2)	81 (32.3) 88 (35.1) 82 (32.7)	0.6
Heart or lung donor	Yes No	214 (58.9) 149 (41.1)	54 (21.8) 194 (78.2)	<0.001
Pancreatic or liver donor	Yes No	340 (93.9) 22 (6.1)	126 (51.2) 120 (48.8)	<0.001
RECIPIENT				

Age recipient	≤40 years 41–59 years 60 or older	72 (19.8) 172 (47.4) 119 (32.8)	38 (15.1) 100 (39.7) 114 (45.2)	0.007
Sex recipient	Male Female	218 (60.1) 145 (39.9)	151 (59.9) 101 (40.1)	0.9
TRANSPLANTATION PROCEDURE				
Organ	Kidney kidney Kidney pancreas Kidney liver	232 (63.6) 127 (34.8) 6 (1.6)	235 (93.3) 14 (5.5) 3 (1.2)	‹0.001
Side	Right Left	129 (35.3) 236 (64.7)	113 (44.8) 139 (55.2)	0.02
Transplantation period	2007–2010 2011–2015	182 (49.9) 183 (50.1)	96 (38.1) 156 (61.9)	0.004
Panel Reactive Antibody	0 ≥1	239 (66.9) 118 (33.1)	178 (71.2) 72 (28.8)	0.3
Warm ischaemic time (WIT1, (min))	‹15 15–19 ≥20	NA	88 (35.3) 99 (39.8) 62 (24.9)	
Lukewarm ischaemic time (LIT1, (min))	‹15 15–19 ≥20	NA	93 (39.6) 66 (28.1) 76 (32.3)	
WIT1 + LIT1 (min)	‹30 30–39 ≥40	NA	89 (38.0) 80 (34.2) 65 (27.8)	
Lukewarm ischaemic time (LIT2 (min))	≤50 51–69 ≥70	97 (26.8) 112 (30.9) 153 (42.3)	105 (41.8) 69 (27.5) 77 (30.7	‹0.001
Lukewarm ischaemic time (LIT—total (min))	‹60 60–79 ≥80	162 (44.8) 104 (28.7) 96 (26.5)	57 (24.3) 70 (29.8) 108 (45.9)	‹0.001
Cold ischaemic time (CIT (h))	≤10 11–14 ≥15	123 (34.0) 135 (37.6) 103 (28.4)	30 (12.0) 117 (46.8) 103 (41.2)	‹0.001
Warm ischaemic time (WIT2 (min))	‹30 30–34 ≥35	170 (46.8) 83 (22.9) 110 (30.3)	105 (41.8) 54 (21.5) 92 (36.7)	0.3
Perfusion fluid	HTK UW Modified UW Other	70 (19.3) 276 (76.2) 14 (3.9) 2 (0.6)	146 (57.9) 90 (35.7) 14 (5.6) 2 (0.8)	‹0.001
Perfusion fluid amount	≤6000 6000–8000 ›8000	194 (54.6) 111 (31.3) 50 (14.1)	53 (22.6) 64 (27.2) 118 (50.2)	‹0.001

Supplemental Table 1. Patient characteristics: DBD versus DCD grafts.
DCD grafts were transplanted in significantly older recipients compared to DBD grafts (resp. 55 versus 52 years) and duration of hospital admission before donation of the graft was significantly longer in DCD versus DBD grafts. Thereby, during the interval 2007-2010 significantly more DBD then DCD grafts were transplanted, this difference was not significant for the 2011-2015 interval. Significantly more DBD grafts were in the category of shortest duration of cold ischemia time (34% versus 12% in DCD grafts).

Supplemental Table 2. Functional outcome of kidney transplantations in our single center (LUMC) cohort.

Single center (LUMC) cohort (n=628)				
	PF	DGF	PNF	Unknown
DBD (n=370)	n=292 (78.9%)	n=73 (19.7%)	n=5 (1.4%)	-
DCD (n=258)	n=102 (39.5%)	n=150 (58.1%)	n=6 (2.3%)	-

Odds Ratio (OR) for DGF in DCD compared to DCD grafts: 5.88 (4.11-8.42); p<0.001).
PF = primary function, DGF = delayed graft function, PNF = primary non-function

Supplemental Table 3. Summary of the defined ischemia periods

ISCHEMIC PERIOD	TIME SLOT DEFINED
Lukewarm ischaemic time 1 (LIT1) (DCD donors)	Switch off – death
Warm ischaemic time 1 (WIT1) (DCD donors)	Death – start cold perfusion
Lukewarm ischaemic time 2 (LIT2) (DCD and DBD)	Start cold perfusion – nephrectomy
Cold ischaemic time (CIT) (DCD and DBD)	Start cold perfusion – start kidney transplantation procedure
Warm ischaemic time 2 (WIT2) (DCD and DBD)	Start kidney transplantation procedure – reperfusion of the graft

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VII

Mitochondrial restoration precedes functional recovery of the dysfunctioning graft after clinical kidney transplantation

Article under peer review

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ABSTRACT

Delayed graft function (DGF) following kidney transplantation negatively affects transplantation outcome. Incident DGF strongly associates with critical metabolic deficiencies due to extensive mitochondrial damage. It was thus hypothesized that factors involved in mitochondrial recovery not only constitute a potential therapeutic target in DGF but also form markers of imminent recovery. To test the latter, a retrospective patient cohort comprising kidney transplant recipients (n=24) that developed DGF following transplantation, was included. Biopsies taken during the period of DGF were analyzed and classified based on the stage of functional recovery. Grafts showing signs of (acute) rejection were excluded. Expression of Drp1 and OPA1, two major regulators of mitochondrial fission and fusion, and activation of the tissue-regenerative Hippo pathway (through quantifying nuclear translocation of Yes-Associated Protein (YAP)-1, were analyzed by using immunohistochemistry. Mitochondrial density was quantified by mitochondrial (manganese-dependent) Super Oxide Dismutase (MnSOD) staining. Tissue sections were semi-quantitatively scored by two experienced observers and their observation was plotted against the time to functional recovery. Results show that recovery of the mitochondrial pool (MnSOD) ($P<0.001$) and activation of the YAP-1 pathway (nuclear translocation) ($P<0.002$) precede functional recovery following DGF. Mitochondrial fission and fusion markers Drp1 and OPA1 do not qualify as markers of imminent functional recovery following DGF. In conclusion, in the context of DGF, both mitochondrial recovery reflected by increased mitochondrial density, and activation of Hippo signaling (YAP-1) qualify as histological markers of upcoming functional graft recovery and may constitute potential therapeutic targets.

INTRODUCTION

Pressing organ-donor shortages have led to a progressive reliance on so-called extended criteria donations, in order to meet the needs for renal transplantation. Compared to standard criteria donor kidneys, kidneys from extended criteria donors are associated with higher rates of primary non-function and delayed graft function (DGF). DGF comprises the situation in which the graft recipient is in need of dialysis in the first week(s) after transplantation, and associates with inferior (long-term) graft survival.[1-8]

Both primary non-function and DGF are characterized by a persistent need of dialysis, due to impaired clearance by the graft. In primary non-function and DGF, serum creatinine is not a reliable marker for monitoring functional recovery, since it is influenced by dialysis. Moreover, urine production in DGF grafts, if any, is often not representative for functional transplant recovery because of residual function of the native kidneys.[9] As a consequence, there is no early prospective marker that discriminates between DGF and primary non-function in the early phases after transplantation.

Incident DGF strongly associates with critical metabolic deficiencies due to extensive mitochondrial damage in the graft.[10–12] Given the imperative role of an adequate energy supply in cellular homeostasis, it was reasoned that re-establishment of mitochondrial function is a prerequisite for functional recovery following DGF. It was hypothesized that factors involved in mitochondrial recovery predict impending recovery, but may also constitute potential therapeutic targets for treating DGF. A dual function that is now referred to as "theranostic"

Mitochondria are subcellular organelles that are organized in integrated subcellular networks. They are not generated the novo, but are constantly being rejuvenated through fission and fusion of the existing cellular mitochondria. The processes of mitochondrial fission and fusion are under strict and dynamic control of the canonical factors Drp1(fission) and OPA1(fusion), in order to meet energy demand and thus to preserve the cellular energy homeostasis.[13]

This study evaluates the potential of the factors Drp1 and OPA1, as well as cellular mitochondrial density (assessed by MnSOD staining) as histological markers of imminent functional recovery following DGF. As of the presumed canonical role of the Hippo-pathway in graft regeneration[14], we compared the potential of the mitochondria-centered factors as predictors of imminent recovery.

We here show, that mitochondrial content (as quantified by MnSOD staining) and activation of the Hippo-pathway (nuclear translocation of YAP-1) precede functional recovery following DGF.

MATERIALS & METHODS

BIOPSY MATERIAL, HISTOLOGICAL STAINING AND ETHICS

Formalin-fixed paraffin embedded renal graft biopsies from patients transplanted between 2008 and 2011 at the Leiden University Medical Center were selected. Grafts that never recovered (primary non-function) or that showed signs of acute rejection were excluded. All selected transplants involved grafts donated after cardiac death (DCD) and displayed DGF following transplantation (*viz. histo-pathological diagnosis 'acute tubular necrosis'*). All biopsies were taken on clinical indication.

Kidneys were classified on basis of the number of days passed between the moment of biopsy and functional recovery (defined as significant fall in serum creatinine and absent need for dialysis). Hence, the time to recovery reflects the number of days passed between the biopsy and functional recovery.

Protocolled biopsies, taken from functioning grafts as part of the PROTECT study (*Aydin et al., 2012*) (biopsies taken 8.3 (0.5) (mean (sem)) days after transplantation) served as reference (n=*6*).

Four micron sections were cut and the slides stained using standard immunohistological staining protocols. Details of the antibodies (Abcam, Cambridge, UK) and LifeSpan Biosciences Seattle, USA) used and the staining conditions are described in Table 1. Tissue sections were visualized using anti-rabbit/mouse peroxidase-conjugated EnVision (DAKO, Glostrup, Germany) and counterstained with hematoxylin (Merck, Darmstadt, Germany).

HISTOLOGICAL SCORING

Slides were semi-quantitatively scored. Each slide was scored based on the intensity of the factor stained as 0 (none), 1 (minimal), 2 (slight), 3 (moderate) or 4 (abundant). Two persons (J.L. and L.W.) independently reviewed and scored the slides. Disagreements were resolved by joint review on a multihead microscope. Data was analyzed with one-way analysis of variance (ANOVA) with a linear regression test. $P<0,05$ was considered statistically significant and was two-tailed. Analyses were performed using IBM SPSS Statistics (version 22).

Table 1. Immunohistochemistry details

Primary Antibody	Clone	Host	Dilution	Retrieval	Secondary Antibody	Dilution	Provider
Anti-Drp1	4F6	Mouse	1/200	EDTA	EnVision+ -HRP (mouse)	Ready to use	Abcam
Antri-OPA1	LS-B6560	Rabbit	1/300	Citrate	EnVision+ -HRP (rabbit)	Ready to use	LSBio
Anti-SOD2	C7	Rabbit	1/125	None	EnVision+ -HRP (rabbit)	Ready to use	Abcam
Anti-YAP-1	76252	Rabbit	1/500	EDTA	EnVision+ -HRP (rabbit)	Ready to use	Abcam

Table 2. Patient characteristics and functional recovery (n=6 per group) (Mean(sem))

DAYS TO FUNCTIONAL RECOVERY AT MOMENT BIOPSY	0-1 day	2-4 days	5-9 days	9-12 days	Reference
Age Donor (years)	40 (5.6)	51 (3.5)	46 (8.5)	58 (2.8)	54 (5.1)
Sex Donor (% male)	67%	16%	67%	75%	40%
WIT1 (min)	19 (1.5)	27 (8.9)	29 (6.5)	18 (2.9)	15 (1.9)
WIT2 (min)	28 (2.6)	30 (2.2)	31 (4.0)	27 (1.8)	35 (2.6)
CIT (min)	1014 (127)	860 (25)	1021 (95)	826 (69)	922 (69)
Age recipient (years)	60 (4.6)	56 (2.4)	56 (5.3)	56 (5.1)	61 (3.1)
Sex recipient (% male)	50%	50%	67%	25%	55%
Time point of biopsy (days post KTx)	9.2 (0.5)	10.5 (0.7)	10.5 (0.2)	11.6 (0.5)	8.3 (0.5)
Number of dialyses following KTx	2.0 (0.7)	4.2 (0.6)	5.5 (0.5)	5 (0.8)	1.2 (0.4)
Hospital admission (days)	18.8 (3.6)	18.3 (1.9)	29.5 (6.4)	31.3 (3.8)	13.2 (0.4)
Serum creatinine preoperative (µmol/L)	644.8 (100.7)	814.2 (134.4)	766.5 (72.4)	645.0 (161.1)	799.8 (68.3)
Serum creatinine 6 months after KTx (µmol/L)	119.0 (6.02)	142.0 (17.8)	158.2 (24.9)	153.5 (24.5)	140.8 (14.7)
Serum creatinine 1 year after KTx (µmol/L)	124.3 (4.9)	132.7 (22.4)	135.8 (13.1)	170.3 (19.8)	138.3 (11.6)

KTx = kidney transplantation

WIT1 = warm ischemia time is the time between cardiac death and the start of cold perfusion of the graft (DCD)

WIT2 = period of warm ischemia during the transplantation procedure

CIT = cold ischemia time is defined as the period of cold storage of the graft starting with cold perfusion of the graft in the donor.

RESULTS

We included 24 human kidney biopsies. All biopsies were collected from DCD kidneys. None of these kidneys showed signs of acute rejection. Six additional kidney biopsies from functioning transplanted grafts were available as reference. Patient and graft characteristics are summarized in Table 2. Examples of MnSOD, OPA1 and Drp1 expression (immunohistochemistry) in the reference grafts are shown in the middle panels of Figures 1 and 2.

Mitochondrial density was quantified by MnSOD staining. Figure 1 shows that mitochondrial density progressively associates with the time to functional recovery ($P<0.001$) (Fig.1C), with sparse staining in the grafts that required 9 or more days to recover (Fig.1A) and gradual increase to normalization immediately prior to functional recovery. (Fig.1B)

OPA1, a pivotal regulator of mitochondrial fusion, followed a similar pattern with minimal expression in grafts with an extended time to recovery (viz. more than 9 days, Fig.2D, 2F), but progressive increases upon imminent functional recovery ($P<0.039$) (Fig.2E, 2F). Drp1 expression, the canonical regulator of mitochondrial fission, was stable for all time points tested (Fig.2A–C).

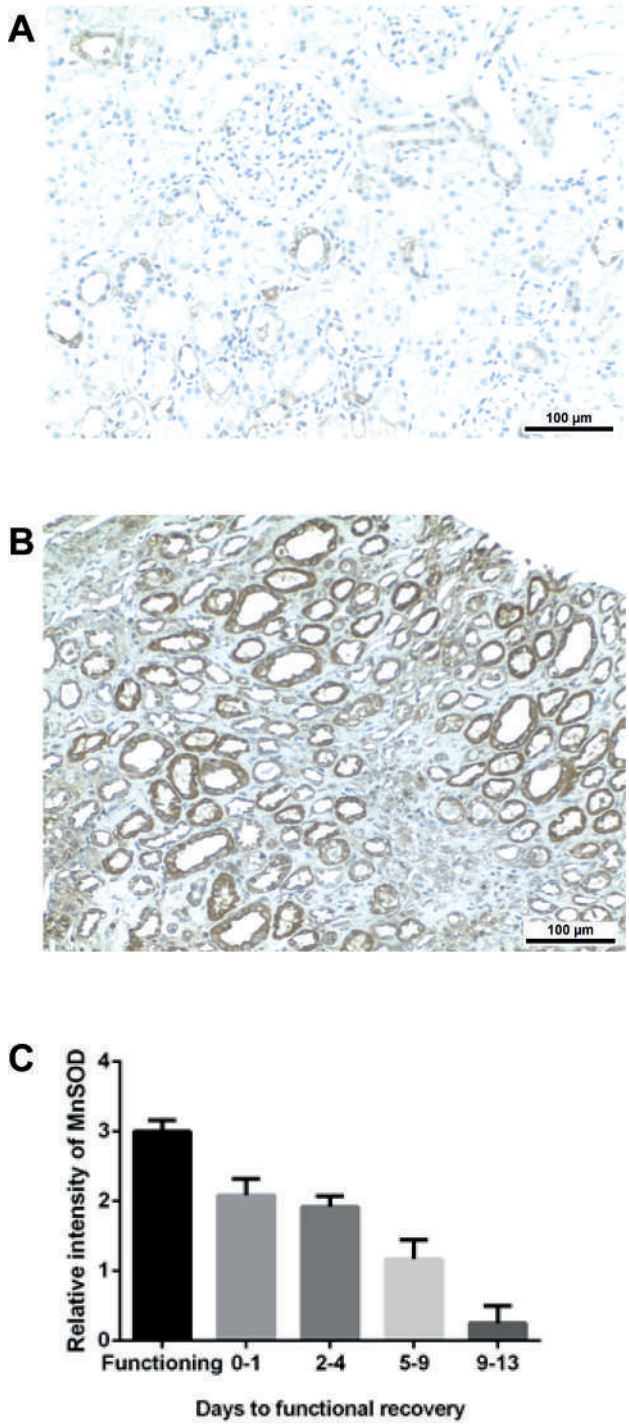

Figure 1. Inverse relationship between mitochondrial density (quantified by MnSOD staining) and time to functional recovery during DGF.
A) Kidney graft severely suffering from DGF (biopsy taken two weeks prior to functional recovery.
B) Functioning (reference) kidney. (20 Fold)
C) Mitochondrial restoration and functional recovery. Increased MnSOD expression proceeds actual functional graft recovery (ANOVA - P<0.001).

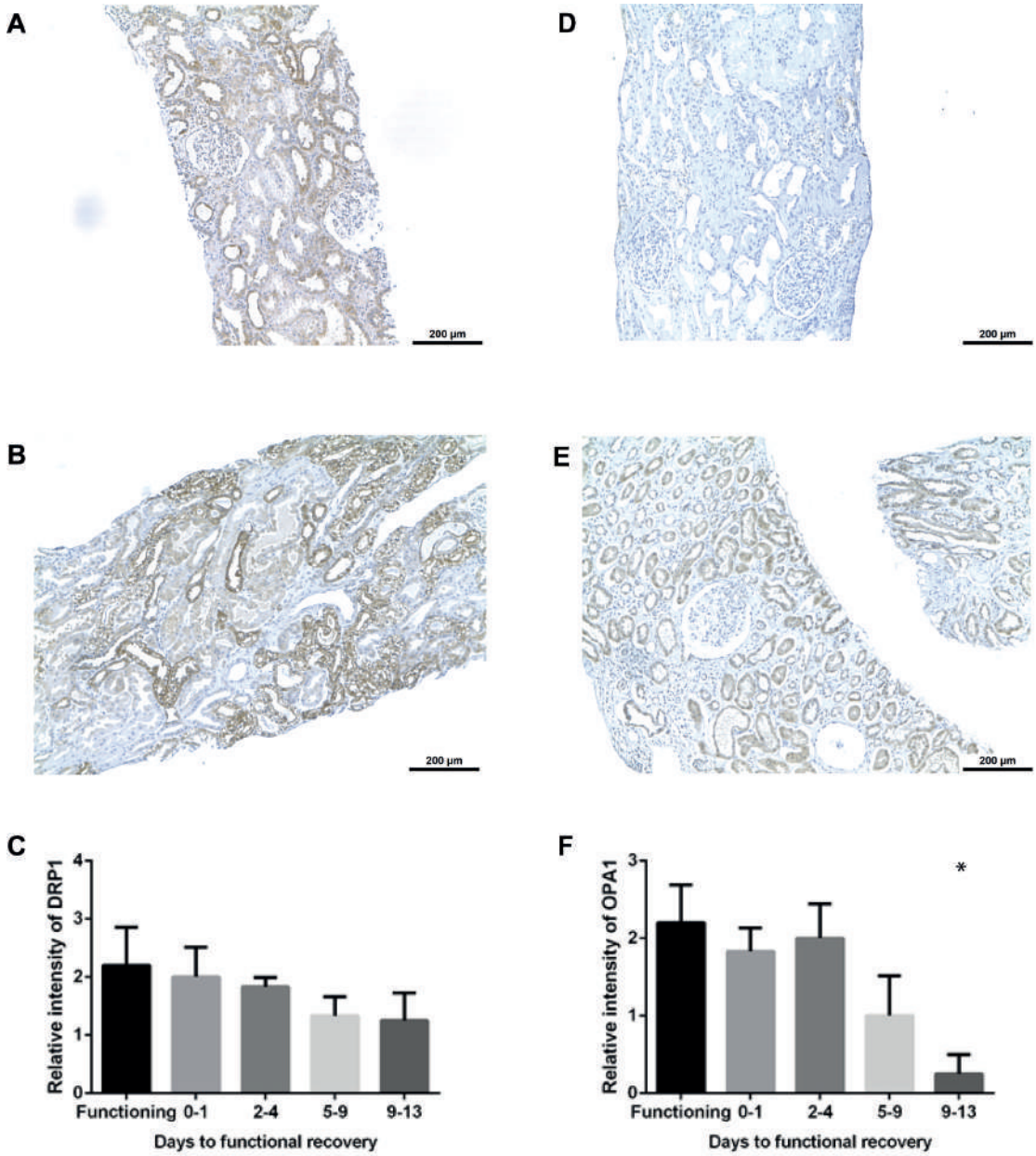

*Figure 2. Mitochondrial dynamics (fission and fusion factors) and time to functional recovery in DGF. Immunohistochemistry of Drp1 (A-B-C) and OPA1 (D-E-F) in grafts suffering from DGF (A-D) and functioning reference grafts (B-E). (Overview Images: 10 Fold) C) Stable expression of the fission factor DRP1. F) Minimal OPA1 expression in grafts with an extended time to recovery and early reconstitution prior to functional recovery (ANOVA - P‹0.039, day 9-13 versus reference *P‹ 0.05).*

The Hippo pathway is recognized as a central regulator of processes involved in renal recovery following ischemia/reperfusion injury.[14] Activation of this pathway results in dephosphorylation of its downstream effector YAP-1, followed by nuclear translocation of YAP-1. Hence, nuclear translation of YAP-1 reflects activation of the Hippo pathway. To test a possible association between activation of the Hippo pathway and imminent functional kidney transplant recovery, we quantified the fraction of YAP-1-positive nuclei, results of which showed a progressive association up to a fraction similar to that in reference kidneys at the time of functional recovery ($P<0.002$) (Fig.3C).

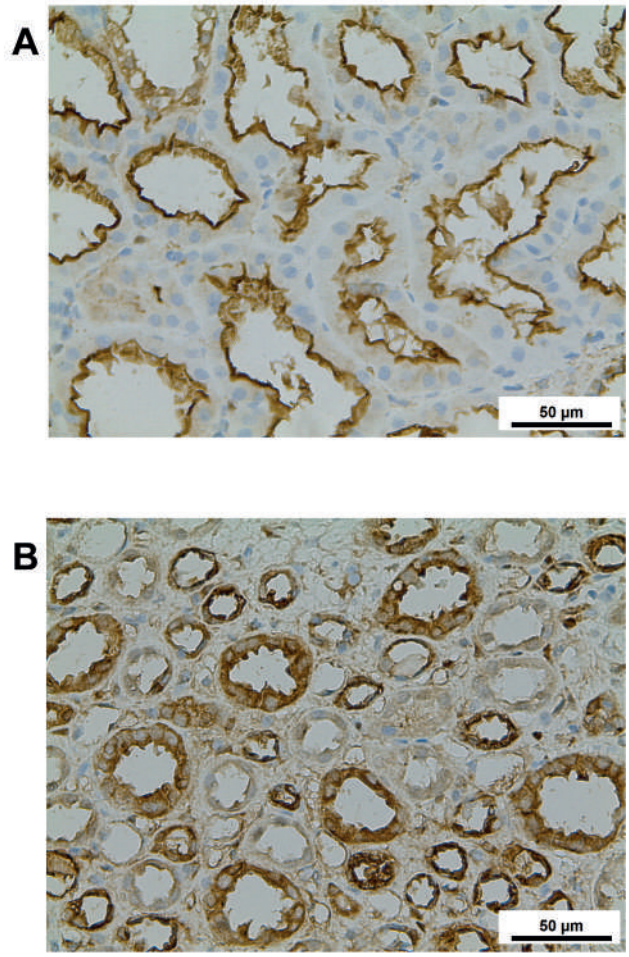

*Figure 3A. Activation of the Hippo-pathway (nuclear translocation of YAP-1) precedes functional recovery during DGF. YAP-1 negative nuclei in non-functioning DGF grafts and positive nuclei (B) in functioning reference grafts (40 Fold). C) YAP-1 nuclear translocation associates with imminent graft recovery (ANOVA - P‹0.002, day 5-9 and 9-13 versus reference *P‹ 0.05).*

DISCUSSION

The presented data identifies MnSOD expression and nuclear YAP-1 translocation as predictors of oncoming recovery in non-functional kidney transplants following transplantation. Hence, these histological markers allow for differentiation between eminent recovery and absent recovery in a period of failing clinical readouts such as urine production and serum creatinine levels.[9] Expression of the mitochondrial fusion factor OPA1 weakly associates and fission factor Drp1 does not predict imminent functional recovery. These observations support an association between mitochondrial reclamation and functional recovery.

Increased reliance on so-called extended criteria grafts in an era of increasing donor shortages has led to a sharp increase in the incidence of DGF and primary non-function following renal transplantation.[2,4-6] At this point, medical interventions minimizing the effects of DGF (i.e. ischemia/reperfusion injury) are urgently needed. Therefore biomarkers to predict functional recovery and clinical outcome are required. Moreover, these biomarkers may be used to quantify effects of future interventions aimed at alleviating ischemia/reperfusion injury and or tissue resilience. In the absence of reliable conventional readouts of graft function this study focuses on a histological approach.

Recently a one-to-one association between a persistent post-reperfusion metabolic deficit and future DGF was identified.[10,15] Further evaluation showed that this was due to profound mitochondrial damage.[10,15] As cellular powerhouses, mitochondria are crucial for adequate ATP production and therefore for cell homeostasis, survival and recovery. We hypothesized that mitochondrial repair precedes functional recovery and that in the context of primary non-function following kidney transplantation mitochondrial stability and recovery constitutes a theranostic.

Mitochondrial homeostasis reflects a highly dynamic process. Mitochondria are not generated de novo, but the mitochondrial pool is constantly rejuvenated through a process of mitochondrial fusion and fission. This process serves quality control but also allows rapid adaptations in order to serve metabolic demands.[13] Recovery of a non-functional graft critically relies on re-establishment of an adequate mitochondrial pool from residual mitochondria. This is supported by the association between MnSOD staining and imminent recovery.[16] MnSOD is a mitochondria specific marker that in our hands outperforms other markers of mitochondrial density such as cytochrome C.[17]

In contrast, the mitochondrial fission and fusion factors Drp1 and OPA1 did not classify as histological markers of recovery. This may reflect differences in protein half-life or alternatively that expression of these proteins is part of metabolic privileged expression profiles.[18–20] Moreover, although OPA1 and DRP1

are canonical factors in the mitochondrial fission and fusion, their functions extend far beyond mitochondrial genesis and include roles in apoptosis.[21] As such, expression of Drp1 and OPA1 may be part of the damage control following ischemia/reperfuson injury.[22] The actual functional recovery is deferred until reinstatement of an adequate energy supply.

Functional recovery following kidney transplantation is under critical control of the Hippo pathway, a highly conserved pathway that controls morphogenesis and regeneration.[14] Signaling through the pathway is (partly) mediated by active dephosphorylation and subsequent nuclear translocation of the Yes-associated protein-1 (YAP-1). Given its presumed cardinal role in kidney regeneration following transplantation[14], we considered an evaluation of Hippo activation (YAP-1 nuclear translocation) as a biomarker of imminent repair relevant. Results mimicking those for MnSOD, show that functional recovery is preceded by a sharp increase in mitochondria content and activation of the Hippo-repair pathway.

All the described observations fit into a model in which DGF upon kidney transplantation is related to mitochondrial damage and a consequently severe deficit of energy. Since repair mechanisms are energy consuming processes, recovery of the energetic machinery, the mitochondria, needs to precede early functional recovery. Subsequently, mitochondrial preservation and recovery [23,24] are considered valid targets against DGF (i.e. ischemia/reperfusion injury). In fact, melatonin, meclizine and cyclosporine are currently evaluated in clinical trials of myocardial ischemia/reperfusion.[25-28]

All in all, results show that MnSOD and YAP-1 are promising histological markers of early functional recovery upon DGF. The temporal dynamics and normalization of the expression level characterize these markers as theranostics for DGF.

Limitations: This is a retrospective study on clinical biopsies from non-functioning grafts, as such it cannot be excluded that the observed changes are by association rather than causative. Functional studies are required to proof the point that mitochondrial recovery is a theranostic rather than a biomarker.

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VIII

Impaired reactive aldehyde metabolism is associated with delayed graft function in human kidney transplantation

Article under peer review

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ABSTRACT

Background: Delayed graft function (DGF) is an early complication following kidney transplantation with an unclear molecular mechanism. Through gene and biochemical analyses, we describe that impaired reactive aldehyde metabolism is associated with DGF in humans.
Methods: Kidney biopsies from grafts with DGF from deceased donor grafts (n=16) were compared with living donor grafts (n=20) by gene pathway analysis. A second series of grafts with DGF (n=10) were compared to living donor grafts (n=10) by measuring aldehyde dehydrogenase (ALDH) expression, function, and reactive aldehyde adduct protein formation.
Results: A decrease in gene and protein expression of mitochondrial ALDH enzymes, including ALDH2, ALDH4A1 and ALDH7A1, were noted in those developing DGF compared to those that did not. Further, ALDH enzymatic activity was reduced in donor grafts developing DGF compared to those that did not (n=8/group, 37±12* vs. 79±5 mg/min/mg tissue, *P‹0.005, respectively). A lower ALDH enzymatic activity correlated to a longer length of hospital stay for the transplant recipient (›14 days 36.5±13.0*, between 7-14 days 44.2±13.0*, and ‹7days 86.8±4.7, mg/min/mg protein, *p‹0.05).
Conclusions: Together, our study associates a reduced ALDH enzymatic activity with DGF following kidney transplantation. Measuring reactive aldehyde load and ALDH enzymatic activity at the time of kidney transplantation can potentially be used as a biomarker to predict DGF.

INTRODUCTION

Delayed graft function (DGF) is the deferred functional recovery of a donor graft kidney following kidney transplantation requiring the temporary need for dialysis. DGF detrimentally affects renal function, graft longevity, and is an important risk factor for acute kidney rejection.[1,2] The reported incidence of DGF in deceased donor kidneys is 25% and can be perhaps as high as 50% for kidneys from cardiac death donor organs.[3,4] Additionally, the incidence of DGF is steadily rising due to the increased use of marginal donor grafts secondary to organ transplant shortages. As a consequence, DGF leads to reduced graft function, prolonged hospital admissions, increased demand of donor kidneys for re-transplantation secondary to rejection, and a higher economic societal burden.[5]

Transplants from living donors are much less susceptible to DGF when compared to grafts from deceased donors.[1] The benefit of receiving living donor kidneys may partially be mediated by shorter ischemia times. However, the differences in molecular biology that may contribute to the outcomes between living donor and deceased donor kidneys have not been extensively studied. Therefore, examining differences between living donor grafts and deceased donor grafts may provide insight to design biomarkers to predict DGF and molecular targets to develop treatment strategies to reduce the incidence of DGF.

As an initial step to identify a mechanism underlying DGF during kidney transplantation, we used human renal biopsies from transplanted deceased donor kidneys who developed DGF and compared the molecular differences using gene arrays to kidneys from living donor transplants (that did not develop DGF). From this approach, we developed a hypothesis that impaired reactive aldehyde metabolism is associated with DGF. Recent findings suggest that reactive aldehydes, produced after the reactive oxygen species-induced attack of lipid membranes, impair cellular functions.[6,7] These toxic aldehydes include acetaldehyde, malondialdehyde (MDA), and 4-hydroxynonenal (4-HNE). 4-HNE can cause irreversible Michael addition adduct formation on proteins at cysteine, lysine or histidine amino acids and 4-HNE cellular adducts can be assessed by specific antibodies.[8] These aldehyde adducts formed on proteins can produce changes in enzyme activity, ion channel gating, and mitochondrial energetics.[7,9-11] Therefore, to further explore our hypothesis in regards to reactive aldehydes and DGF, we then used a second set of kidney biopsies to examine reactive aldehyde levels and assays to measure aldehyde dehydrogenase expression and activity in kidney biopsies.

MATERIALS & METHODS

PATIENT ENROLLMENT

Prior to initiation of the study, the study protocol was approved by the medical ethics committee at the Leiden University Medical Center. Written informed consent was obtained from each patient.

Paired renal cortical biopsies were obtained at the end of the cold ischemic period (prior to implantation) and 45 min after reperfusion of the kidney in the recipient (Figure 1A). Kidney biopsies from an initial 18 donor kidneys were used to conduct the whole genome array portion of the study. In this portion of the study, we obtained 10 paired renal biopsies from living donor patients (which did not develop DGF) and 8 paired renal biopsies from deceased donor patients that went on to develop DGF.

For follow-up we then obtained an additional 10 paired renal biopsies from living donor kidneys and 10 paired renal biopsies from deceased donor kidneys that developed DGF. For the additional paired biopsies, the biopsy taken prior to transplant was used to validate the gene array findings and the biopsy taken during reperfusion was used to analyze protein expression and enzyme activity (Figure 1B and Figure 1C). Details regarding enrollment and patient demographics are described in detail within the Supplemental Material section (Supplemental Table 2 and Supplemental Table 3).

Renal allograft transplantations were perfused and stored with either University of Wisconsin solution or Custodiol® HTK (histidine–tryptophan–ketoglutarate) solution. None of the grafts were machine perfused. For renal transplantation, all patients were induced by propofol, sufentanil and atracurium. Patients were intubated in addition to a central venous catheter used for intraoperative monitoring. During the procedure, patients were maintained on a propofol and sufentanil infusion. Patients received basiliximab (day 0 and 4) as immunosuppressive induction. Patients were maintained on tacrolimus or cyclosporine A, mycophenolate mofetil and steroids for immunosuppression. Biopsies were taken from the upper pole of the kidney. For tissue biopsies obtained prior to transplantation, a small cortical incision was made. For biopsies taken after reperfusion, a spring-loaded automatic biopsy needle was used (16 Ga Travenol). Tissue was snap frozen in liquid nitrogen and stored at -80° C. Tissue was labeled and stored with a unique identifier and stored. For the genome array studies, all samples were analyzed together after the groups were collected. The validation studies were also performed after the tissue was obtained selectively using only tissue from deceased donors who developed DGF after transplantation.

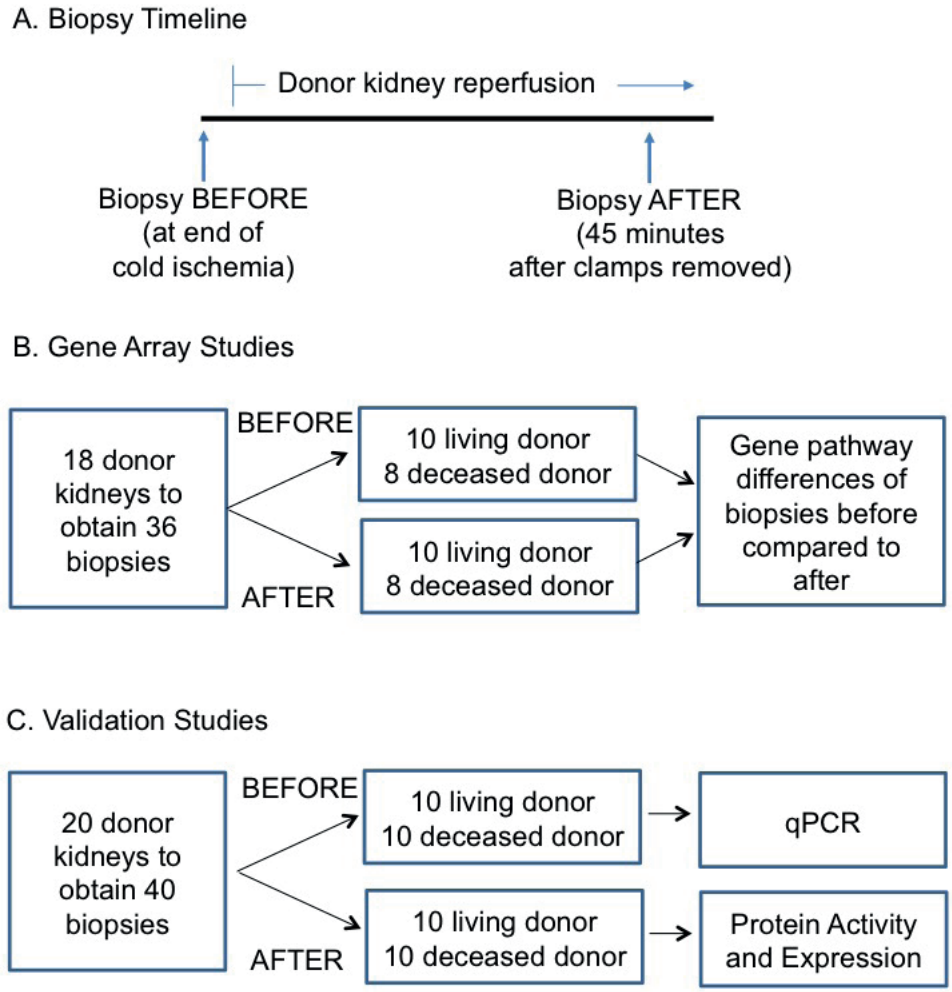


Figure 1. Timeline and allocation of renal biopsies obtained for the study. A. Two biopsies were taken for each transplanted kidney. One biopsy was obtained prior to reperfusion of the kidney at the end of the cold ischemia period. The second biopsy was obtained 45 minutes after the kidney was reperfused. B. Initially, the first 36 biopsies were used for a genome array, consisting of biopsies from 10 living donor kidneys and 8 deceased donor kidneys that developed DGF. C. Forty additional biopsies were obtained for validation from 10 transplanted kidneys from living donors and 10 transplanted kidneys from deceased donors that developed DGF. Biopsies taken prior to transplantation were used to validate the gene array studies. Biopsies taken after the kidneys transplanted were used for western blot and enzyme activity assay.

The transplanted patients were also followed during their hospital stay and the length of hospital stay after transplantation was documented. Patients were considered as developing DGF if they were in need of dialysis within the first week after transplantation. For those requiring dialysis, acute rejection was excluded as a cause of DGF by renal biopsy. Surgical complications of the transplant procedure were also excluded as a source of DGF. Further, if transplant recipients required one episode of dialysis after transplantation due to incident hyperkalemia, these grafts were also not included as having DGF.

GENOME ARRAY

Paired biopsies were taken with one biopsy prior to transplantation and one biopsy during transplant reperfusion. From these renal biopsies, total RNA was extracted using RNAzol (Campro Scientific, Veenendaal, The Netherlands) and glass beads. 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 manufacturer's instructions at the Service XS facility in Leiden.

The tissue biopsies collected from the two groups (kidneys from living donors and kidneys from deceased donors) were run on separate gene arrays. The 2 paired tissue biopsies obtained for each transplanted kidney resulted in running 36 gene arrays for the tissue biopsies that were collected. The gene arrays obtained were analyzed by a statistician at the University of Leiden blinded to the identification of the groups of tissue biopsies collected. An average replicate value was calculated and log2 ratios were computed by comparing the matched pair biopsies and the gene expression differences for each transplanted kidney prior to and after reperfusion. One value per gene was calculated for the average expression of multiple probes with the same Entrez gene identification; resulting in 15093 unique gene profiles.

BIOCHEMICAL ASSAYS

For validation of gene array studies, 20 transplanted kidneys (10 living donor and 10 deceased donor kidneys) were used to perform further biochemical analysis, which included qPCR, western blot, and enzymatic activity assays.

For qPCR, RNA was isolated from kidney biopsies taken at the end of the ischemic period. Kidney biopsy lysates were made by sonification and RNA was isolated

by use of Ambion© RNAqueous Kit. To substantially reduce the possibility of DNA contamination in the preparations, the isolated total RNA was subject to precipitation with lithium chloride and DNase digestion (Ambione DNase free). cDNA was made using the Takara© Primescript cDNA synthesis kit with oligo dT primers. qPCR reactions were performed in a final volume of 20 ml that contained 15 ml of Fast SYBR© Green Master Mix (Life Technologies), 1000nM primer (ALDH2, ALDH7A1 or GAPDH) or 500nM primer (ALDH4A1), and 10ng cDNA. Primer characteristics are described (Supplemental Table 1). The cycling protocol was 20 seconds at 95°C, followed by 40 cycles of 3 seconds at 95°C and 30 seconds at 61°C. The melt curve protocol was 15 seconds at 95°C, followed by a minute at 60°C, followed by a gradual temperature increase from 60°C to 95°C (+0.03°C per 15 seconds) in 42 minutes.

For western blot analysis, kidney biopsies taken 45 minutes after reperfusion were homogenized in mannitol-sucrose buffer (pH 7.4) with protease inhibitor in a glass homogenizer. Protein counts were obtained by Bradford assay and samples were normalized to mg protein. Western blot was performed as described.12 Primary antibodies used included ALDH2 (Santa Cruz), ALDH4A1 (Abcam), ALDH7A1 (Abcam) and ALDH1A1 (Abcam) at 1:1000. Secondary antibodies were used at 1:3000. Density of bands was measured by Image-J and normalized to GAPDH.

To determine ALDH enzyme activity, 25 mg of protein were used. ALDH enzyme activity was measured spectrophotometrically (340 nm) by analyzing the reaction of NAD+ to NADH as previously described12. The activity assay was performed at 25°C in 50 mM sodium pyrophosphate buffer (pH 9.4), 2.5 mM NAD+ and 10 μM acetaldehyde was used as substrate. ALDH enzyme activity was converted to mmole NADH/min/mg of protein.

STATISTICAL ANALYSIS

Sample sizes were chosen for this study based on previous experience at the University of Leiden has on conducting clinical studies regarding ischemia/reperfusion injury in human kidney transplantation.[13,14] SPSS 22.0 (SPSS, Chicago, IL) was used for statistical analysis. Primary outcome of the genome array was defined a priori at initiation of the analysis. The 15093 unique gene profiles obtained from the whole genome array were analyzed by ingenuity pathway analysis (Redwood City, CA, USA). Living donor kidneys were compared with deceased donor grafts that developed DGF. For pathway analysis, data is represented as P-values for the change in expression comparing the paired biopsies; fitting into predefined pathways. With values provided as composite P-values based upon the o-hypothesis for each given pathway. P-values are expressed as the –log P-value. Additionally, 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. For the validation of gene array studies, primary outcome was defined prior to the biochemical assays and data was compared between the two groups using Students t-test.

RESULTS

For the gene array portion of the study, changes in gene pathway P-values were calculated for the living donor kidneys and the deceased donor kidneys that developed DGF. This was conducted by comparing each gene array of a post-transplant biopsy to a paired pre-transplant biopsy gene array and sequential run an Ingenuity pathway analysis. We selected the 10 signaling pathways with the highest differences in P-values in kidneys from living donors compared to the kidneys from deceased donors and evaluated the genes within these identified pathways[15] to determine whether specific genes may have caused the differences between living and +DGF deceased donor grafts. (Table 1)
Distinct genes were up-regulated in these pathways for only the living donor kidney biopsies at reperfusion when compared to the kidney biopsies of grafts that developed DGF (Table 1). Of the top 10 most enriched pathways in living donor grafts compared to those kidneys from deceased donors that developed DGF, 4 genes, all in the aldehyde dehydrogenase (ALDH) family of 21 enzymes, were commonly occurring.[16]

PATHWAY	*Difference in value*	*Genes only upregulated in living donors*	*Genes only upregulated in deceased donors +DGF*	*Common*
Serotonin Degradation	6.59	ALDH4A1, ALDH2, ALDH7A1, ALDH3A2, ADH6, UGT2B7, UGT2B10, UGT2A3, UGT1A9, AKR1A1, SMOX, DHRS4	None	None
Tryptophan Degradation	5.54	ALDH4A1, ALDH2, ALDH7A1, ALDH3A2, AKR1A1, DDC, SMOX	None	None
Histamine Degradation	5.34	ALDH4A1, ALDH2, ALDH7A1, ALDH3A2, HNMT, ABP1	None	None
Ethanol Degradation II	5.03	ALDH4A1, ALDH2, ALDH7A1, ALDH3A2, ADH6, AKR1A1, ACSS2, DHRS4	None	ACSL3
NRF-2 Mediated Oxidative Stress Response	4.82	AKR7A2, AKR1A1, FTL, NQO2, ABCC2, MAF, GSTA5, SCARB1, FMO1, GSTA1, GSTA2, GSTA3, MGST1, PRKCQ, ACTB, ACTG1, MGST2, MAP2K3, SQSTM1, AOX1, EIF2AK3, EPHX1	UBB, JUNB, DNAJA1, DNA	FOS, JUN, JUND, DNAJA4, DNAJB11, MAFF

Xenobiotic Metabolism Signaling	4.63	ALDH4A1, ALDH7A1, ALDH3A2, ALDH8A1, FTL, UGT2B7, NQO2, ABCC2, MAF, GSTA5, CYP3A7, HS6ST2, SMOX, FMO1, GSTA1, GSTA2, GSTA3, MGST1, PRKCQ, UGT2B10, UGT8, PPP2R5A, MGST2, MAP2K3, EIF2AK3, UGT1A9	CITED2, MAP3K8, TNF, HSP90AB1, HSP90AA1	None
Noradrenaline and adrenaline degradation	4.33	ALDH4A1, ALDH2, ALDH7A1, ALDH3A2, ALDH8A1, ADH6, AKR1A1, SMOX, DHRS4	None	None
LPS/IL-1 Mediated Inhibition of RXR function	4.14	ALDH4A1, ALDH7A1, ALDH3A2, ALDH8A1, GSTA1, GSTA2, GSTA3, GSTA5, APOE, MGST1, SLC27A2, ACOX2, ABCC2, CYP3A7, IL1R2, MGST2, SCARB1, NR5A2, HS6ST2, FMO1, SMOX	ALAS1, HMGCS1, TNF	ACSL3, JUN, NR0B2
Glutathoine-mediated detoxification	4.12	GSTA1, GSTA2, GSTA3, GSTA5, MGST1, MGST2, GGH	None	None
Oxidative Ethanol Degradation III	3.98	ALDH4A1, ALDH2, ALDH7A1, ALDH3A2, ACSS2	None	ACSL3

Table 1. Gene array differences from living compared to +DGF deceased donor kidney biopsies. Whole genome array changes before and after transplantation were compared from each living donor and deceased donor kidney using Ingenuity pathway analysis. The difference in P-values between living donor kidneys and deceased donor kidneys gene arrays sets was the highest for the 10 signaling pathways listed. Further sub-analysis of these signaling pathways showed 80% of these 10 signaling pathways with 3 genes, ALDH3A2, ALDH7A1, and ALDH4A1, upregulated in living donor kidneys that were not upregulated in kidneys that developed DGF. One gene, ALDH2, was upregulated in 60% of these 10 pathways. Specific genes that were only upregulated in living donor kidneys and deceased donor kidneys are listed. Further, genes commonly upregulated in both living and deceased donor kidneys are also listed.

The major source of reactive aldehyde production, particularly during reperfusion of organs, is at the mitochondria.[7] Therefore, we focused on examining ALDH2, ALDH7A1, and ALDH4A1 genes in addition to their protein expression and activity for further analysis. Each of these targets is mitochondrial, can protect against cellular stress, and in experimental assays metabolize reactive aldehydes.[7,17–19] Gene array expression of the mitochondrial ALDH enzymes ALDH2 and ALDH7A1

was significantly higher in the living donor grafts compared to the grafts with DGF (ALDH2: 1919.4±86.7 versus 1314.5±76.6*, ALDH7A1: 1739.9±65.5 versus 1230.4±83.1*, *p=0.001) with ALDH4A1 just at statistical significance (1324.1±34.7 versus 1102.9±105.9; p = 0.05, Figure 2A-C). As a comparison, no differences were seen for the cytosolic ALDH enzyme ALDH1A1 (Figure 2D). Further, when analyzing all 20 genes in the family of ALDH enzymes in the gene array (ALDH3B2 was not in the array), all other ALDH enzymes not initially identified by Ingenuity pathway analysis, besides ALDH1L1, remained unchanged during kidney transplantation (Supplemental Figure 1).

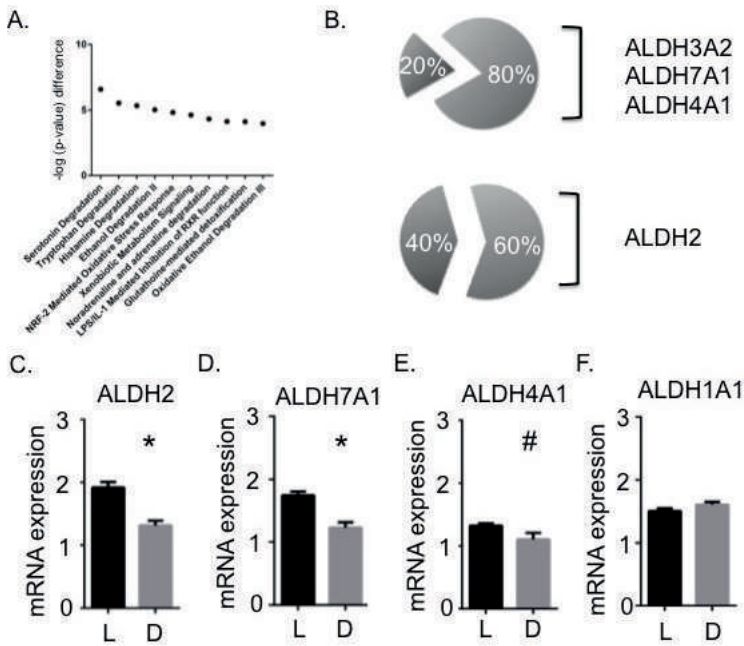


Figure 2. Summary of gene array studies. A. Whole genome array changes before and after transplantation were compared from each living donor and +DGF deceased donor kidney using Ingenuity pathway analysis. The difference in P-values between living donor kidneys and deceased donor kidneys gene arrays sets was the highest for the 10 signaling pathways listed. B. Further sub-analysis of these signaling pathways showed 80% of these 10 signaling pathways with 3 genes, ALDH3A2, ALDH7A1, and ALDH4A1, upregulated in living donor kidneys that were not upregulated in kidneys that developed DGF. One gene, ALDH2, was upregulated in 60% of these 10 pathways.

*C. Of these genes, ALDH2 and ALDH7A1 were statistically significant between living donor kidneys compared to kidneys developing DGF (*P‹0.01). ALDH4A1 nearly reached statistical significance (#P=0.05). This is in comparison to other ALDH family gene members, such as ALDH1A1, which did not change between living donor kidneys compared to kidneys that developed DGF.*

To validate the gene array findings initially identified and further examine in particular the mitochondrial enzymes ALDH2, ALDH4A1 and ADLH7A1 through biochemical analysis, we obtained biopsies from an additional 10 living donor grafts that did not develop DGF and 10 deceased donor kidney grafts that developed DGF. Initially, we performed qPCR on all 10 biopsy samples that were obtained prior to kidney transplantation. The details of the primer design and validation for ALDH2, ALDH4A1, ALDH7A1 and ALDH1A1 are described in the supplemental material (Supplemental Table 1 and Supplemental Figure 2). For qPCR analysis significant differences were noted when normalizing to GAPDH and calculating a delta Ct for living donor kidneys compared to deceased donor kidneys (Supplemental Figure 3, n=10/group, ALDH2: 3.3±0.8 vs. 5.7±0.8*, ALDH7A1 3.7±0.4 vs. 5.6±0.6* and ALDH4A1: 2.5±0.3 vs. 4.6±0.6**, *p‹0.01, **p‹0.001, reported as delta Ct values normalized to GAPDH). In relation to fold expression differences in percent relative to the living donor biopsies, qPCR analysis showed deceased donor (DGF) grafts had significantly lower expression of ALDH2, ALDH7A1 and ALDH4A1 (Figure 3, n=10/group, *P‹0.01). As a comparison, ALDH1A1 was measured and unchanged between groups for either method of analysis (Supplemental Figure 3, Figure 3). Further, we quantified levels of protein expression by western blot for ALDH2, ALDH7A1 and ALDH4A1 for biopsies taken 45 minutes after reperfusion. Both ALDH7A1 and ALDH4A1 had significant changes in expression between our two groups (Figure 4, ALDH7A1: 1.2±0.1* vs. 0.7±0.07, *p‹0.001, ALDH4A1: 1.7±0.3* vs. 0.9±0.1, *p‹0.017). Western blot for ALDH2 showed a relative decrease in expression that did not reach statistical significance (Figure 4, ALDH2: 5.2±1.1 vs. 4.0±0.6).

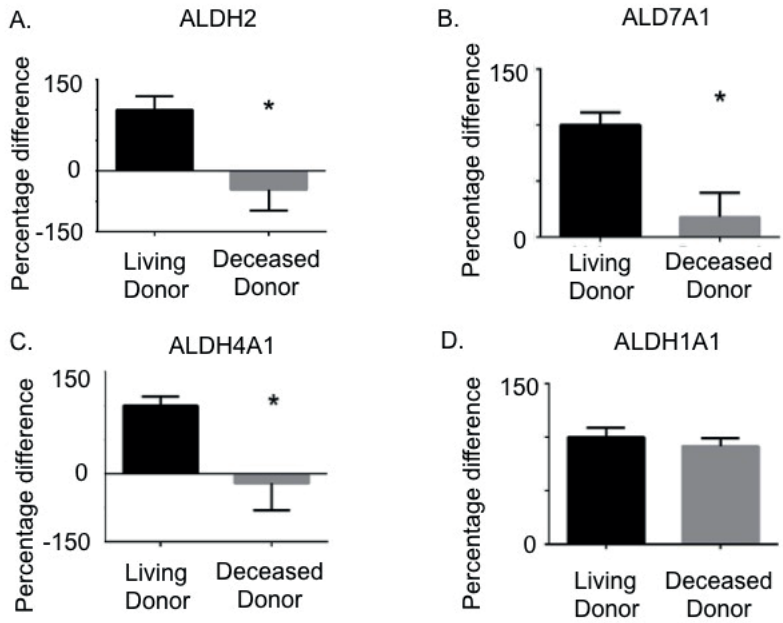


*Figure 3. qPCR Validation studies for kidney biopsies taken prior to transplant. A. ALDH2 B. ALDH7A1 C. ALDH4A1 D. ALDH1A1 (used for reference). ALDH2, ALDH7A1 and ALDH4A1 by qPCR all were significantly elevated in living donor kidneys compared to kidneys with DGF (n=10/group, *P<0.05). Ct values of each gene were normalized to GAPDH to calculate the delta Ct value. To visualize higher expression in living donor grafts; average delta Ct value for living donors was set at 100%. A negative value in the kidneys which developed DGF is an average delta Ct that is more than twice different as compared to the living donor control group.*

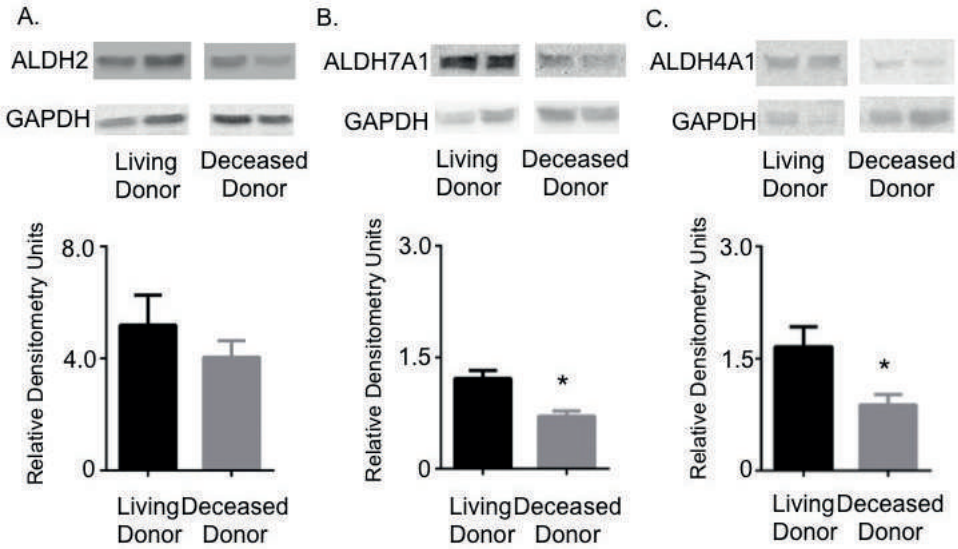


*Figure 4. Western blot from kidney biopsies taken after reperfusion of transplanted kidneys. A. ALDH2 B. ALDH7A1 C. ALDH4A1. All western blots were normalized to GAPDH. Both ALDH7A1 and ALDH4A1 were significantly different in kidneys of living donors compared to kidneys of deceased donors that developed DGF (n=8/group, *P<0.05).*

Due to the qPCR and western blot results, we further tested in the biopsies taken after reperfusion whether total ALDH enzymatic activity differed between living donor kidney and deceased donor (DGF) kidney biopsies. Biopsies of living donor kidneys taken 45 minutes after reperfusion have significantly higher ALDH enzyme activity compared to deceased donor (DGF) grafts for metabolizing reactive aldehydes. The total activity was significantly higher for the living donor kidney biopsies compared to the deceased donor (DGF) kidney biopsies (Figure 5A: 78.6±4.7 vs. 36.9± 11.5* mg/min/mg protein, *p ‹ 0.005).

Since these ALDH enzymes are responsible for metabolizing aldehydes within the cell, we further tested in these tissue samples the extent of reactive aldehyde adduct formation which is occurring in living donor and deceased donor (DGF) kidneys. The amount of 4-HNE protein adducts in the reperfused kidneys were significantly higher for deceased donor (DGF) kidneys compared to living donor kidneys (Figure 5B, n=6/group, 4-HNE adducts: 1.2±0.2 vs. 1.9±0.3* p‹ 0.028).

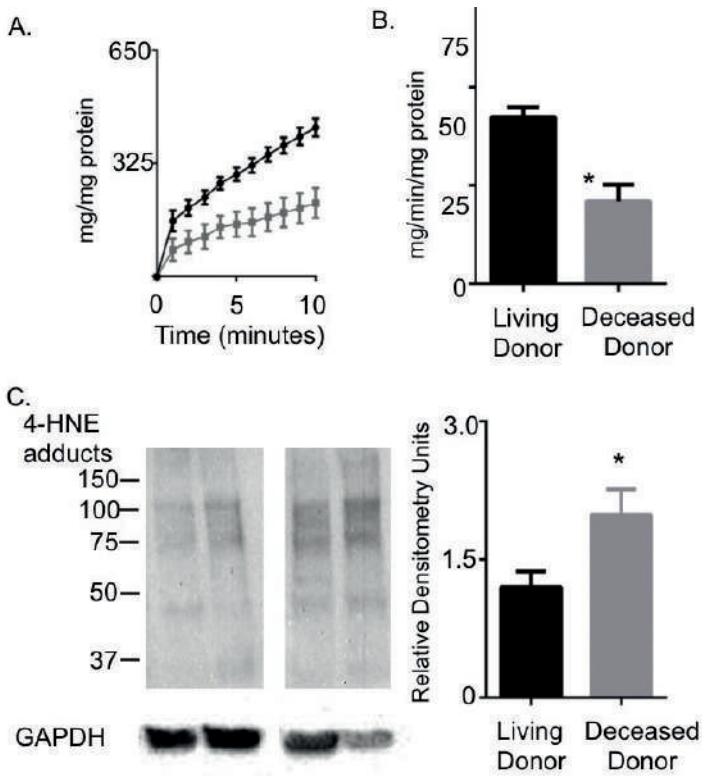


*Figure 5. ALDH activity and reactive aldehyde adducts from kidney biopsies taken after reperfusion of transplanted kidneys. A. ALDH enzymatic activity when challenged with acetaldehyde. B. Cumulative ALDH enzymatic activity. C. 4-hydroxynonenal-induced protein adducts Western blots were normalized to GAPDH. Black bars or lines = living donor kidneys, grey bars or lines = kidneys with DGF, n=8/group, *P<0.01, relative densitometry units.*

When comparing ALDH enzymatic activity to length of hospital stay for all patients transplanted, we discovered that a higher ALDH enzymatic activity was associated with a shorter transplant recipient hospital stay (Figure 6, 86.8±4.7 if length of stay was 7 days or less vs. 44.2±13.0* for length of stay between 7 and 14 days, vs. 36.5±13.0* for length of stay greater than 14 days, mg/min/mg protein, *p ‹ 0.05).

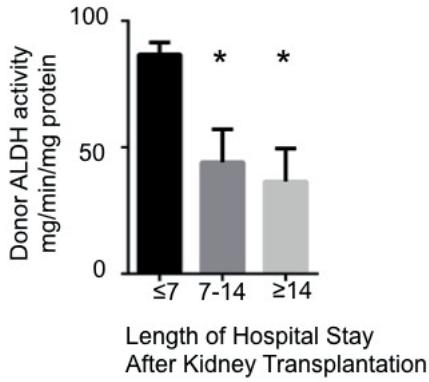


*Figure 6. ALDH activity is associated with length of hospital stay. ALDH enzymatic activity is higher for those with 7 days or less hospital stay (n=6) compared to those patients with a hospital stay between 7 and 14 days (n=5) or greater than 14 days (n=5). *P‹0.05 compared to those with length of stay 7 days or less.*

DISCUSSION

Here we describe an important role for mitochondrial aldehyde dehydrogenase enzymes in the human kidney to metabolize reactive aldehydes produced during organ transplantation. We show an association between altered levels of ALDH enzymatic activity with development of DGF. These initial findings may potentially lead to developing a cellular biomarker based on reactive aldehydes to predict DGF.

Presently, several biomarkers are being investigated for their ability to predict DGF including neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1), interleukin18 (IL-18), klotho, cystatin C and liver type fatty acid binding protein (L-FABP).[20–22] Recently, NGAL blood levels taken from brain-dead kidney donors prior to kidney graft harvesting could not predict the development of DGF.[22] Additionally, the idea of combining several biomarkers to detect DGF was proposed, with a possible triple biomarker approach of malondialdehyde, cystatin C, and serum creatine.[23] Although our results were from kidney biopsies and not measured from circulating blood levels, we suggest here that measuring ALDH enzymatic activity to metabolize reactive aldehydes in addition to 4-HNE levels may potentially be more useful in predicting DGF. Based on the results of our study, showing 4-HNE irreversible adducts occur more in deceased donor kidneys that developed DGF, a panel of reactive aldehydes which include 4-HNE could provide useful information for medical management post-operatively of patients receiving a kidney transplant.

Further, it is also important to determine how genetic polymorphisms of ALDH2 and ALDH7A1 may affect the chance of DGF. In particular, 560 million people in the world have a genetic variant of ALDH2, ALDH2*2, which severely limits the metabolism of reactive aldehydes.[24] This genetic variant is present for those of East Asian descent and after alcohol consumption cause facial flushing and an increased heart rate. Although no study has yet to link an ALDH2*2 variant as a predictor of DGF, this may be due to organ transplantation (and in particular kidney transplantation) numbers are traditionally low in East Asia compared to the rest of the world secondary to cultural reasons.[25] Further, a population of Ashkenazi Jews may also have a decreased ALDH2 enzymatic activity due to a polymorphism in the promoter region of the enzyme.[26] The effects of either the donor or recipient having a genetic polymorphism in ALDH2 will require further study.

Additionally, a genetic polymorphism in ALDH7A1 is considered to have a founder effect in the Dutch, most commonly causing an amino acid substitution

of glutamic acid at amino acid 399 for glutamine.[27] In the severest form, when a person is autosomal recessive for this genetic variant in ALDH7A1, pyridoxine-dependent seizures may occur.[27] However, very little is known regarding whether being heterozygous for the E699Q genetic variant may affect cellular function in times of stress such as that seen for an organ during transplantation. This may be important in the context of kidney transplantation since in experimental models, overexpressing ALDH7A1 enzyme protects from both cellular toxicity and hyperosmotic stress.[17,28] Further studies are needed to determine how genetic variants in ALDH7A1 both in the donor organ and the recipient may influence both cellular injury and DGF in renal transplant. Research regarding whether the use of more specific activators of ALDH enzymes, such as the ALDH2 specific activator Alda-1, may be effective in preventing DGF warrants further study.[7,12]

Our study does have potential limitations that need to be considered when interpreting the data presented. We only focused on mitochondrial associated ALDH enzymes, although ALDH3A2 and ADLH1L1 also had an increased gene expression for living donor kidneys compared to deceased donor kidneys. However, these two enzymes are not known for a role in metabolizing reactive aldehydes in cells. Furthermore, decreased protein expression might be due to loss of cell and mitochondrial integrity. Additionally, it should be considered that donor death itself induces metabolic dysregulation and mitochondrial dysfunction. The study is also an association study and will require further validation both in experimental models and in the clinical realm. However, recent evidence does suggest in rabbits that during machine perfusion and cold storage of rabbit kidneys that higher levels of 4-HNE are produced and is associated with an increase in renal cell apoptosis and reversed by increasing ALDH2 expression.[29]

Together, we suggest that DGF is related to a reduced ALDH enzymatic activity from mitochondrial associated ALDH enzymes ALDH2, ALDH7A1, and ALDH4A1 in human tissue biopsies. This reduced ALDH activity results in an accumulation of 4-HNE-induced protein adducts, leading to cellular changes that are cytotoxic to the cell and are associated with DGF (Figure 7). Improving ALDH activity forms a molecular target in the development of treatment strategies to reduce the incidence of DGF follwing kidney transplantation and preserving ALDH enzymes or increasing ALDH activity may potentially decrease the incidence of DGF by increasing reactive aldehyde metabolism.

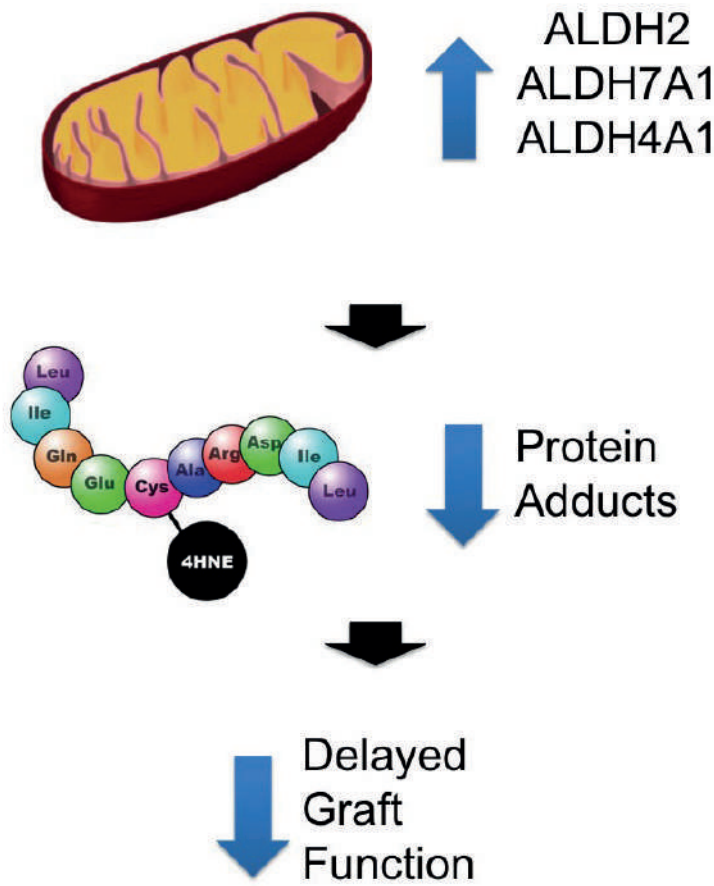
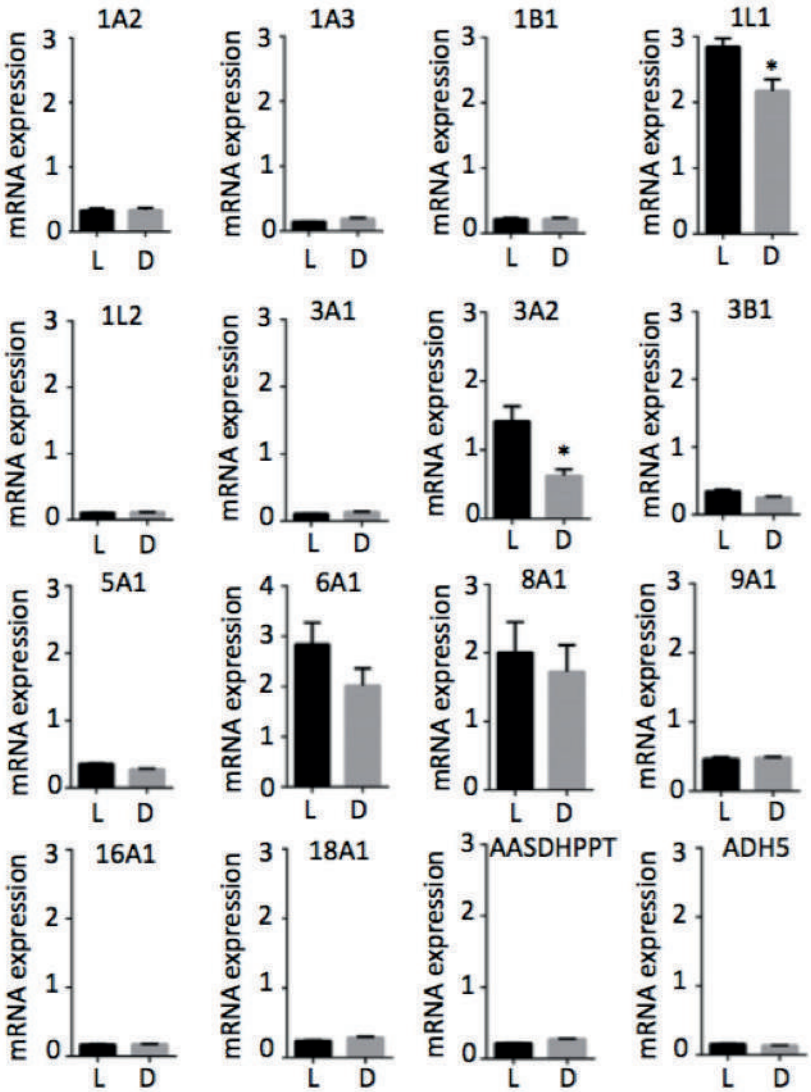
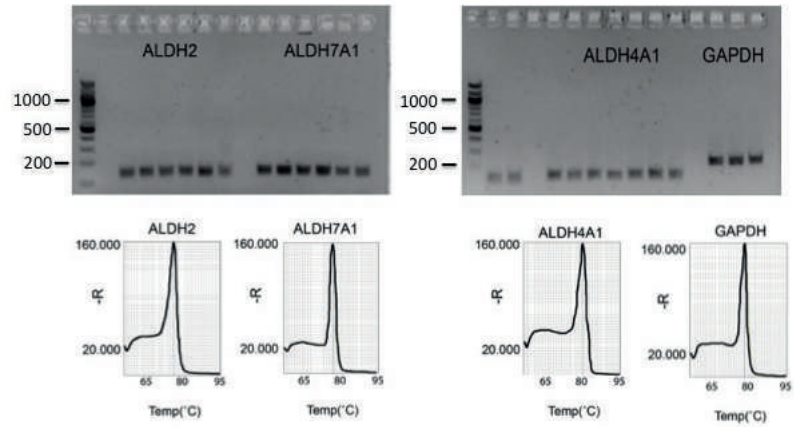


Figure 7. Summary figure. ALDH enzymes are important in clearing toxic reactive aldehydes including 4-HNE in transplanted kidneys. The ALDH enzymes decrease 4-HNE-induced protein adduct formation, and overall reduce the amount of reactive aldehydes that are associated with DGF.

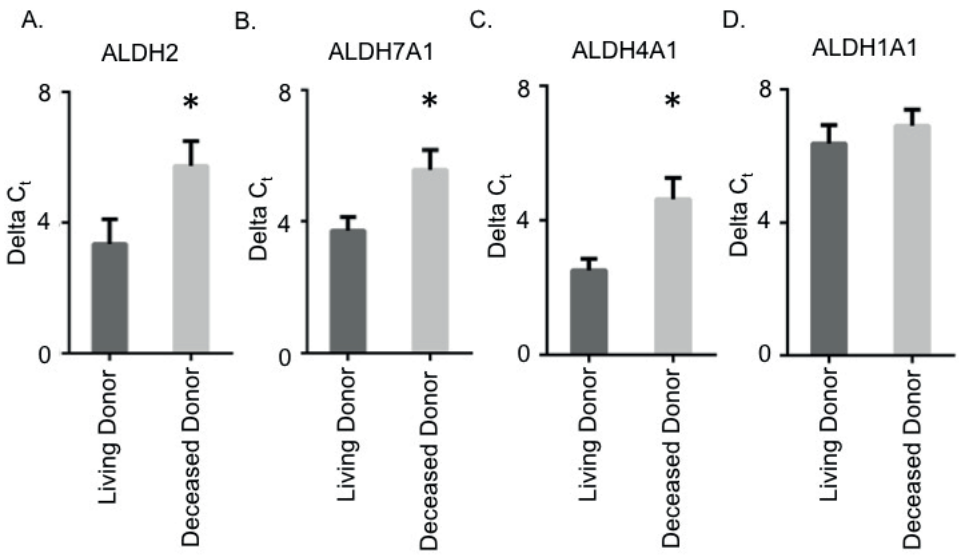
SUPPLEMENTARY FIGURES



*Supplemental Figure 1. ALDH family of enzymes gene array results. 16 additional ALDH enzyme family genes. Of the 16 genes, only ALDH1L1 and ALDH3A2 showed significant differences between the living donor kidneys compared to the deceased donor kidneys. *P<0.01.*



Supplemental Figure 2. qPCR validation studies. Gene products run on DNA gel after qPCR for each primer set used. Further representative melt curve produced for each qPCR product.



*Supplemental Figure 3. Delta Ct values for kidney biopsies prior to transplantation. A. ALDH2 B. ALDH7A1 C. ALDH4A1 D. ALDH1A1. Each qPCR product was compared to the Ct value for GAPDH. Differences in Ct values from ALDH genes compared to GAPDH showed significant differences in delta Ct in living donor kidneys compared to deceased donor kidneys for ALDH2 ALDH7A1 and ALDH4A1 (n=10/group, *P<0.01).*

SUPPLEMENTARY TABLES

Target	NCBI code	Forward Primer	Reverse Primer
ALDH2	NM—000690.3	CCGAGGTCTTCTGCAACCAG	AAGGCCTTGTCCCCTTCAG
ALDH7A1	NM—001182.4	CTTGCCCCCATAGACCACTG	GCACAGATCCGAGTTGGGAA
ALDH4A1	NM—170726.2	GGTCCTTGCTCTCCACGATG	CTGCAGTGATTGATGC-CAACTC
ALDH1A1	NM—000689.4	ATCAAAGAAGCTGCCGGGAA	GCATTGTCCAAGTCGGCATC
GAPDH	NM—002046.5	GAGAAGGCTGGGGCTCATTT	AGTGATGGCATGGACTGTGG

Supplemental Table 1. Primer design. mRNA sequences were obtained from NCBI (http://www.ncbi.nlm.nih.gov/protein), Primer-BLAST was used to develop primers (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Serial cloner program (version 2.6.1 ©Franck Perez [SerialBasics]) was used to construct the primers.

Patient Characteristics for Gene Array Studies

	Living donor kidney graft (n=10)	Deceased donor kidney graft (n=8)
Age recipient (yrs)	49 ± 5	56 ± 4
Sex recipient (% males)	60%	50%
Age donor (yrs)	52 ± 2	49 ± 6
Sex donor (% males)	30%	75%
Ischemia time (min)	213 ± 12	1001 ± 96*
Hospital Stay (days)	8 ± 1	15 ± 2*
Recipient – cause of renal failure		
*Glomerulonefritis *Polycystic kidney disease *DM type 2 *Obstructive uropathy *Maligne hypertension *Renal failure e.c.i.	40% 20% 10% 10% 0% 20%	12.5% 25% 12.5% 0% 0% 50%
Donor cause of death		
*Living donor *CVA *SAB *TRAUMA *CA-OHCA-AMI *Suicide *Miscellaneous	100%	 0% 25% 37.5% 25% 0% 12.5%

Histocompatibility (HLA mismatches, %)		
0	10%	12,5%
1	10%	25%
2	10%	25%
3	20%	25%
4	20%	12.5%
5	20%	0%
6	10%	0%

*Supplemental Table 2. Patient characteristics for gene array studies. A total of 18 patients were included. When comparing recipients of a living donor transplant to recipients of a deceased donor transplant, signi cant differences were noted for developing DGF, duration of ischemia time, and length of post-transplantation hospital stay. *P‹0.01*

Patient Characteristics for Validation Studies

	Living donor kidney graft (n=10)	Deceased donor kidney graft (n=8)
Age recipient (yrs)	58 ± 4	56 ± 4
Sex recipient (% males)	70%	60%
Age donor (yrs)	58 ± 2	57 ± 4
Sex donor (% males)	50%	70%
Ischemia time (min)	221 ± 18	900 ± 88*
Hospital Stay (days)	8 ± 2	15 ± 3*
Recipient – cause of renal failure		
*Glomerulonefritis	40%	30%
*Polycystic kidney disease	20%	20%
*DM type 2	0%	20%
*Obstructive uropathy	10%	10%
*Maligne hypertension	10%	10%
*Renal failure e.c.i.	20%	10%
Donor cause of death		
*Living donor	100%	
*CVA		20%
*SAB		20%
*TRAUMA		20%
*CA-OHCA-AMI		30%
*Suicide		10%
*Miscellaneous		0%

Histocompatibility (HLA mismatches, %)		
0	0%	10%
1	10%	10%
2	0%	30%
3	10%	40%
4	20%	0%
5	40%	10%
6	20%	0%

*Supplemental Table 3. Patient characteristics for validation studies. A total of 20 patients were recruited. When comparing recipients of a living donor transplant to recipients of a deceased donor transplant, signi˜cant differences were noted for developing DGF, duration of ischemia, and length of post-transplantation hospital stay. *P‹0.01*

SUPPLEMENTARY MATERIALS

PATIENT ENROLLMENT AND DEMOGRAPHICS

Fifty-four patients who received a kidney transplant were enrolled to obtain the donor kidney tissue biopsies for this study. Of those, two patients enrolled were excluded due to bleed diathesis. For the gene array portion of the study, six of the patients receiving a deceased donor kidney did not develop DGF and were not included in this study. For the validation portion of the study, eight of the patients receiving a deceased donor kidney did not develop DGF and were not included in this study.

The patient demographics for the gene array study are summarized (Supplemental Table 2). All patients were of European ancestry. For the biopsies analyzed, 10 were from living donors and 8 were from deceased donors that went on to develop DGF. No significant differences in age were identified between patients receiving a kidney from a living donor versus patients receiving a kidney from a deceased donor (49±5 versus 56±4 years). The length of cold ischemic time of the graft was significantly different between living and deceased kidney donors. Moreover, none of the living donor grafts developed DGF. The length of hospital stay for those with a deceased donor transplanted kidney developing DGF doubled. None of the patients developed graft failure required permanent dialysis.

Similar to the first part of our study, no significant differences in patient age or donor age were noted between groups of the validation part of this study. Significant differences in ischemia time between living and deceased donors, in addition to hospital stay were identified. None of the living donor grafts developed DGF. All the biopsies used for this portion of the study were from deceased donor grafts that developed DGF after transplantation (Supplemental Table 3).

PCR PRIMER DESIGN AND VALIDATION

To develop primers to measure gene expression of ALDH2, ALDH4A1, ALDH7A1 and ALDH1A1 in human biopsies, we initially designed and optimized primers using human embryonic kidney cells (HEK293). Primer sequences are provided (Supplemental Table 1). Optimization of the primers involved both analysis of the melt curve in addition to running a DNA gel to confirm the proper size and single PCR product was formed from the primers designed (Supplemental Figure 2).

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IX

Short vs. prolonged statin therapy differentially affects the myocardial response to injury after cardiac surgery:

The PreOperative STatin InterVEntion trial

Article under peer review

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R.J.M. Klautz, Jan H.N. Lindeman

ABSTRACT

Background: Preclinical studies suggest that acute administration of statins is superior to continued statin therapy in repressing the inflammatory response upon tissue injury. Human studies are scarce and results are controversial. Aim of this study is to determine whether acute preoperative treatment with simvastatin is more effective than prolonged preoperative treatment in mitigating the inflammatory response following on-pump cardiac surgery.
Methods: Patients scheduled for isolated valve surgery, not being on a statin regimen, were included (n=12) and randomized into acute (40 mg simvastatin 12 hours and 2 hours before surgery) or prolonged treatment regimen (40 mg simvastatin for at least two weeks preoperatively). Sequential arteriovenous sampling over the heart was performed to selectively assess the myocardial response. Plasma cyto/chemokines were assessed in a multiplex platform, and CRP, troponin and CPK levels in a certified clinical chemistry lab. The study was terminated prematurely after interim analysis indicated futility.
Results: The different treatment strategies did not affect the myocardial inflammatory and systemic post-operative response as no differences were found for any of the 30 cyto/chemokines in the multiplex platform, nor in the postoperative CRP response. However, prolonged statin treatment was associated with reduced levels of myocardial damage markers (troponin T ($P<0.027$) and CPK ($P<0.037$)).
Conclusion: This study does not confirm experimental evidence that acute statin therapy is superior to prolonged therapy in mitigating the inflammatory response following on-pump cardiac surgery. Unexpectedly, both myocardial damage markers troponin T and CPK were reduced in the prolonged treatment group.

INTRODUCTION

Cardiac surgery using a cardiopulmonary bypass (CPB) machine elicits an inflammatory response, leading to endothelial damage, free radical production, complement and thrombocyte activation, and cytokine release.[1-3] These effects are thought to negatively influence surgical outcome. Besides their cholesterol-lowering function, statins are known to exert potent anti-inflammatory effects.[4] As a consequence it is hypothesized that via these pleiotropic, anti-inflammatory actions, statins mitigate the inflammatory response caused by CPB.
Indeed, preclinical studies show promising results of peri-operative statin therapy in repressing the inflammatory response and limiting the infarct size.[4-6] However, data from clinical studies are less outspoken. Although this may reflect differences between experimental models and clinical reality, it is known that the anti-inflammatory potential of statins is most outspoken during acute treatment [7,8], and that the effect (partially) wanes during continued treatment.[9] Clinical studies so far, all report the effect of prolonged statin therapy on surgery outcomes[10,11] or do not differentiate between acute and continued treatment.[12]
Considering the preclinical observations that the anti-inflammatory potential of acute statin therapy is superior to maintenance therapy we hypothesized that short-term preoperative statin treatment is superior to continued treatment in mitigating the inflammatory response after on-pump cardiac surgery. The latter was tested in this exploratory randomized trial.

MATERIALS & METHODS

PATIENT POPULATION

The local medical ethics committee of the Leiden University Medical Center approved the study protocol. Patients received written and oral information before giving their informed consent. In order to achieve a homogenous study population, this study only included patients scheduled for isolated mitral valve surgery (or in combination with tricuspid valve surgery). Patients undergoing minimal invasive procedures or ablation for arrhythmia, patients with manifest atherosclerosis and those receiving treatment with statins were excluded. Three weeks before surgery, patients were randomized into the short or prolonged treatment group. This trial was registered at the 'Nederlands trial register', NTR2673.
The trial was terminated prematurely as interim analysis performed after inclusion of 12 patients indicated futility.

INTERVENTION

In the prolonged group 40 mg simvastatin (oral intake daily) was administrated at least for 14 days prior to surgery. The short-term treatment group received one dose the evening before and a second dose the morning before surgery. We have previously shown the anti-inflammatory potential of 40 mg simvastatin.[13] The dose of statins used in this study corresponds to doses used in everyday practice.

DATA COLLECTION

The details of anesthesia and surgical procedures, as well as plasma measurements and handling, are similar to that described in detail in our previous article.[3] In summary, systemic blood samples were obtained at the outpatient clinic prior to start of the statin intervention and the day before surgery from a vein, mostly antecubital. To selectively measure the myocardial response to injury we used the technique of arterial-venous concentration differences. To be more specific, sequential paired arterial (radial artery) and myocardial venous blood samples (coronary sinus) were collected simultaneously over the reperfused heart at 0, 30, 60 min, and 2, 6 and 24 hours after reperfusion (i.e. removing the aortic cross-clamp). Plasma preparation was performed as described earlier.[3]
A multiplex assay platform (X-plex, Biorad, Veenendaal, the Netherlands) was used to simultaneously measure selected cytokines and chemokines (TNF-a, IL-1B, IL-1ra, IL-2, IL-4, IL-5,, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-16, IL-17 and IL-18; IFN-γ, IP-10, MCP-1 (CCL-1), MIP-1α (CCL-3), MIP-1β (CCL-4) , RANTES (CCL-5), Eotaxin (CCL-11), G-CSF, GM-CSF, bFGF, PDGF-bb, and VEGF-A). We have previously shown that in the context of cardiac surgery plasma levels of these

factors exceed the sensitivity of the assay.
Peripheral Troponin T and CPK levels (reference values ‹0.050 µg/L and ‹145 U/L, respectively) were measured systemically before surgery (baseline), directly after surgery, and 6 and 12 hours after surgery in our certified clinical chemistry lab. Peripheral CRP levels (reference value ‹10 mg/L) were measured before surgery (baseline), directly after surgery, and 24 and 48 hours after surgery in our certified clinical chemistry lab.

STATISTICAL ANALYSIS

SPSS 22.0 (SPSS Inc, Chicago, III) was used for statistical analysis. Patient characteristics were analyzed by a t-test and expressed as mean ± SEM. For the arteriovenous plasma measurements, the area under the curve (AUC) was estimated, and compared between the treatment groups through a linear mixed model analysis for the total timeslot of measurements. 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 between groups was calculated and the null hypothesis (AUC = 0) was tested by a Wald-test based on the estimated parameters of the linear mixed model. P-values ‹0.05 were considered significant.

RESULTS

STUDY POPULATION

Between January 2011 and October 2014, twelve patients were included in this single center study. In total, twenty-eight patients were approached. Ten patients refused to participate (refused study medication). Two patients were withdrawn from the study because of noncompliance since they did not take their medication, and for one patient statin therapy was started by the attending physician. Additionally, three patients were excluded because arteriovenous sampling failed due to dysfunction of the coronary sinus catheter (see Patient flow chart in Sup. Fig.1). Baseline characteristics of the included patients were similar (Table 1). None of the patients died during the study period.

Table 1. Patient characteristics

	Short treatment (n=6)	Prolonged treatment (n=6)	P-value
Age (years)	62.7 ± 2.8	68.0 ± 3.8	0.29
Gender (no. of males)	4	4	1.00
Body mass index (kg/m2)	25.5 ± 1.0	27.1 ± 1.3	0.35
Medical history (no.) ·Hypertension ·Diabetes mellitus ·Chronic kidney disease	 2 0 0	 2 0 0	
Current medication (no.) ·Beta-blocker ·Insulin ·Antiplatelet or anticoagulant ·Calcium-channel blocker ·ACE inhibitor	 4 0 1 0 3	 3 0 2 1 3	
Scheduled surgery (no.) ·Mitral valve surgery ·Mitral and tricuspid valve surgery	 4 2	 3 3	
Days of preoperative statin treatmen	2 ± 0.0	23 ± 2.7	‹0.005

Table 1. Patient characteristics showing no significant differences between the groups except for days of preoperative statin treatment, as analyzed by a t-test expressed as mean ± SEM.

INFLAMMATORY RESPONSE

Systemic (arterial) and myocardial (arteriovenous differences) inflammatory responses in the short and prolonged treatment groups were similar. In fact, no differences were found for any of the cyto/chemokines tested. The dynamics of systemic and myocardial responses for the pro-inflammatory cytokines IL-6 and MCP-1 are illustrated in Figure 1A. The dynamics of all other cyto/chemokines are shown in Sup. Fig. 2. Similarly, no differences were found for plasma CRP levels at 24 and 48 hours after cardiac surgery (resp. P=0.29 and P=0.56; Figure 1A).

MYOCARDIAL DAMAGE

Differences were found for the biochemical damage markers troponin T and CPK. Figure 1B shows similar baseline values for the two patient groups but significantly higher Troponin T and CPK levels in the 12 hours following surgery in the short-term treatment group (differences in AUC for troponin T: P=0.004 and CPK: P=0.0003).

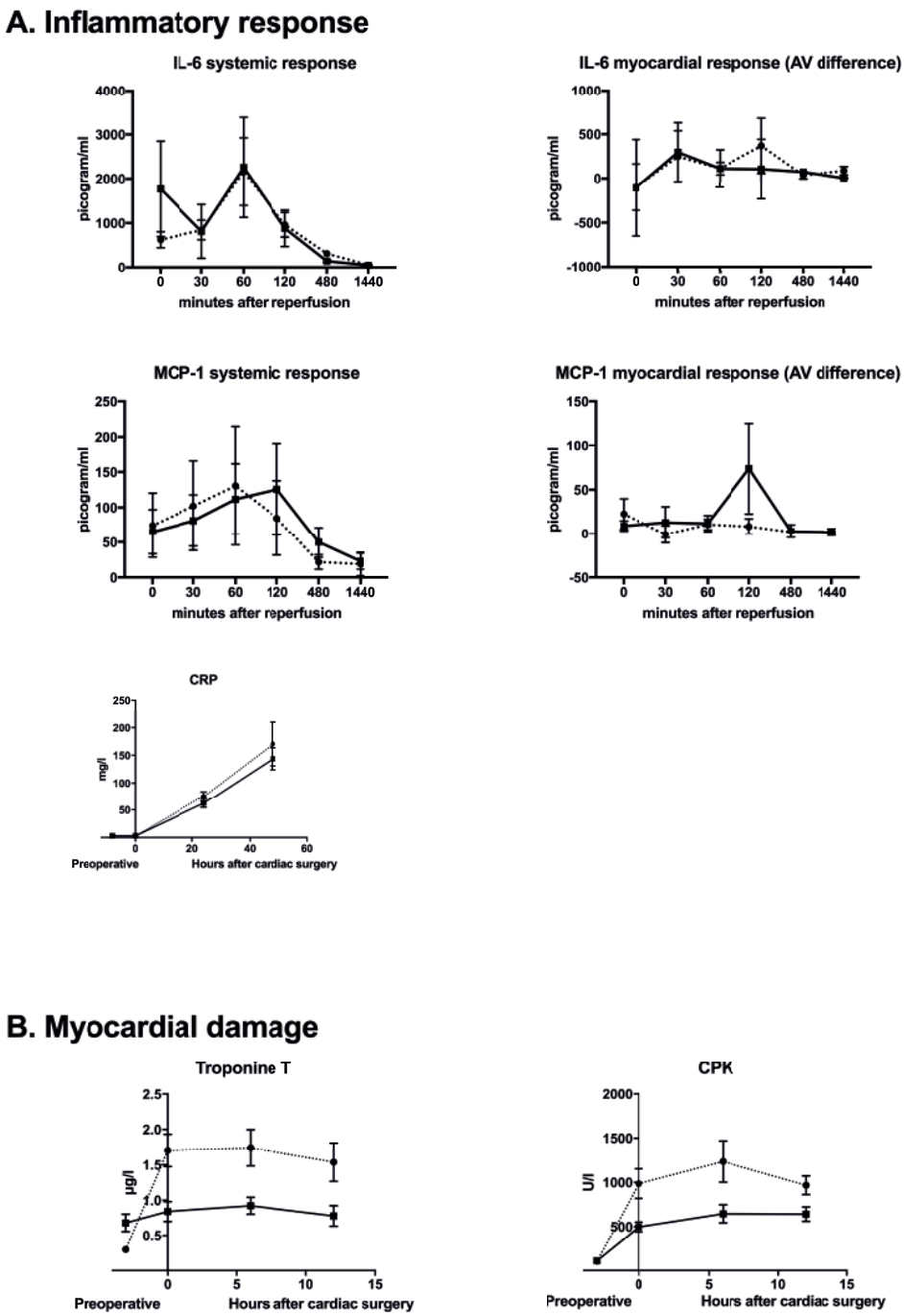


Fig. 1A. Pro-inflammatory cytokines (IL-6 MCP-1) and CRP levels. Levels of pro-inflammatory cytokines did not differ between treatment groups (IL-6: P=0.79, MCP-1: P=0.83) by comparing area under the curve.

Nor did the myocardial release, measured by arteriovenous differences over the reperfused heart (IL-6: P=0.53, MCP-1: P=0.50; Sup. Fig.1 provides myocardial release of the other measured cytokines). Systemic levels of CRP did not differ between the two treatment groups (P=0.11).
Legend: acute treatment group: dashed line, prolonged treatment group: solid line.

Fig. 1B. Levels of myocardial damage markers perioperatively.
Lower troponin T and CPK levels were observed in patients that received simvastatin 40 mg daily at least two weeks prior to surgery at 0, 6 and 12 hours postoperatively as compared to acute administration (red. day before and day of cardiac surgery). Troponin T: P=0.004; CPK: P=0.0003 (area under the curve was compared between the two treatment groups).
Legend: acute treatment group: dashed line, prolonged treatment group: solid line.

DISCUSSION

Results from this exploratory trial performed during planned myocardial ischemia/reperfusion do not confirm preclinical conclusions that acute preoperative statin treatment is superior to continued treatment in suppressing the post-reperfusion inflammatory response.

Preclinical studies convincingly show beneficial effects of statin therapy in limiting tissue damage following myocardial ischemia/reperfusion.[14-16] Positive effects are thought to be mediated by limiting the post-reperfusion response; an effect that is thought to involve increased endothelial NO production via activation of the PI3K-Akt-eNOS pathway.[4,17,18] Yet, clinical studies all fail to confirm these promising preclinical observations. This discrepancy between preclinical models and clinical reality may reflect interspecies differences and/or differences between the model context and real life ischemia/reperfusion. However, it may also relate to the fact that clinical studies and clinical evaluations are performed during continued statin therapy. Experimental data show that aspects of the statin anti-inflammatory potential fade during prolonged therapy, an effect attributed to weaning (desensitization) of the responses as result of metabolic adaptations and compensatory mechanisms.[5]
On this basis it was hypothesized that the negative conclusions from clinical trials may reflect the weaning phenomenon of continued statin therapy. It was therefore decided to perform an exploratory trial in patients with planned ischemia/reperfusion (on-pump valve surgery with cross-clamping of the aorta). As of unanticipated slow inclusion rate (mainly due to patients' unwillingness to participate because of concerns on side effects of statins or polypharmacy), it was decided on an interim analysis to test for futility. Conclusions obtained met all requirements for futility: results show parallel responses for *all* inflammatory mediators tested, and indicated suppressed post-operative troponin and CPK levels responses in the prolonged statin therapy group. Consequently the data fully refute the study hypothesis, and the decision was made to terminate the trial for reasons of futility.

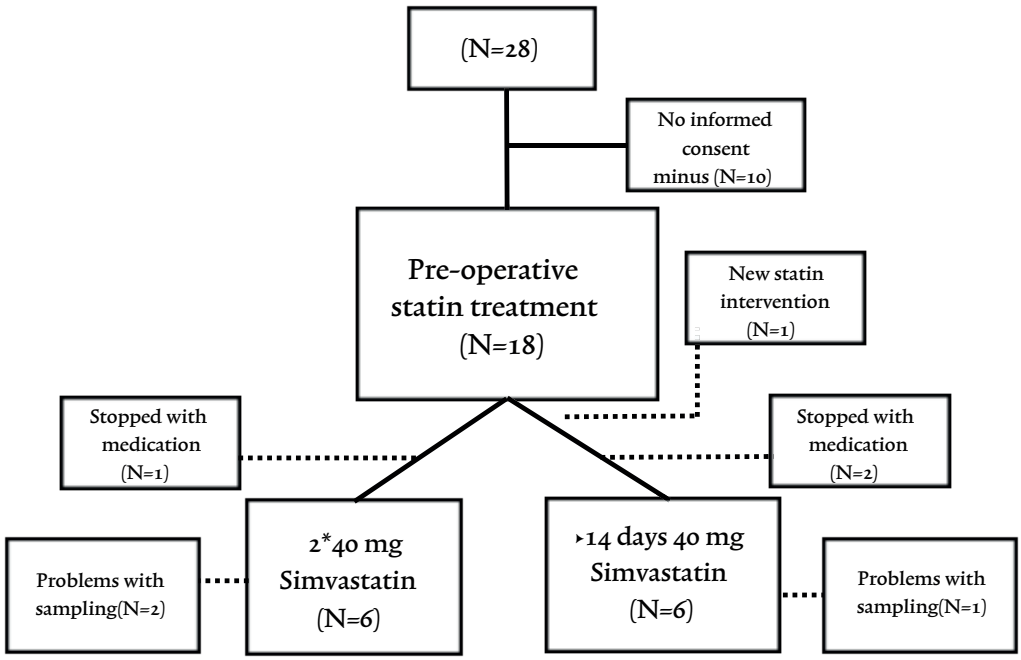
Although the findings from small studies are sensitive to a type II statistical error, we consider the observation of superior post-operative suppression of myocardial damage markers in the prolonged statin therapy realistic. Not only was the effect isolated, and observed for both, unrelated damage markers tested; the observations also fit in the reported beneficial effects of statin treatment on outcome (early mortality and duration of ICU/hospital admission) after cardiac surgery.[19,20]
A notable observation from this trial is that this beneficial effect appears

independent of the myocardial inflammatory response.

Moreover, the purpose of our trial was to test the hypothesis that acute statin therapy is superior to prolonged therapy, hence a reference group not receiving statin therapy is missing. As such we cannot quantify the impact of statin therapy per se on the post-reperfusion inflammatory response. Yet, the anti-inflammatory potential of statins has been firmly established in other contexts.[13] Moreover, on basis of this study it cannot be excluded that the apparent superiority of long-term statin therapy over acute statin therapy reflects a negative impact of the acute statin exposure. Finally, this trial has been performed with a hydrophilic statin (simvastatin). Since differences in cardio-protective effects have been reported between hydrophilic and lipophilic statins[21] results from this trial may not, or to a different extent, apply to lipophilic class of statins.

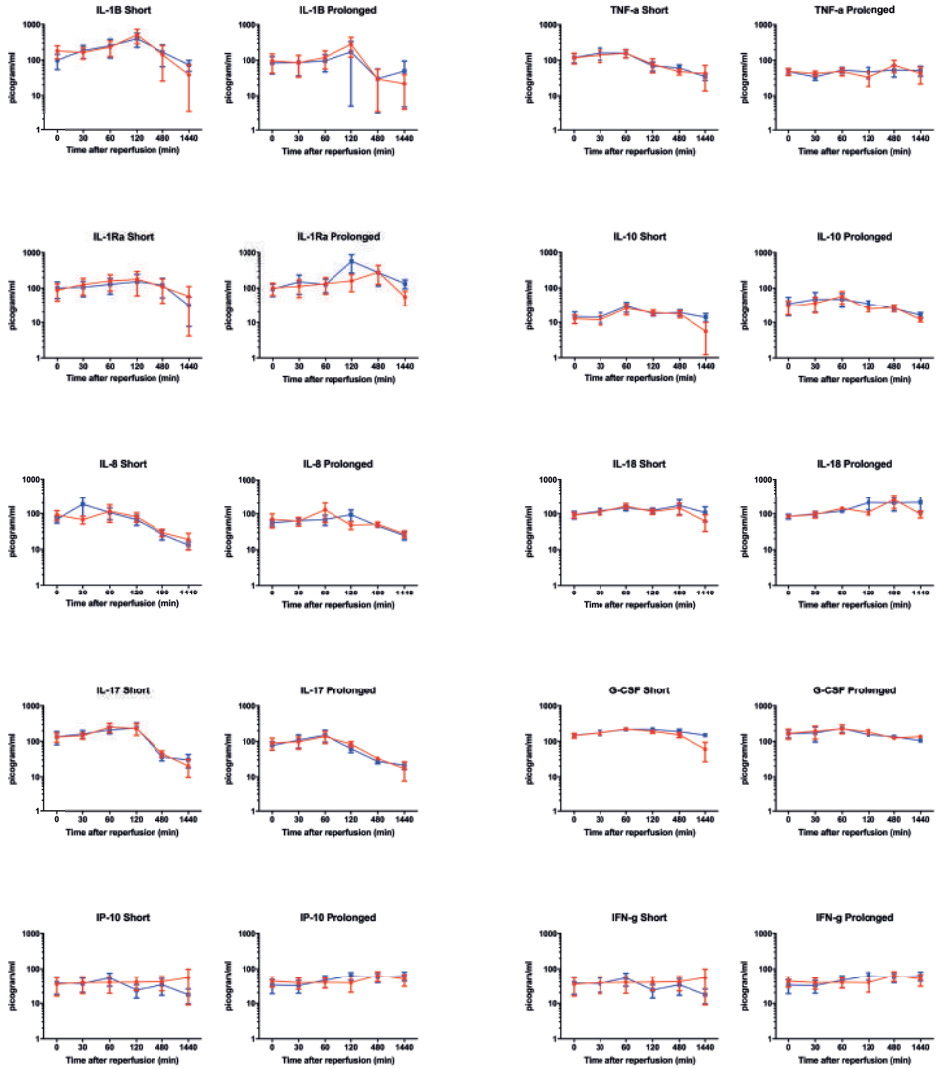
In summary, this trial does not support the hypothesis that acute statin therapy, by virtue of (partial) weaning of the anti-inflammatory potential during prolonged therapy, is superior to continued treatment in alleviating post-reperfusion inflammatory responses. On the contrary, indications were found for superior myocardial protection for prolonged statin therapy.

SUPPLEMENTARY FIGURES



Supplemental Figure 1. Flow-chart of our patient inclusion

Myocardial cytokine release



Supplemental Figure 2. Myocardial release of cytokines
Release (arteriovenous difference) of pro-inflammatory cyto/chemokines by the reperfused myocardium was absent. No differences were found between acute and prolonged treatment groups.
Legend: Red lines resemble arterial levels, blue lines venous levels (mean (SEM)). Left columns: acute treatment group; Right columns: prolonged treatment group.

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Donor pretreatment in clinical kidney transplantation: a critical appraisal

Clinical Transplantation

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ABSTRACT

Kidney transplantation represents one of the medical achievements of the 20th century. However, its continued success is limited by the increasing shortage of donor grafts. As a result more kidney grafts from marginal donors are being considered for transplantation, with concomitantly more initial graft injury and limited organ and patient survival. This has led to an increased need for interventions aiming to optimize and preserve graft quality. Interventions within the donor may protect against ischemia/reperfusion injury and therefore donor pretreatment is a promising strategy to increase graft function and survival. During the last decade, diverse donor pretreatment interventions have been explored in animal studies. Moreover, the first human trials concerning donor pretreatment in kidney transplantation have provided encouraging results. Unfortunately, it remains difficult to determine how and where to intervene in the multifactorial and complex processes that affect the donor kidney. Moreover, ethical matters play a critical role in donor interventions, and pretreatment should principally not have any potentially unfavorable effects on other organs to be transplanted or on the living donor. This review provides an overview of promising therapeutical strategies for donor pretreatment in kidney transplantation and discusses the clinical trials that have been conducted thus far.

INTRODUCTION

Kidney transplantation is the preferred treatment of patients with end-stage renal disease.[1-3] The previous decade is characterized by a steady increase in the number of kidney transplantations. This increase largely reflects improved medical therapy for renal failure. Besides the fact that more patients are being considered eligible for kidney transplantation, improved survival after transplantation led to the emergence of patients requiring re-transplantation, due to progressive loss of graft function in the long run.[4-6] The augmented demands for kidney transplantation resulted in organ shortage and as a consequence, waiting lists for kidney transplantation are ever increasing. In June 2012 more than 99 000 American citizens were on the waiting list for a kidney transplant.[7-9]

Kidney grafts can be derived from living or deceased donors. Of deceased donors, more organs are recovered from donation after brain death than from donation after cardiac death. However, the current organ shortage necessitates expansion of the donor pool by the increased use of marginal donor kidneys. Since these marginal kidneys have worse long-term outcome, it has been proposed that they may benefit most from pre-transplantation interventions that preserve or even improve graft quality.[10]

Before and during the process of transplantation the graft is exposed to various noxious events, including donor brain death, cold preservation and ischemia/reperfusion (I/R) injury, all potentially contributing to the functional deterioration of the graft. The importance of these harmful mechanisms is illustrated by the superior results of living donor transplantation. Despite generally more accepted HLA mismatches, living donor transplantation is associated with minimal delayed graft function and improved long-term outcomes. This observation suggests that non-HLA-specific factors such as donor health and duration of the ischemic period before transplantation have substantial impact on short and long-term graft function.[11-13]

Consequently, interventions in the donor, aimed at minimizing pre-transplantation graft injury, may potentially have large effects in preventing acute and long-term graft dysfunction. This review will focus on the prevention of harmful processes that initiate graft damage in the donor. Various intervention strategies for donor pretreatment that have been tested in clinical kidney transplantation or animal experiments involving kidney transplantation will be discussed.

PROCESSES IN BRAIN DEATH

To date, the majority of deceased kidney grafts are derived from donation after brain death. Unfortunately, brain dead donor kidneys have a worse graft and patient survival as compared to living donor kidneys. Brain death leads to dysregulation of the autonomic nerve system, inducing many pathophysiological processes in the human body. Brain death is usually provoked by a period of increased intracranial pressure exceeding the mean arterial pressure and thereby blocking brain perfusion. The physiological responses to this increased pressure and brain damage can have effects on multiple organ systems. The most prevalent derangements are cardiovascular. With increasing intracranial pressure, a compensatory arterial hypertension is induced, sometimes with bradycardia. Next, the catecholamine storm sets in, with sympathetic stimulation, vasoconstriction, raised systemic vascular resistance, and tachycardia. After the catecholamine storm, there is a loss of sympathetic tone and peripheral vasodilatation. So, brain death results in severe hemodynamic instability and the resulting hypotension, if untreated, leads to hypoperfusion of all organs. This phase is well-known for the damage it can inflict in organs to be transplanted.

Other common clinical problems associated with donor brain death may include diabetes insipidus, disseminated intravascular coagulation, arrhythmias and pulmonary edema. Injury to the hypothalamus and the pituitary gland causes disturbances of hormonal homeostasis and thermoregulation.

On the microvascular level, brain death is associated with the induction of adhesion molecule expression and endothelial cell activation.[14] The hemodynamic, neurogenic, hormonal and microvascular disturbances lead to a generalized inflammatory response in the donor. This is characterized by the release of cytokines into the circulation which can trigger an inflammatory response in all organs[15,16] with tissue infiltration by granulocytes,[16] monocytes and lymphocytes.[17] All these physiological derangements should be limited as far as possible to maintain optimal graft condition before donation. Donor management is the primary approach to do so.

DONOR MANAGEMENT

After the diagnosis of brain death, there is a change from curative patient care to optimizing organ function for subsequent transplantation. This donor management is the active care of the donor from the time of diagnosis of brain death until procurement of organs, and involves correction of the widespread physiological changes that occur during brain death. If possible, early recognition of the potential organ donor and aggressive correction of the non-physiological state, even before consent to organ donation, are crucial to optimize post-transplantation graft function.

In order to standardize management, donor goals have been developed. These aim to maintain physiology close to normal values and were based on measurements performed routinely in patients in the intensive care unit. They include objectives to maintain body temperature, ensure adequate oxygenation, circulating volume, cardiovascular stability, and adequate urine output. Indeed, in a prospective study, the application of a standardized donor management protocol increased the number of retrieved and transplanted organs per donor substantially.[18]

One part of donor management is providing cardiovascular support. This support principally includes stabilization of hemodynamics in the donor. Treatment of hypertension associated with the catecholamine storm may significantly increase available grafts for transplantation.[19] In the consecutive hypotensive period, the first priority is to maintain an adequate intravascular volume. Fluid therapy should however, be carefully titrated. Recent studies recommend restrictive fluid management, since this restriction increases the number of transplantable lungs without influencing kidney graft function or survival after transplantation.[20, 21]

In stabilizing the donor, hormone replacement can aid by correcting the loss of pituitary function after brain death. Posterior pituitary function is very commonly lost, leading to diabetes insipidus with associated fluid and electrolyte changes. Anterior pituitary function may be preserved or only partially affected. Most hormone replacement therapies use a combination of methylprednisolone, vasopressin and thyroid hormone In a retrospective study, the fraction of donors that received replacement therapy with these three hormones, had an increase in the number of procured organs by 22,5%.[22]

Aggressive donor management increases the number of organs available for transplantation and has minimized loss of potential donors due to cardiovascular collapse in the process of brain death. However, most studies deny a major effect on graft quality and survival of donor management. Therefore, grafts may benefit from additional interventions that more specifically prevent organ damage before procurement. This donor pretreatment aiming to further maximize organ quality is an evolving field that constitutes the next step in optimizing kidney graft survival.

DONOR PRETREATMENT

Donor pretreatment is the active treatment of the donor in order to improve organ quality before and after transplantation. It distinguishes itself from donor management by the fact that donor management concentrates on stabilizing the donor to normal physiological ranges, while pretreatment is aiming to inhibit potentially harmful processes. Many therapies used as donor pretreatment have been investigated for their ability to reduce or prevent renal I/R injury in

animal experiments. Few interventions have been studied in clinical trials, which are summarized in Table 1. Here we discuss the most frequently applied and promising approaches to donor pretreatment.

Table 1. Human clinical trials of donor pre-treatment and result on outcome in kidney transplantation

Intervention	*Design*	*n*	*Main result*	*References*
Dopamine	Single-blind RCT	264 brain dead donors	Dopamine pre-treatment decreased the incidence of dialysis post-transplantation. No change in acute rejection or patient or graft survival after three yr	Schnuelle et al.[42]
Steroids	Double-blind RCT	306 brain dead donors	Donor pre-treatment with corticosteroids did not reduce the incidence or duration of DGF	Kainz et al.[60]
PUVA	Non-randomized	59 deceaeddonors	PUVA pre-treated grafts had a signi˜cantly lower number of rejection episodes, other outcome parameters were not different	Oesterwitz et al.[70]
Hyperoxia	Double-blind RCT	60 living donors	Donor oxygen pre-treatment the day before transplantation improved kidney function at 10 d after transplantation	Montazeri et al.[79]

RCT, randomized controlled trial; DGF, delayed graft function; PUVA, psoralen plus ultraviolet A.

Ischemic preconditioning

Over the past decades several studies were performed, exposing an organ to brief periods of ischemia to protect against subsequent periods of ischemia and reperfusion. This phenomenon of ischemic preconditioning has been first described in 1986 in the heart.[23] Since then, many animal studies, mainly in rats, have reported beneficial effects of donor ischemic preconditioning before kidney transplantation.[24–27] Our present understanding of the molecular mechanisms causing these effects is still largely incomplete. Experimental studies have shown that the protective effects of renal ischemic preconditioning are mediated by adenosine[28], nitric oxide[29, 30] and subsequent activation of signalling networks involving protein kinases and transcription factors. Recently, more complex mechanisms have been proposed as well, including cellular actions of regulatory T cells and endothelial progenitor cells.[31, 32] It is generally acknowledged that the

mechanism of ischemic preconditioning may differ between species and organs and it still remains controversial whether ischemic preconditioning is beneficial in large animals as well. In fact, studies on kidney transplantation in dogs and renal I/R injury in pigs both failed to confirm beneficial effects of renal ischemic preconditioning and although the first description of ischemic preconditioning dates from almost thirty years ago, the technique has not yet been translated into the clinical setting.[33, 34]

In more recent studies, remote ischemic preconditioning conferred protection to I/R injury by preceding ischemia and reperfusion of another organ or tissue. In animal experiments, the donor kidney can be protected after transplantation by remote ischemic preconditioning of the hindlimb.[35] Remote ischemic preconditioning suggests the involvement of humoral mediators and consequently protection is both dialyzable, transferable, and receptor-mediated.[36] Remote ischemic preconditioning has the advantage that it is more easily applicable in clinical transplantation than ischemic preconditioning of the graft itself. At present, clinical studies in which remote ischemic preconditioning of the lower limb is explored to improve outcome of kidney transplantation have been initiated and patients are being recruited (www.clinicaltrials.gov).

Catecholamines

Before transplantation, the graft is exposed to harmful periods of warm and cold ischemia. Dopamine is capable of protecting endothelial cells from damage during cold preservation by inducing protective enzymes, such as heme oxygenase-1 (HO-1).[37, 38] Dopamine could therefore be a promising donor pretreatment, rendering the kidney graft more resistant to I/R injury. In a rat allogeneic kidney transplantation model it was shown that donor dopamine pretreatment diminished histological damage, monocyte infiltration and cytokine expression in the kidney graft. Moreover, both short- and long-term graft function significantly improved.[39, 40] Other catecholamines, like dobutamine and norepinefrin, did not influence post transplantation kidney function.[41]

This preclinical experimental evidence resulted in a clinical trial of donor dopamine pretreatment.[42] Almost 300 brain dead donors were randomized to receive low dose dopamine pretreatment or placebo. Donors had to be stable on low dose noradrenalin and dopamine was continuously infused at a standard rate. The main outcome measure, need for dialysis during the first week after transplantation, was significantly reduced in recipients of a dopamine pretreated graft. However, dopamine pretreatment did not affect graft or patient survival. Donors in the dopamine group showed a significant but clinically not relevant increase in systolic blood pressure. In addition, effects of dopamine pretreatment were more pronounced with increasing cold ischemia time, supporting the

hypothesis that the beneficial effects of dopamine are mediated by its protective effects on the endothelium. Moreover, dopamine pretreatment may therefore even have the largest effects in marginal donors.[42]

Heme oxygenase-1

The ischemic period before transplantation can induce oxidative stress, which on its turn induces the release of the cytoprotective enzyme HO-1. Both HO-1 and carbon monoxide (CO), a product of HO-1 metabolism, are potential candidates for donor pretreatment. Induced expression of HO-1 in rat kidney donors led to decreased cell infiltration, downregulation of inflammatory genes and diminished histological signs of chronic rejection, resulting in increased graft function and survival after transplantation.[43]

The effects of HO-1 may be mediated by its downstream CO production, since induction of CO in the donor improved graft function similarly to HO-1, and both HO-1 and CO were able to diminish donor immunogenicity.[44-47] However, cellular mechanisms may also be involved in the protective effect of HO-1. HO-1 induction in the donor decreased early post-transplant alloreactivity, donor-derived dendritic cells, and T-cell reactivity in the recipient.[48] Moreover, donor pre-treatment with HO-1 has been shown to improve microcirculation after transplantation.[49] Ultimately, both HO-1 and CO appear promising opportunities for donor pretreatment. Whether their application is feasible and beneficial in clinical practice remains to be seen.

Anti-complement therapy

The inflammatory storm in the process of brain death may induce complement activation in the donor, thereby causing damage in the kidney graft before transplantation.[50] Inhibition of complement activation in the donor could therefore be beneficial for the kidney graft. The complement system is part of the innate immune system and it can be activated through the classical, alternative and lectin pathway. Soluble complement receptor 1 (sCR1) acts as an inhibitor of the common part of all three complement pathways. Pretreatment of donor rats with sCR1 around induction of brain death prevented the increase in circulating C3d and significantly improved renal function immediately after transplantation.[51] These first encouraging results should be confirmed in further animal experiments applying other complement inhibitors, before translation to clinical application can be made.

Erythropoietin

Erythropoietin (EPO) was originally identified for its role in erythropoiesis, but is now known for its anti-apoptotic and cytoprotective effects as well. These

protective effects are mediated by different receptors and mechanisms than the ones regulating the hematopoietic effects and may defend the kidney from I/R injury, potentially even when administered to the donor. This is particularly interesting considering the conceivably unfavorable side effects of systemic EPO treatment to the recipient.
Two very recent animal studies addressed donor pretreatment with EPO.[52, 53] In a rat model, brain dead donors were pretreated with EPO or carbamylated EPO (cEPO), which lacks the hematopoietic effects of EPO. Although kidneys were not actually transplanted, short term graft function was analyzed in an isolated perfused kidney set-up. Both EPO and cEPO diminished the influx of polymorphonuclear cells into the kidney and grafts showed a normalization of creatinine clearance in this isolated perfused kidney model.[53] In a study with larger animals, involving porcine kidney transplantation, cardiac dead donors were pretreated with EPO. In as little as 4 hours after reperfusion, renal injury and inflammation decreased and renal function improved in the EPO pretreated group.[52]

Immunosuppressive and anti-inflammatory agents
The period of donor brain death is well known for causing a systemic, non-specific inflammatory reaction, and organs from brain dead donors are influenced by this inflammatory state.[17, 54-57] Moreover, the ongoing inflammatory reaction after transplantation is responsible for reperfusion induced tissue damage.[58]
When translating findings of animal experiments into human therapies, the general immunosuppressive effects of corticosteroids could be suitable to suppress the inflammatory response in brain dead donors. Methylprednisolone is frequently administered in donor management as part of hormone replacement therapy, but may also be administrated at a higher dose than normally given for replacement therapy, in order to suppress inflammatory response. Recently, limited data from a large randomized, blind, placebo-controlled trial on donor steroid pre-treatment became available. The results show that expression of pro-inflammatory genes in brain dead donors normalized after steroid pretreatment. Although the incidence and duration of delayed graft function did not change with steroid pretreatment, the follow-up period was fairly short and information on longer term graft function is not available as yet.[59, 60, 60] Nevertheless, reducing the pro-inflammatory storm after brain death remains a promising approach, as illustrated by the improved kidney graft survival in rats after donor pretreatment with JNK signal transduction inhibition.[61]
Preconditioning of rat donors with calcineurin inhibitors cyclosporine A or tacrolimus decreased structural damage and resulted in improved graft function after kidney transplantation.[62] Moreover, pretreatment with tacrolimus combined

with rapamycin even improved outcome synergistically.[63] Not all studies could confirm these results, potentially explained by gross differences in experimental set-up and dosing of immunosuppressives between studies.[64]
The basic mechanism behind immunosuppressive donor pretreatment remains unknown. The responsible mechanism appears not to be, as expected, an additional inhibition of the alloimmune response. An effect on renal I/R injury is more likely, since the protective effects have been observed in syngeneic kidney transplant models and appear related to acute renal stress.[65]

Other pretreatment therapies
Photosensitizer + UVA (PUVA) treatment was applied in a series of studies nearly 30 years ago. Donor rats were pretreated with a photosensitizer and during hypothermic storage the kidney was irradiated with UVA. PUVA pretreatment improved graft survival in rats, with a dose-response relationship of the irradiation period.[66] The positive effect is ascribed to decreased graft immunogenicity.[67-69] To validate these results, a clinical study was performed in 1986 showing that PUVA pretreated grafts had a significantly lower number of rejection episodes, although all other outcome parameters were not different.[70] Altogether, PUVA pretreatment showed some successes but since then results have never been validated or reproduced by others.
Diverse rodent studies report on donor pretreatment with various substances with antioxidant capacities. Amongst others N-acetylcysteine (NAC), melatonin, danshen, and taurine were applied in animal kidney donors. Most studies showed improvement in biochemical parameters or secondary endpoints only, although some also demonstrated increased survival.[71-74] Nevertheless, unconfirmed results of these single studies on various antioxidants will probably not have a great impact on clinical donor pretreatment in the next few years, unless confirmed or applied in clinical studies.

Within the last few years, miscellaneous studies using diverse donor pretreatment strategies have been published with varying results. Vagus nerve stimulation was applied as donor pretreatment in brain dead donors for its potential anti-inflammatory effects. Vagus nerve stimulation decreased the expression of pro-inflammatory genes, decreased TNF-α production, diminished monocyte infiltration and more importantly, improved post-transplantation graft function.[75] Another study showed that donor statin pretreatment increased graft function and reduced renal inflammation in a rat kidney transplantation model.[76] Others showed that ICAM-1 inhibition in rat kidney donors improved graft survival, although effects were even larger when ICAM-1 was inhibited in the recipient or during preservation.[77] Disappointing results in animal studies have also been

reported; glutamine donor pretreatment did not affect the post-transplantation renal function in rats.[78] Finally, in humans a remarkable randomised clinical trial showed that hyperoxic donor pretreatment resulted in improved urine production and creatinine clearance after transplantation.[79] Previous animal experiments involving renal I/R without transplantation showed identical results.[80, 81] It may be speculated that hyperoxia induces oxidative stress in the donor, which enhances endogenous antioxidant mechanisms of the kidney. Another explanation is that hyperoxia leads to an improved oxygen reserve capacity of the kidney that protects the energy metabolism during the ischemic period. Finally, there have been reports on successes of hyperthermic donor preconditioning in rodent experiments. Two studies of the same group described beneficial effects of donor hyperthermia on kidney function and graft survival after transplantation. Hyperthermia induced renal expression of heat shock proteins was held responsible for the beneficial effects.[82, 83]

FUTURE PERSPECTIVES

Although the principle of donor pretreatment is not new, the clinical trend to use more marginal donors for transplantation only recently necessitated the search for new ways to optimize donor organ quality. Much preclinical research on donor pretreatment has been done, with promising results. The first human trials have recently shown protective effects of donor pretreatment with dopamine and corticosteroids, and these are likely to be of great influence in the coming years. Despite all the promising results from preclinical studies, the amount of clinical trials studying donor pretreatment is regretful. Moreover, studies specifically aimed at effects in marginal donor grafts are scarce. It has been suggested that the great amount of groups of interests involved in transplantation, the difficult ethical debate concerning informed consent of deceased donors and the effect of pretreatment on other organs considered for transplantation hampers the translation into the clinical setting.[9, 84] In the near future we expect results from some ongoing clinical trials, studying the effects of glucose, ischemic preconditioning, HO-1 induction and dopamine. Donor pretreatment targeting the immune system or oxidative stress responses remain interesting topics, but still have to prove themselves in the clinical setting.

In this review we focused on kidney transplantation. Research on transplantation of other organs will also provide new targets for pretreatment of kidney donors. For example, animal experiments with 17β-Estradiol as donor pretreatment showed improved outcome after transplantation of different organs.[85] Donor pretreatment with metformin improved acute and chronic rejection in cardiac transplantation in mice.[86] Ultimately, it is to be expected that a combination of agents will be used as tailored donor pretreatment, and timing may turn out to be

crucial in the success of donor pretreatment.

ETHICAL CONSIDERATIONS

In trying to translate donor pretreatment strategies to the clinical setting, ethical issues may be raised, particularly involving deceased donors. Most issues are related to the fact that manipulations will not benefit the donor directly in any way. Living donors provide the simplest situation, where the donor and recipient are fully aware of the donation procedure and are able to provide detailed consent, with prolonged time for consideration and reflection. It is obvious that donor pretreatment may never harm the living donor. The situation is more difficult for deceased donors. The act of participating in an organ donor register or carrying a donor card is considered as consent to organ donation, but it is not clear whether such consent would extend to invasive pretreatment strategies, and when they could be started. There is a need to establish whether pretreatment could be seen as no different from other medical interventions, such as the administration of heparin to donors. One can argue that consent to organ donation suggests consent to all techniques required to allow optimal quality of grafts. However, as long as donor pretreatment is applied in the experimental setting, trials will need informed consent of all parties involved.

In the declaration of Helsinki is stated that all participants of scientific research should know the aims, methods, anticipated benefits and potential risk of a study, before giving their informed consent.[87] How this should be implemented in deceased donors would naturally be by consent of the family. Although posing this extra question to the familiy may cause reluctance in doctors, it is important to respect and protect the deceased and to maintain public trust in organ donation program. Field experts discussed the lack of uniform standards when it comes to consent in donor pretreatment. They conclude that uniform standards are needed to overcome institutional pluralism.[88]
Indeed, several guidelines to deal with consent issues in performing research on donor management and donor pretreatment have been published recently. The Consensus Panel on Research with the Recently Dead (CPRRD) stated that research in organ transplantation donors is only allowed when it does not interfere with organ procurement and the donor must have given informed consent. In the absence of a statement from the donor, the family is allowed to give informed consent.[89]

However, donor pretreatment does not only influence the donor and all its organs suited for transplantation; it also involves all the recipients of the transplanted organs. It can be argued that recipients are no research subjects in studying

donor pretreatment, because this could only affect them indirectly. But conform the declaration of Helskinki, all research subjects have to give consent before performing an experiment. Indeed, ideally recipients would be asked for informed consent while being placed on the waiting list. However, recipients that do not consent to a pretreated kidney should not be disadvantaged when pretreated organs are being allocated. Consent would not be voluntary anymore then. Moreover, in the allocation process, some of the recipients remain unknown until the donor management period or even after organ procurement. It is suggested that an exception of recipients consent could fall under 'emergency research consent waiver'; because there is only a very small time window for asking permission. Off course this exception of consent is more easily accepted when the risk of the experiment is minimal.[88] It is clear that recipient consent to donor pretreatment poses many ethical and logistical questions. For now, it seems that institutional review boards have to determine what interventions are allowed and how donors and recipients provide informed consent.

The timing of pretreatment may be crucial, particularly with regard to agents that require a significant length of time prior to donation, to provide the beneficial effect. Thus, there may be requirements to commence administration of the agent before lack of objection is obtained. Although this might be an unusual principle, this type of approach has already been approved by ethical committees and has been used in clinical trials on organ donors.[90]

Deceased donors are often multiple organ donors. Intervention on behalf of one organ may be harmful to other potentially transplantable organs or at least affect them differentially. Donor pretreatment should be carefully tested for the effects on all potential grafts. If multiple organs from one donor are being sent to different transplant centers, the institutional board of the recipient centers should have been consulted before acceptance of the organ. Whether all recipients should provide informed consent when the intervention is targeted at another organ, and no adverse effects on the particular organ are demonstrated, is a matter of debate. Since matching of donor and recipients may sometimes be complete during or after the organ retrieval, it seems unreasonable that individual recipients or review boards can veto against donor pretreatment.

In conclusion, the great potential benefits of donor pretreatment are clear and preclinical results are promising. Clinical trials however are hampered by the intriguing and extensive ethical issues that are raised by experimental treatment of deceased donors and how we should handle issues on informed consent of donor and recipients. At present, one of the biggest challenges for donor pretreatment

trials is how they could be implemented in the current donation and organ allocation systems. It is stated that multi-disciplinary organisation with all those involved in the logistics of deceased donor procedures is fundamental to perform studies in donor intervention and that these studies are essential to raise graft quality to the next level.[91]

CONCLUSION

In these times of scarcity of donor organs, it is absolutely necessary to search for therapeutical options to render more marginal donor organs suitable for transplantation and to improve graft quality. During and before the process of transplantation the graft is exposed to various noxious events, which will lead to functional deterioration. Prevention of injury already in the donor could facilitate transplantation of more marginal donor grafts and provide better outcomes. Donor management is the first step to prevent derangements in the donor and has been much improved by standardization. Further, more specific improvement of graft condition is the aim of donor pretreatment. Donor pretreatment by various strategies, including ischemic preconditioning, HO-1 induction, anti-inflammatory and anti-complement interventions, erythropoietin and catecholamines have all been proven successful in animal experiments. Although many of these promising results in animals have yet to be confirmed in human kidney transplantation, the first pretreatment strategies have already shown encouraging beneficial effects in clinical studies. Current clinical studies are however limited by the large amount of ethical considerations on both the donor and recipient side. The lack of international guidelines makes institutional review boards and research groups repeat the same ethical questions and discussions on this topic worldwide.

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Part III

Summary

Future Perspectives

XI

SUMMARY AND FUTURE PERSPECTIVES

Leonie G.M.Wijermars

SUMMARY

Kidney transplantation is considered one of the greatest medical achievements of the last century. Although developments have resulted in significant improvements in both patient and graft survival rates, ischemia/reperfusion (I/R) injury continues to negatively impact the success rate of kidney transplantations. Despite numerous experimental studies indicating promising results in the prevention and treatment of I/R injury, to date no clinical interventions are available. This highlights the importance of unravelling the pathophysiological mechanisms causing I/R injury in humans.

This thesis explores the pathophysiology of I/R injury. *Chapter 1* outlines the objective of the thesis and describes why kidney transplantation is a predictable, accessible and clinically relevant model for studying I/R injury in humans. In addition, the latest research regarding the pathophysiology of I/R injury is reviewed. Previous studies by de Vries et al. demonstrate that the culprit mechanisms commonly assumed (such as thrombocyte, endothelial, neutrophil and complement activation) are not critical mechanisms in the acute phase of reperfusion injury.[1–3] In addition, the lack of effective antioxidant therapies and absence of radical oxygen species (ROS)-mediated-damage biomarkers suggest that the role of ROS in initiating clinical I/R injury may be lesser and perhaps not as critical as generally thought.[4]

In *Chapter 2* the presumed key role for ROS in I/R injury is further examined. The hypoxanthine-xanthine oxidase axis is assessed as a potential source of ROS. The demonstration of low and stable xanthine oxidase (XO) activity in ischemic and reperfused human kidneys indicated that the enzyme xanthine oxidase is not a source of ROS in human I/R injury. The absence of end products of xanthine oxidase reactions (i.e. uric acid and oxypurinol) further validates this finding. In humans, allantoin is the stable end product of the antioxidant activity of uric acid. The fact that no allantoin was released from the reperfused grafts, not even from DGF kidneys, fundamentally challenges the notion that I/R injury in human kidneys is driven by (XO produced) ROS.

In situ enzymology did show substantial XO activity in ischemic rat kidneys, contrasting the absence of activity in human biopsies. This interspecies difference explains why preclinical studies targeting the XO system did protect against I/R injury - while there was no effect in human studies. *Chapter 3* describes differences in I/R injury between species, indicating a translational gap between experimental models (e.g. rodents) and the clinical situation.[5,6] A widely acknowledged

letter in Nature stated a new, promising explanation for the mechanism of I/R injury: "ischemic accumulation of succinate drives reperfusion injury through mitochondrial ROS".[7] This theory was deducted from studies using mice and results described in Chapter 3 indicate that succinate-driven reactive oxygen formation does not occur in human kidney transplantation. This profound difference between mice and humans could be attributed to mitochondrial susceptibility to I/R. Human mitochondria were far more vulnerable than mice mitochondria, with human mitochondria having impaired capacity to oxidize succinate after exposure to brief ischemia. Thus succinate driven I/R injury appears to be a murine phenomenon and consequently addresses the difficulty of translating findings in mice to humans with regards to I/R injury research.

In the absence of evidence for involvement of the canonical pathways in clinical I/R injury, new approaches were used to unravel underlying mechanisms. Unbiased transcriptomic and metabolomic analyses were performed in the setting of clinical kidney transplantation. DGF development was used as the readout for I/R injury. *Chapter 4* describes measurements of arteriovenous(AV) concentration differences over the reperfused graft. These AV measurements show persistent lactate release in the acute phase of reperfusion in grafts that later developed DGF. Conversely control grafts did not show persistent lactate release and renal acidosis after reperfusion, indicating immediate reestablishment of aerobic respiration in control grafts. Biopsies taken at the end of the ischemic period and 45 minutes after reperfusion validated the results of the plasma measurements. Metabolomic analysis of these biopsies shows an almost immediate increase of tissue glucose/lactate rate in grafts with adequate functional recovery, on the contrary DGF grafts showed persistently low glucose/lactate rates. These observations implicate defective mitochondrial oxidative phosphorylation (OXPHOS) in DGF grafts. Transmission electron microscopy confirmed mitochondrial damage, with imaging showing mitochondrial morphology recovery in grafts that functioned adequately after reperfusion and deterioration in DGF grafts. These findings imply that DGF development is preceded by a profound post-reperfusion metabolic deficit that results from severe mitochondrial damage.

The results of Chapter 4 suggest that mitochondrial dysfunction and sequential energetic deficits are key drivers of I/R injury. It was realized (and concluded following the cohort study in Chapter 6) that almost all kidney grafts suffering from DGF start recovering within a few days to sometimes weeks after transplantation. As recovery is energy-dependent, this raises the question which metabolic pathways would still function in DGF grafts. To address this question, *Chapter 5* compares metabolic adaptation to ischemia and reperfusion in DGF

versus control grafts.

In summary, the differences in metabolic adaptation between DGF and control grafts were identified in 5 functional clusters: (I) metabolic collapse (power outage); (II) β-oxidation; (III) glycolysis/glutamine oxidation and autophagy; (IV) Krebs cycle (entry) defects, and (V) phospholipolysis/cell damage. This metabolome-broad approach shows that DGF grafts are hallmarked by energetic exhaustion ('metabolic meltdown'). Unlike control grafts, kidneys that developed DGF showed a decrease in phosphocreatine levels and continuous production of hypoxanthine and xanthine after reperfusion. This indicates ATP/GTP catabolism and a failure of ATP production in grafts that later develop DGF. This apparent metabolic deficit appears despite abundant activation of β-oxidation, glycolysis/glutamine oxidation and possibly autophagy. The accumulation of the Krebs-cycle entry-products acetylcarnitine and pyruvate point to Krebs cycle (entry) defect(s) in DGF grafts and release of these metabolites indicates that these carbon flows exceed the capacity of the Krebs cycle. Release of the Krebs cycle intermediate α-ketoglutarate, in combination with the absence of succinate recovery implies graded defects of oxoglutarate dehydrogenase activity in DGF grafts.

Findings in chapter 5 extend conclusions from experimental studies and our earlier reports. It shows that incident DGF not only associates with mitochondrial dysfunction, but that damage extends beyond the membrane bound respiratory complexes and also involves complexes located in the mitochondrial cytosol. The fully preserved β-oxidation in DGF grafts implies that these effects are specific, and not merely reflect gross mitochondrial damage. Subsequent to this mitochondrial dysfunction, the ATP deficit leads to inability to maintain homeostasis, which results in a state of on-going tissue damage after reperfusion. This is reflected by the continuous release of phospholipids and uracil, which mark membrane and cell damage respectively.

Since DGF resembles a state of energetic crisis, attempts to mitigate DGF should focus on sustaining a minimal level of metabolic competence. In order to identify candidate targets, the post-reperfusion metabolome of renal I/R injury was established. It is hypothesized that ATP could be provided both by enhancing glycolysis (via inhibiting glutaminolysis) and the catabolism of inosine. This is discussed in the 'Future Perspectives' section of this thesis.

To further explore factors determining the short- and long-term outcomes of kidney transplantation, graft function and survival were analysed in *Chapter 6*. Analysis of the Netherlands Organ Transplant Registry (NOTR) indicates a

50% higher incidence of primary non-function (PNF), and an almost tripled incidence of delayed graft function (DGF) in kidneys donated after cardiac death (DCD) (n=2891), compared to kidneys donated after brain death (DBD) (n=4084). The higher incidence of DGF in DCD compared to DBD grafts results in reservations regarding the use of DCD grafts in kidney transplantation. However, after excluding the grafts with primary non-function (7,9% of all DCD and 4.5% of all DBD grafts) 10-year graft survival was similar for both donortypes. Further evaluation shows that duration of cold ischemia longer than 24 h disproportionally mitigates graft survival of DCD grafts ($P<0.001$).

It was shown that incident DGF negatively impacts graft survival in DBD grafts, while it does not so in DCD grafts. It was realized that this differential impact of DGF on DBD grafts might reflect biological differences between the graft-types. Indeed, functional recovery curves show an exponential "catch-up" in DCD grafts that fully compensates for the initial graft loss, thus resulting in similar long-term graft survival for both donortypes. Hence, the current negative conception regarding the survival rate of DCD donors might need revision.

Following conclusions in Chapter 3, 4 and 5, *Chapter 7* proceeds on the role of mitochondria. No biomarkers currently exist to predict functional graft recovery during DGF. With mitochondrial dysfunction underlying DGF, it was hypothesized that mitochondrial regeneration precedes functional recovery. Therefore mitochondrial regeneration was expected to be a potent biomarker for functional recovery. To validate this hypothesis, graft biopsies taken during the period of DGF were examined (n=30). Mitochondrial SOD and YAP-1 (activation of Hippo signalling) qualified as histological markers of upcoming functional graft recovery. It came to our attention that these markers may also hold the key to potential therapeutic targets.

To further explore mitochondrial targets in clinical I/R injury, *Chapter 8* studies the role of mitochondrial aldehyde dehydrogenase (ALDH) enzymes. ALDHs are enzymes catabolizing toxic aldehydes, which originate from lipid peroxidation in I/R injury. Epidemiological studies show that deactivating point mutations in the ALDH2 gene lead to increased damage following myocardial I/R injury.[8,9] Results of Chapter 8 show that ischemia and reperfusion in control grafts is marked by enrichment of (Ingenuity) pathways involving ALDH genes - this enrichment is not found in grafts that later develop DGF. Additionally, in the acute phase of reperfusion, significantly higher ALDH activity is found in controls compared to DGF grafts. Based on these results, decreased ALDH enzymatic activity can potentially be used as a biomarker to predict DGF. Above that, mitochondrial

ALDH enzymes are seen as a promising target and improving ALDH activity may potentially decrease the incidence of DGF.

Chapter 9 investigates an alternative intervention that may be effective in limiting I/R injury. The results of the 'POSITIVE' study are described: Pre-Operative STatin InterVEntion and cardioprotection for mitral valve surgery. During mitral valve surgery, ischemia of the myocardium is induced by cardiopulmonary bypass (CPB) and its reperfusion sets in when the CPB clamps are released. Results of this exploratory trial show that pre-treatment with statins at least two weeks prior to on-pump cardiac valve surgery reduces myocardial damage without affecting the inflammatory response. Further studies are needed to explore whether donor pre-treatment with simvastatin will be effective in mitigating renal I/R damage.

Chapter 10 summarizes all experimental and clinical studies that have been performed on donor pre-treatment in kidney transplantation. Several strategies showing promising results in animal studies are described (e.g. ischemic preconditioning, HO-1 induction, anti-inflammatory interventions, anti-complement interventions, epo and catecholamines) - however a clear lack of clinical studies to support these results remains (ischemic preconditioning[10,11]; epo[12]; cyclosporine[13,14]; melatonine[15]). While recognizing the ethical issues regarding including deceased donors as a research subject when studying donor pre-treatment, it is anticipated that the insightful gains that could be realized outweigh these concerns and thus enable us to overcome this moral dilemma.

FUTURE PERSPECTIVES

This thesis investigates the mechanisms underlying I/R injury in human kidneys, responsible for the development of DGF. The main conclusion (Chapters 4 and 5) is that mitochondrial dysfunction is the key driver. This is supported by a recent scientific statement published by the American Heart Association, which states that mitochondria play a critical role in cardiovascular pathologies like I/R injury.[16]

The insight that DGF grafts are in a state of deep energetic crisis (Chapter 5) indicates that that an ATP-dependent intervention given after reperfusion will not be effective. This finding sheds a whole new light on the field of I/R injury research. It is postulated that future attempts to mitigate DGF should focus on sustaining a minimal level of metabolic competence. This can be accomplished in two ways:

(I) by therapeutics targeting ATP-production pathways that are still functional
(II) through preventive strategies protecting mitochondria during I/R.[17]

I. THERAPEUTICS: TARGETING INTACT ATP-PRODUCING PATHWAYS

To identify intact ATP-producing pathways to deal with the deficit described above, the post-reperfusion metabolome of renal I/R injury (i.e. DGF grafts) was established. The metabolome of DGF grafts can be described as follows: continuous lactate, alanine and asparagine release in reperfused DGF grafts signals persistent post-reperfusion aerobic glycolysis. This process also involves extensive glutaminolysis indicated by the glutamine uptake and glutamate release into the circulation. Recovery of post-reperfusion hydroxybutyrate levels indicate intact β-oxidation in DGF grafts. Interestingly, data for lipid oxidation showed a low, albeit persistent, post-reperfusion release of short-chain carnitines (≤C6) from DGF grafts. This indicates peroxisomal β-oxidation in DGF grafts, since this is limited to large and medium chain lipids.
Despite these intact pathways providing Krebs cycle entry products (glycolysis, glutaminolysis, β-oxidation), DGF grafts do not produce sufficient ATP due to impaired oxidative phosphorylation as result of Krebs cycle defect(s). From the pathways described above, aerobic glycolysis is the only pathway that yields ATP (2*ATP).

Throughout the process, it was noticed that both glutaminolysis and peroxisomal lipid oxidation could limit the ATP production of aerobic glycolysis. Glutaminolysis and lipid oxidation produce H+, leading to acidification of the

graft and causing the pH-sensitive enzyme lactate dehydrogenase (LDH) to be ineffective. In this manor, glutaminolysis and peroxisomal lipid oxidation can potentially restrict the ATP production normally generated by LDH. LDH is essential for aerobic glycolysis since it breaks down pyruvate into lactate. Therefore, it is hypothesized that inhibiting these pathways will allow for the production of more ATP on a net basis due to the removal of H+ production (acidification) which hampers the functioning of LDH. Thereby, peroxisomal lipid oxidation is accompanied by stoichiometric hydrogen peroxide production, which may perpetuate I/R injury. This is another reason why inhibiting this pathway could be a potential target for therapy.

Another hypothetical pathway that could increase ATP production is through the breakdown of inosine to hypoxanthine, which yields 8*ATP.[18] This pathway is effective in erythrocytes: aerobic glycolic cells devoid of mitochondria. Since mitochondria are dysfunctional in DGF grafts and inosine is lost in post-reperfusion biopsies, supplying inosine to the reperfused graft could potentially be an effective option to produce ATP. Along these lines, inosine is superior to glucose in preserved cellular ATP content of eukaryotic cells during hypoxia[19] and ameliorates tissue damage in experimental ischemia/reperfusion models.[20,21]

II. PREVENTIVE STRATEGIES; PRESERVING MITOCHONDRIA

In addition to maximizing the ATP production in DGF kidneys, a higher level of effectiveness will be achieved by also focusing on preventive strategies. Since mitochondrial damage underlies DGF, preventive strategies should focus on preserving mitochondria.[17] Mitochondrial preservation and recovery[22–25] are currently considered to be valid targets for intervention[26,27] and results from clinical studies are anxiously awaited. This thesis describes two other approaches to preserve mitochondria. Chapter 4 describes the protective effect of the cardiolipin-binding peptide SS-31 (Bendavia) during I/R. Chapter 8 hypothesizes about the protective role of detoxifying mitochondrial ALDH enzymes.

The organ transplantation process is exceptionally suitable for preventive strategies[28] as there are several points of intervention before I/R.

Donor pre-treatment is one option. The timeframe of donor pre-treatment encompasses a highly controlled situation in which the donor is monitored at the ICU until the moment the organs are procured for donation. During this time mitochondria could be successfully targeted prior to the induction of I/R injury. However, performing clinical studies with potential donors brings an extra level

of complexity and is accompanied by ethical difficulties (as is discussed in Chapter 10).
Another option for implementing a preventive strategy is during machine perfusion.[28,29] The time between donation and transplantation (i.e. when the ischemic donor graft is perfused on a pump) could provide an ideal opportunity to target mitochondria.[17]

CLINICAL APPLICATIONS

One of the most important conclusions of this thesis is that DGF is caused by metabolic incompetence due to mitochondrial dysfunction. To identify the clinical variables associated with DGF and transplantation outcome, a retrospective cohort study was performed (Chapter 6). DCD donortypes are associated with increased incidence of DGF and PNF, indicating superior short-term outcome of DBD grafts. Results of functional graft recovery imply a difference in biology between DBD versus DCD grafts - indicating a superior recovery potential of DCD grafts. This superior recovery potential of DCD grafts results in the most important clinical conclusion, namely that after restriction of duration of cold ischemia time to 24 h or less, 10-year graft survival is similar in DBD and DCD grafts. Currently reservations exist regarding the use of DCD grafts in kidney transplantation.[30–32] In fact, only 10 out of 27 European countries presently accept DCD donor grafts in their kidney transplantation programs [33,34], while only 10% of all deceased donor kidney transplantations in the USA are DCD donor grafts.[35] The results of this thesis suggest that hesitancy towards the use of DCD grafts might not be justified, if cold ischemia duration does not exceed 24 h. This could help alleviate current difficulties caused by donor shortages and reduce the 3,5 years average waiting time for a kidney transplant in the Netherlands (NTS data).

FUTURE RESEARCH

Research/study design

Several reports recently stated that the majority of I/R injury research had missed its purpose as they comprised of non-translatable animal studies.[36–38] Statements from the NIH[39], FDA[40] and several other (transplantation) expert institutions[6,37] confirm the translational gap between preclinical research and I/R injury in humans, disapproving non-translatable studies.
By understanding and investigating the mechanisms of I/R injury in humans, this thesis indicates that human mitochondria are more vulnerable to I/R than rodent mitochondria – this could explain the significant translational gap between experimental models and clinical I/R.[5] Based on the results of Chapter 3, it is hypothesized that pigs are a better model than rodents as their mitochondrial vulnerability to I/R is more similar to humans. Additionally, pig's metabolic

adaptation to I/R shows similarities to humans as it is characterized by the release of betaine, methionine and serine.[41] It is recommended that future studies be performed on the translation of porcine to human I/R injury.

Another recommendation (Chapter 5) is that metabolomic studies should not only rely on tissue data as this provides a static view of the actual situation and is therefore (potentially) biased. Tissue metabolomics should be combined with plasma metabolomics which will provide a more dynamic and just point-of-view of metabolic processes.

Study focus / questions to be answered
In the near future research has to localize the mitochondrial defects of grafts that later develop DGF. Labelled Krebs-cycle intermediates can be used to identify which step of the Krebs cycle is dysfunctional. When the defect is known, targeted preventive or therapeutic measures can be developed to prevent DGF or enhance the regeneration of grafts that developed DGF.
Another challenge will be unravelling epidemiological questions and possibly connecting these to graft biology. Results of Chapter 6 show that incident DGF impairs graft survival of DBD grafts, but not of DCD grafts. Dynamical studies show superior recovery potential of DCD grafts and this difference in resilience between the two donortypes needs to be investigated. Interestingly, in both donortypes increased donor age was an independent risk factor for inferior graft function. The question rises whether there is a connection between (mitochondrial) senescence (aging) and increased vulnerability to I/R or decreased resilience/regeneration capacity. Creating the bigger picture where clinical (patient/epidemiological) data is combined with cell biology will be the pursuit of future research.[42,43]

LIMITATIONS
For this thesis, human kidney transplantation was chosen to study I/R injury. It provides a situation of planned, complete organ ischemia and reperfusion and therefore is preferable for studying I/R injury than clinical stroke or myocardial infarction. However, this model does have some limitations. Due to limited size of the patient groups it was not possible to address specific points such as different donor and recipient characteristics – however larger samples sizes would not expect to change the conclusions, but only result in smaller confidence intervals. Furthermore the effects of prolonged cold ischemia, as found in the transplantation setting, are not directly translatable to other clinical situations like myocardial infarction and stroke since these are caused by so-called warm ischemia. Another concern is that the mechanism of I/R injury as found in the

kidney, may not directly be translatable to other organs, since metabolic profiles, preference substrates and mitochondrial respiratory may differ. In fact it is known that differences in vulnerability for I/R injury exists across various organs.

SUMMARY

This thesis provides insights into the mechanisms of renal ischemia/reperfusion injury based on human kidney transplantation (i.e. DGF). A severe energetic crisis differentiates DGF kidneys from adequately functioning controls. Although intact β-oxidation, aerobic glycolysis and glutaminolysis provide Krebs Cycle intermediates, these intermediates are not able to enter the mitochondrial Krebs cycle. Hence, dysfunctional mitochondria disable efficient ATP production leading to the metabolic incompetence that causes DGF. This finding sheds a whole new light on I/R injury and explains why ATP-dependent therapeutics remain ineffective.

A major difference in the vulnerability of mitochondria to ischemia and reperfusion between rodents and humans was found. This could explain the current differences in effectiveness of therapies in the experimental versus the clinical setting and highlight the translational gap. Big cohort studies as described in Chapter *6* give insights in donor, recipient and transplant-procedure variables and challenge the reluctance towards the use of DCD donor kidneys. Superior recovery potential of DCD compared to DBD grafts was established and future studies need to deepen the knowledge on these biological differences. This thesis shows that recovery of the mitochondrial pool and activation of the Hippo pathway precede functional recovery. The downstream end-products of these pathways (MnSOD, Yap-1) can be used as markers to predict functional recovery. New preventive strategies could limit I/R injury by preserving mitochondria (such as hypothetical treatments with the peptide SS-31 or activation of the mitochondrial enzyme aldehyde dehydrogenase).

The big challenge will be the identification of pathways and targets that effectively preserve mitochondrial function and prevent the energetic crisis underlying DGF. Large translational studies should be combined with clinical and cell-biological data to understand the link between I/R injury and long-term outcomes. This will overcome the detrimental effects of I/R injury on graft function and survival - thereby increasing the success rate of kidney transplantation.

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NEDERLANDSE SAMENVATTING

Ischemie/reperfusie(I/R) schade is de paradoxale toename van weefselschade wanneer ischemisch weefsel opnieuw doorbloed wordt. Deze vorm van schade schade speelt een cruciale rol in onder andere myocard infarcten, cerebrovasculaire accidenten en orgaantransplantaties.
Al meer dan 50 jaar wordt er veel onderzoek verricht naar I/R schade. Uit evaluatie van deze onderzoeken blijkt dat veelbelovende resultaten uit diermodellen moeilijk transleerbaar zijn naar de kliniek. Dit benadrukt het belang om een stap terug te nemen en te focussen op de mechanismen van I/R schade in de mens. In de studies beschreven in dit proefschrift dienen niertransplantaties als humaan model om de mechanismen van I/R schade te onderzoeken. Omdat zowel ischemie als reperfusie tijdens orgaantransplantaties gepland zijn (in tegenstelling tot bijvoorbeeld infarcten) en er directe toegang is tot het gereperfundeerde orgaan, biedt de situatie van niertransplantaties de uitgelezen mogelijkheid om I/R schade in de mens te onderzoeken.

Door het persisterende donortekort worden steeds vaker organen van marginale donoren gebruikt voor transplantatie. Transplantatie van deze organen leidt in toenemende mate tot orgaanschade en heeft daardoor, zowel op de korte als lange termijn, verminderende transplantaat functie en overleving tot gevolg. Interventies die I/R schade kunnen voorkomen of verminderen zijn dan ook van cruciaal belang.

Op basis van de uitkomsten van niertransplantaties is er een graduele verdeling te maken in de mate van I/R schade. Levende donor niertransplantaties leiden vrijwel nooit tot “delayed graft function” (DGF – het vertraagd op gang komen van de nier na transplantatie èn de uitkomstparameter van I/R schade), terwijl meer dan de helft van de overleden donor niertransplantaties DGF tot gevolg heeft. Levende donor niertransplantaties worden om deze reden als referentiegroep gebruikt in de beschreven studies.

Voorgaande studies, waarin niertransplantatie gebruikt werd als model voor humane I/R, konden geen bewijs vinden voor de rol van veronderstelde mechanismen van I/R schade zoals trombocyt, endotheel, neutrofiel en complement activatie.(*Thesis D.K. de Vries*) Ook konden er geen biomarkers van vrije radicaal schade worden aangetoond in de acute fase na reperfusie van het transplantaat. Dit terwijl vrije zuurstof radicalen altijd in één adem genoemd worden met I/R schade. Bronnen van vrije zuurstof radicalen zijn de gereactiveerde mitochondriale elektronentransportketen, NADPH oxidase in neutrofielen en xanthine oxidase (XO) in endotheel cellen. De rol van mitochondriale dysfunctie in I/R schade wordt beschreven in hoofdstuk 2, 4 en 5 van dit proefschrift. Neutrofiel

activatie als veroorzaker van I/R schade werd eerder al uitgesloten door de Vries et al.(*Thesis D.K. de Vries*) Hoofdstuk 2 belicht de hypoxanthine-XO-as en laat zien dat XO als bron van vrije radicalen geen oorzaak is van I/R schade in de mens. Middels in situ enzymologie werd er een lage XO activiteit gemeten, zowel aan het einde van de ischemische periode als in de acute fase van reperfusie. Deze lage XO activiteit werd geconfirmeerd door de afwezigheid van door XO geproduceerd urinezuur en oxypurinol in de gereperfundeerde nier. Bovenal werd er geen allantoïne uitgescheden door de gereperfundeerde transplantaten, zelfs niet door nieren met klinische I/R schade (DGF). Allantoïne is het stabiele eindproduct van de antioxidant activiteit van urinezuur in de mens. Aangezien er geen allantoïne wordt gevormd ondermijnt dit direct de veronderstelde aanwezigheid en rol van vrije zuurstof radicalen in humane I/R schade. Opvallend was dat de in situ enzymologie wel een hoge XO activiteit toonde in ischemische ratten nieren; een uiting van interspeciële verschillen in het XO systeem.
Hoofdstuk 3 neemt deze interspeciële verschillen verder onder de loep. Zoals uitgebreid besproken in de inleiding van dit proefschrift (Hoofdstuk 1), bestaat er een enorme translationele kloof tussen experimentele modellen en de klinische realiteit. Dit blijkt uit de talloze veelbelovende resultaten uit preklinische studies die bij vertaling naar humane studies falen op klinische eindpunten. In een breed gedragen Nature artikel, gepubliceerd in 2014 (*Chouchani et al.*), werd een nieuw mechanisme van I/R schade gevindiceerd: accumulatie van succinaat tijdens ischemie leidt tot de terugstroom van elektronen naar complex I van de mitochondriale elektronentransportketen, waardoor er tijdens reperfusie vrije radicalen ontstaan die I/R schade veroorzaken. In de bestudeerde muismodellen werd dit mechanisme in verschillende organen gevalideerd. In hoofdstuk 3 laten wij zien dat er van dit mechanisme geen sprake is in de mens, aangezien er geen succinaat accumulatie plaats vindt tijdens ischemie. Sterker nog, in tegenstelling tot de muis, werden patiënten met I/R schade (+DGF) juist gekenmerkt door uitputting van hun succinaat reserves. Daarnaast werden duidelijke interspeciële verschillen gevonden met betrekking tot de mitochondriale vatbaarheid voor ischemie en reperfusie; waarbij humane mitochondria het meest kwetsbaar zijn en al na een relatief korte periode van ischemie verminderde capaciteit hebben om succinaat te oxideren. Resultaten in Hoofdstuk 3 laten zien dat succinaat gedreven I/R schade een muis-fenomeen is en legt opnieuw het knelpunt van translatie van experimentele modellen naar de mens bloot.

Kortom, van verschillende veronderstelde mechanismen van I/R schade kon de rol in humane I/R, in de klinische situatie van niertransplantaties, niet aangetoond worden.
In de zoektocht naar aanwijzingen voor alternatieve, onderliggende mechanismen

beschrijft Hoofdstuk 4 een objectieve benadering middels 'transcriptomics' en 'metabolomics'. Tijdens de transplantatieprocedure werden de nier arterie en vene gecannuleerd, zodat over de tijd gepaarde samples over het orgaan afgenomen konden worden, vanaf het eerste moment van reperfusie. Arterioveneuze (AV) metingen laten zien dat DGF wordt gekenmerkt door de persisterende productie van lactaat, het eindproduct van aerobe glycolyse. Daarentegen zijn transplantaten die geen DGF ontwikkelen direct na reperfusie in staat om over te schakelen naar een aeroob metabolisme. Deze transplantaten scheiden geen lactaat uit en de nieren verzuren niet. De bevindingen in plasma werden gevalideerd middels de analyse van nierbiopten. Deze biopten werden gepaard afgenomen: aan het einde van de ischemische periode en 45 minuten na reperfusie. In biopten van nieren die adequaat herstelden werd direct na reperfusie een stijging van de glucose/lactaat ratio gemeten. In DGF transplantaten bleef deze ratio daarentegen laag, passend bij defecte oxidatieve phosphorylatie (OXPHOS) in de mitochondriën. Beeldvorming middels transmissie elektronen microscopie liet inderdaad herstel zien van mitochondriale morfologie in transplantaten die adequaat functioneerden en verslechtering van de mitochondriale morfologie in DGF nieren. Defecte mitochondriale OXPHOS leidt vervolgens tot totale metabole uitputting in DGF nieren. Dit wordt geïllustreerd door persisterende hypoxanthine excretie (het eindproduct van ATP katabolisme) en de afwezigheid van opregulatie van metabole genen.

Hoofdstuk 5 vormt een vervolg op de bevindingen van hoofdstuk 4 en verdiept zich in het energiek functioneren van het transplantaat na reperfusie. Met een metaboloom-brede benadering wordt middels arterioveneuze metingen en biopten voor en na reperfusie de status van DGF en daarmee renale I/R schade in de mens beschreven.
Het metabole profiel van DGF nieren is samen te vatten in 5 metabole clusters: (I) metabole ineenstorting/energetische crisis, (II) beta-oxidatie, (III) glycolyse/ glutamine oxidatie en autofagie, (IV) Krebs cyclus intrede defecten en (V) phospholipolyse/cel schade.

De post-reperfusie status van DGF transplantaten wordt gekenmerkt door het verlies van phosphocreatine en voortdurende productie van xanthine en hypoxanthine, de eindproducten van ATP/GTP katabolisme. Dit duidt op het plaatsvinden van een totale energetische crisis. DGF nieren laten zien dat alles in het gereperfundeerde orgaan in staat wordt gesteld om de mitochondriale Krebs cyclus draaiende te houden. Herstel van 3-hydroxybutyraat waarden in post-reperfusie biopten indiceert dat de beta-oxidatie intact is, evenals de pyruvaat synthese middels glycolyse en transaminase van glutamaat. Ondanks de vorming

van deze Krebs cyclus substraten vindt er excretie plaats van acetyl-CoA en pyruvaat vanuit de gereperfundeerde DGF nier. Dit duidt erop dat deze substraten niet in staat zijn de Krebs cyclus binnen te treden, waarna ze worden uitgespoeld uit het transplantaat. Excretie van het Krebs cyclus intermediair α-ketoglutaraat, in combinatie met de afwezigheid van herstel van succinaat concentraties, impliceert een defect in oxoglutaraat dehydrogenase activiteit in DGF nieren. De defecten in mitochondriale oxidatieve phosphorylatie leiden tot ATP tekorten. Dit leidt tot het onvermogen om cel homeostase te behouden wat resulteert in een staat van voortdurende weefselschade na reperfusie. Dit wordt weerspiegeld door de continue excretie van phospholipiden en uracil, welke respectievelijk membraan en cel schade kenmerken.
Kortom, ondanks intact glycolyse, glutaminolyse en beta-oxidatie, produceren DGF nieren onvoldoende ATP door gebrekkige oxidatieve phosphorylatie die wordt veroorzaakt door defecten in de Krebs cyclus. Van de bovenstaande, functionele processen produceert aerobe glycolyse 2*ATP. Echter, glutaminolyse en peroxismale beta-oxidatie kunnen de ATP productie van aerobe glycolyse beperken. Glutaminolyse en beta-oxidatie produceren namelijk H+. Dit leidt tot verzuring van het transplantaat en zorgt ervoor dat het pH-gevoelige enzym lactaat dehydrogenase (LDH) ineffectief wordt. Op deze manier kunnen glutaminolyse en beta-oxidatie de ATP productie van LDH tijdens aerobe glycolyse, welke pyruvaat omzet naar lactaat, limiteren. Het wordt daarom verondersteld dat het remmen van glutaminolyse en beta-oxidatie in DGF transplantaten netto-netto meer ATP oplevert, omdat LDH en daarmee aerobe glycolyse optimaler kunnen functioneren.
Een andere mogelijke route die de ATP productie zou kunnen vergroten is middels de afbraak van inosine tot hypoxanthine (8*ATP). Middels deze metabole route produceren erythrocyten, levende cellen die ATP genereren in de afwezigheid van mitochondriën, effectief energie. Omdat de mitochondriën in DGF nieren dysfunctioneel zijn, zou de afbraak van inosine naar hypoxanthine hypothetisch ATP op kunnen leveren.
Concluderend is de status van DGF een gevolg van extreem energetisch falen veroorzaakt door dysfunctie van de mitochondriale Krebs cyclus. Verder onderzoek zal uit moeten wijzen of het preserveren/preconditioneren van het transplantaat met de beschreven energetische substraten zal leiden tot verbetering van de energie status en daarmee tot vermindering van I/R schade. Verwacht wordt dat preventie van mitochondriale schade, vooraf aan ischemie en reperfusie, effectiever zal zijn in het limiteren van I/R schade.

Al met al omvat Hoofdstuk 5 één van de belangrijkste conclusies van dit proefschrift: I/R schade (in humane niertransplantaties) wordt veroorzaakt door

mitochondriale dysfunctie, wat leidt tot energiek falen. Om uit te zoeken welke klinische factoren predisponeren voor dit metabool falen en invloed hebben op de korte en lange termijn uitkomsten van niertransplantaties, worden transplantaat functie en overleving geanalyseerd in Hoofdstuk 6. Analyse van data van de Nederlandse Orgaantransplantatieregistratie (NOTR) laat zien dat nieren gedoneerd na hartdood (DCD: Donation after Circulatory Death; n=2891) een 50% hogere incidentie hebben van primaire non-functie (PNF) en een bijna verdrievoudiging van incidentie van DGF, vergeleken met transplantaten gedoneerd na hersendood (DBD: Donation after Brain Death; n=4084). Deze hoge incidentie van DGF in DCD versus DBD nieren leidt tot terughoudendheid in het gebruik van DCD transplantaten. Echter, wanneer we alle nieren die PNF ontwikkelden na transplantatie excluderen (7,9% van alle DCD en 4,5% van alle DBD transplantaten), is de 10-jaar transplantaat overleving gelijk voor beide donortypen. Verdere analyse wijst uit dat wanneer de koud ischemische periode langer duurt dan 24 uur, dit leidt tot disproportionele verslechtering van DCD transplantaat overleving.
Ook wijst analyse uit dat wanneer DBD nieren DGF ontwikkelen na transplantatie, dit een negatieve invloed heeft op transplantaatoverleving, terwijl de ontwikkeling van DGF geen negatieve invloed heeft op de overleving van DCD transplantaten. Er werd gerealiseerd dat achter de discrepantie in impact van DGF op transplantaatoverleving mogelijk een biologisch verschil tussen de twee verschillende donortypen schuil gaat. Inderdaad, wanneer we het functionele transplantaat herstel vergelijken tussen de twee donortypen zien we een exponentiele inhaalslag van DCD transplantaten. Deze inhaalslag compenseert volledig voor het initiële transplantaatverlies en resulteert daardoor in vergelijkbare lange termijn transplantaat overleving van DBD en DCD transplantaten.
Waarom DGF in DBD nieren wel een negatieve invloed heeft en in DCD nieren niet en waarom DCD nieren een hoger herstel vermogen lijkten te hebben, is niet evident. Enerzijds zou het zo kunnen zijn dat DCD nieren beter beschermd zijn tegen de nadelige effecten van DGF. Een mogelijkheid is dat de initiële warme ischemie die optreedt bij DCD donoren een extreme vorm is van ischemische preconditionering waardoor beschermende mechanismen geactiveerd worden. Anderzijds kan het ontbreken van het hersendood zijn van de donor in DCD donoren een positief effect hebben op de lange termijn overleving. Uitgebreide studies zullen moeten uitwijzen welke oorzaken onderliggend zijn aan het verschillende 'effect' van DGF in de twee donortypen. De belangrijkste klinische conclusie van dit proefschrift is dat de negatieve kijk op de transplantaat overleving van DCD nieren nodig aan revisie toe is. We kunnen concluderen dat de terughoudendheid om DCD nieren toe te laten tot nationale transplantatie-

programma's herzien moet worden, wanneer men rekening houdt met de duur van de koude ischemie. De verwachting is dat orgaantekorten hierdoor verminderen en wachtlijsten zullen slinken.

Vanuit de conclusies van Hoofdstuk 3,4 en 5, gaat Hoofdstuk 7 verder in op de rol van mitochondriën. Op dit moment bestaan er geen biomarkers die de mate van functioneel herstel van het transplantaat gedurende de periode van DGF kunnen voorspellen. Omdat dysfunctionele mitochondriën onderliggend zijn aan DGF, word er verondersteld dat herstel van de mitochondriale machinerie vooraf gaat aan functioneel herstel en dat markers van mitochondriale regeneratie mogelijk biomarkers voor functioneel herstel kunnen zijn. Hoofdstuk 7 beschrijft een retrospectieve studie waarin biopten van nier transplantaten, die zich op dat moment in de fase van DGF bevinden, door middel van immunohistologie zijn geanalyseerd. De studie laat zien dat herstel van de mitochondriale pool, wat zich kenmerkt door toename van Mitochondriaal SOD in het biopt, voorafgaat aan functioneel herstel. Daarnaast voorspelt activatie van de Hippo-pathway, een ver in evolutie teruggaande pathway die een rol speelt in organogenese, functioneel herstel via translocatie van eind effector YAP-1 naar de celkern. Markers voor fission and fusion, het samenvoegen en splisten van mitochondriën waarmee de mitochondriale pool wordt vernieuwd, kwalificeerden zich niet als marker voor functioneel herstel. De resultaten van deze studie kunnen, naast de diagnostische rol als marker voor functioneel herstel, ook bekeken worden in het licht van therapie. In het kader van een zo genoemde theranostic: een combinatie van therapie en diagnosticum. Mogelijk dient activatie van de Hippo-pathway of het versnellen van mitochondriaal herstel ook een therapeutisch doel, namelijk versneld functioneel herstel van het transplantaat. Toekomstige studies zullen dit moeten uitwijzen.

Doorgaand op het concept van mitochondriale bescherming als doelwit tegen I/R schade, wordt de rol van mitochondriale aldehyde dehydrogenase (ALDH) enzymen bediscussieerd in Hoofdstuk 8. Deze ALDH enzymen spelen een rol in het wegvangen van schadelijke aldehydes die ontstaan bij reperfusie van ischemisch weefsel. De resultaten in Hoofdstuk 8 laten zien dat wanneer we genexpressie profielen van controle nieren vergelijken met DGF nieren, in controle nieren 'pathways' waarin ALDH enzymen betrokken zijn verrijken, terwijl deze pathways niet verrijken in DGF nieren. Dit suggereert een beschermende rol van ALDH enzymen in I/R schade of een rol voor aldehyde dehydrogenase enzymen in een adequate reactie op I/R. Daarnaast komt de beschermende rol van ALDH2 in I/R schade sterk naar voren in epidemiologische studies. De deactiverende puntmutatie in het ALDH2 gen, die zeer prevalent is in het Aziatische ras, leidt tot

een toename van schade bij myocard infarcten. Hoofdstuk 8 beschrijft de validatie van de genexpressie profielen middels qPCR en WesternBlot. Daarnaast hebben DGF nieren in de acute fase van reperfusie een significant lagere ALDH activiteit vergeleken met controle nieren. Hieruit wordt verondersteld dat verminderde ALDH activiteit gebruikt kan worden als biomarker om DGF te voorspellen. Bovendien zijn mitochondriale ALDH enzymen een veelbelovend doelwit om potentieel de incidentie van DGF te verminderen.

Hoofdstuk 9 beschrijft een alternatieve interventie die mogelijk I/R schade kan verminderen. Het beschrijft de resultaten van de 'POSITIVE' studie: Pre-Operatieve STatine InterVEntion en cardiale bescherming in mitraalklep chirurgie. Patiënten die een mitraalklep-plastiek ondergingen, waarbij via cardiopulmonaire 'bypass' het myocard intermitterend ischemisch en gereperfundeerd was, werden willekeurig verdeeld in twee patiëntengroepen. Onderzocht werd of dagelijks 40 mg statine minimaal 2 weken voor de ingreep of twee maal 40 mg statine 24 uur voor de ingreep, myocardschade kon verminderen. Metingen middels Multiplex vonden geen effect op postoperatieve inflammatoire markers. Wel werd er een effect gevonden van pre-operatieve statines op circulerende schademarkers van het hart: CPK, en Troponine T waren significant lager in patiënten die 2 weken lang statines gedoseerd kregen. Verdere studies zullen moeten uitwijzen of donor voorbehandeling met simvastatine effectief is in het verminderen van renale I/R schade.

Hoofdstuk 10 beschrijft een ander moment van interventie: niet gedurende de ischemie of na de reperfusie, maar voor het intreden van ischemie in de donor. Dit hoofdstuk vat alle experimentele en klinische studies samen die gedaan zijn naar donor voorbehandeling. Verschillende strategieën laten veelbelovende resultaten zien in dierexperimentele studies (o.a. ischemische preconditionering, HO-1 inductie, anti-inflammatoire interventies, anti-complement interventies, erytropoëtine en catecholamines), echter ontbreken de resultaten van klinische studies veelal. Een belangrijke conclusie die volgt uit dit overzichtsartikel is dat medische onderzoeken naar donor voorbehandeling zich bevinden in complexe ethische spanningsvelden. Hoewel er zeker geanticipeerd moet worden op deze ethische kwesties realiseren wij ons ook dat de factoren van de nieuwe inzichten die deze studies geven en de positieve effecten die zij kunnen hebben op het vergroten van de donorpool, zwaar moeten wegen in de morele dilemma's.

De studies in dit proefschrift bieden inzicht in mechanismen van renale I/R schade (DGF), bestudeerd vanuit humane niertransplantaties. Een energieke crisis onderscheidt DGF nieren van adequaat functionerende controle transplantaten.

Ondanks intacte Krebs cyclus substraat productie door beta-oxidatie, aerobe glycolyse en glutaminolyse in DGF nieren, zijn deze substraten niet in staat om de Krebs cyclus in te stromen. Kortom, door dysfunctionele mitochondriën ontbreekt een efficiënte ATP productie, wat leidt tot de metabole incompetentie die onderliggend is aan DGF. Deze theorie doet een nieuw licht schijnen op I/R schade en kan verklaren waarom ATP-afhankelijke therapieën in het verleden ineffectief zijn gebleken.
Resultaten lieten zien dat de mitochondriale vatbaarheid voor schade van ischemie en reperfusie enorm verschilt tussen mensen en knaagdieren. Dit verschil in vatbaarheid zou mogelijk kunnen verklaren waarom sommige therapieën wel effectief zijn in de experimentele, maar niet in de klinische situatie.
Een grote cohort studie geeft inzicht in donor, ontvanger en transplantatie-procedure variabelen en verwerpt de terughoudendheid naar het gebruik van DCD donor nieren. Een superieur herstel vermogen werd gevonden in DCD vergeleken met DBD transplantaten en toekomstige studies moeten uitwijzen welke biologische verschillen hier onderliggend aan zijn. Resultaten in dit proefschrift kwalificeren MnSOD en Yap-1 als biomarkers voor functioneel herstel tijdens DGF en potentiële strategieën die I/R schade kunnen verminderen moeten focussen op preservatie van mitochondriën (hypothetisch met het peptide SS-31 of activatie van mitochondriale aldehyde dehydrogenase enzymen).
Het identificeren van pathways en doelwitten die effectief mitochondriale functie preserveren zal voor de toekomst een grote en belangrijke uitdaging zijn, om zo de energieke crisis onderliggend aan DGF te voorkomen. Grote translationele studies zullen klinische en celbiologische data moeten combineren om de link tussen I/R schade en lange termijn uitkomsten te begrijpen. Op deze manier kunnen de desastreuze effecten van I/R schade op lange termijn transplantaat functie en overleving overwonnen worden, waardoor het aantal succesvolle niertransplantaties zal toenemen.

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CURRICULUM VITAE

Leonie Wijermars was born together with her twin brother in Zwolle on December 28th of 1989. She completed high school in Zwolle in 2007 at the Thomas à Kempis College at the "Gymnasium" level and on her 17th she started studying Industrial Design at the Technical University of Delft. In 2008, she decided to pursue her passion in Medicine at the University of Leiden.
During her Bachelor's, she quickly realized the importance of medical development and under supervision of dr. J.H.N. Lindeman she started working as a researcher on the 'Positive' study at the Cardiothoracic-surgery department of the Leiden University Medical Center.
After completing her Bachelor of Medicine with a clinical internship at the Komfo Anoyke Teaching Hospital in Kumasi, Ghana, she started as a PhD candidate at the Transplantation Surgery department of the LUMC. The results of which have been published in this thesis (promotor: Prof. dr. J.F. Hamming, co-promotores: dr. J.H.N. Lindeman and dr. A.F.M. Schaapherder). In 2015, she spend 6 months working in the laboratory of dr. E. Gross at the University of Stanford for which she received a number of personal grants including those from the Dutch Kidney foundation, foundation 'De Drie Lichten', the Michaël van Vloten fund, ESOT (European Society for Organ Transplantation) and the dr. Catharine van Tussenbroek fund.
During her PhD, in 2013 she organized the 'LUMC PhD-day', after which she became a board member of the LAP (LUMC Association for PhD candidates). In December 2015, she became a part of the organizing committee of the SEOHS (Symposium for Experimental Surgery Research). She officially started her clinical rounds that same year, during which she did an internship at the Dutch Health Council, an independent governmental advisory board to the Dutch Parliament. On the 27th of October 2017, she completed her Medical Doctor certifications "With Honors" and became a resident otorhinolaryngology, head and neck surgery at the Academic Medical Center (AMC) on the first of January 2018 (under prof. dr. F.G. Dikkers).

Leonie Wijermars werd op 28 december 1989, samen met haar tweelingbroer, geboren in Zwolle. In 2007 behaalde zij haar Gymnasium diploma aan het Thomas à Kempis College te Zwolle, waarna zij op 17-jarige leeftijd begon aan een studie Industrieel Ontwerpen aan de Technische Universiteit te Delft. In 2008 besloot zij deze studie te verruilen voor de studie Geneeskunde aan de Universiteit van Leiden.
Tijdens de bachelorfase raakte zij geïnteresseerd in wetenschappelijk onderzoek en begon zij onder leiding van dr. J.H.N. Lindeman aan de 'Positive' studie op de afdeling thoraxchirurgie van het Leids Universitair Medisch Centrum.
Nadat zij haar bachelor geneeskunde afrondde met een klinische stage in het Komfo Anoyke Teaching Hospital te Kumasi, Ghana, zette zij haar wetenschappelijk werk voort als promovendus op de afdeling heelkunde (transplantatie-chirurgie) van het LUMC, waarvan de resultaten in dit proefschrift zijn beschreven (promotor: Prof. dr. J.F. Hamming, co-promotores: dr. J.H.N. Lindeman en dr. A.F.M. Schaapherder). In 2015 deed zij 6 maanden onderzoek in het laboratorium van dr. E. Gross aan de Universiteit van Stanford, waarvoor zij verschillende persoonlijke beurzen ontving van onder andere de Nierstichting, stichting 'De Drie Lichten', het Michaël van Vloten fonds, het Jo Kolk Studiefonds, het ESOT (European Society for Organ Transplantation) en het dr. Catharine van Tussenbroek fonds.
Gedurende haar promotietraject organiseerde zij in 2013 de 'LUMC PhD-day', waarna zij aantrad als commissie lid van het LAP (LUMC Association for PhD candidates). In december 2015 organiseerde zij met 9 andere Commissie Leden het SEOHS (Symposium Experimenteel Onderzoek voor Heelkundige Specialismen), waarna zij startte aan haar coschappen. Tijdens haar coschappen liep zij enkele weken stage bij de Gezondheidsraad, het onafhankelijk wetenschappelijk adviesorgaan van de Nederlandse regering en parlement. Op 27 oktober 2017 behaalde zij Cum Laude haar artsexamen en op 1 januari 2018 begon zij aan de opleiding Keel- Neus- en Oorheelkunde in het Academisch Medisch Centrum te Amsterdam (opleider prof. dr. F.G. Dikkers).

ACKNOWLEDGEMENTS

Zoals stelling 10 behorend aan dit proefschrift luidt: “promoveren is teamwork”, zijn er ontzettend veel mensen die hebben bijgedragen aan de totstandkoming van dit proefschrift. Ik wil eenieder die mij, op welke wijze dan ook, de afgelopen jaren heeft ondersteund heel hartelijk danken en een kleine selectie wil ik graag in het bijzonder noemen.
Allereerst wil ik alle patiënten die bereid waren mee te werken aan het beschreven wetenschappelijk onderzoek heel hartelijk danken. Zij deden dit volkomen onbaatzuchtig en ‘for-the-greater-good’. Zonder hen was dit proefschrift er niet geweest.

Mijn promotor, Prof. J.F. Hamming, wil ik heel hartelijk danken voor zijn heldere blik en oprechte betrokkenheid. Vanaf het moment dat u het ‘promotorschap’ van Prof. van Bockel overnam stond uw deur altijd voor mij open. U hield overzicht wanneer ik de diepte in dook en zorgde er op de moeilijke momenten voor dat de tanden nog even op elkaar gingen. Veel dank voor uw steun en visie!

Mijn copromotor Jan Lindeman. Beste Jan, jij hebt mij de beginselen van de wetenschap bijgebracht. Je creatieve, eigenzinnige kijk op de wereld, het altijd zien van een uitweg of verklaring blijft mij intrigeren. Dank ook voor het in contact brengen met Dorottya en Kirsten, twee inspirerende doctors/dokters die de eerste stappen hebben gezet in deze onderzoekslijn van het ontrafelen van I/R schade.

Mijn copromotor Sandro Schaapherder. Beste Sandro, dank voor de mogelijkheid die jij hebt geboden om mijn promotieonderzoek te doen op de afdeling Transplantatie chirurgie. Door jouw bijdrage kan er zoiets als translationeel onderzoek bestaan! Ook wil ik alle transplantatiechirurgen van het LUMC hartelijk danken voor hun betrokkenheid bij mijn onderzoek en hun inzet om bloed en biopten te verzamelen.

Daarnaast zijn er nog heel veel mensen die mijn tijd in het LUMC onvergetelijk hebben gemaakt. Lieve kamergenootjes van D6-35, ook al zat ik soms verstopt onder m’n roze koptelefoon, wat hebben we een fijne tijd samen gehad! Ik dank alle mensen op het Heelkunde Lab voor experimentele hulp en gezelligheid! En in het bijzonder alle mensen van de Quax groep voor de gezelligheid tijdens de lunch en borrels!

Dan zijn er te veel vriendinnetjes die ik niet allemaal persoonlijk kan bedanken. Lieve OD-appeltjes, Bondies, Mooie Medico’s, Zwollywood-vriendinnetjes, huisgenootjes van de JPH en Villa Falcka – dank voor al jullie interesse, luisterende oren en lieve kaartjes gedurende mijn promotie en vooral voor alle fantastische

momenten, late uurtjes, mooie feestjes die helemaal niets met promotie of ziekenhuis te maken hadden!

Lieve Sab, wat een mooie tijden hebben we meegemaakt op D6-35. Je was mijn steun en toeverlaat tijdens de lange dagen van wetenschap bedrijven in het LUMC en wat hebben we prachtige herinneringen aan onze roze Elisabeth en samen met Mark als 'axolotl sub' in de Eigenzorg. Dank voor alles en fantastisch dat je vandaag als paranimf naast me staat!

Lieve Er, allerallerliefste broer. Wij zijn nog nooit zonder elkaar geweest en jij kent mij door-en-door. Je inspireert me, daagt me uit en houdt me scherp. Ik ben vreselijk op trots op alles wat je doet en hoe je in het leven staat. Dank dat je ook vandaag naast me staat!

Lieve papa en mama, dank voor het eindeloze, rotsvaste vertrouwen dat jullie in Er en mij hebben. Jullie vangnet is oneindig groot en dat maakt dat wij kunnen vliegen.
Zonder jullie nuchtere kijk op de wereld en grenzeloze hulp was ik nooit zo ver gekomen.

Lieve familie van Nauta Lemke/Sorrentino, dank voor jullie interesse, steun en mental support de afgelopen jaren, ontzettend warm om een tweede familie in Den Haag en Miami te hebben!

Allerallerliefste Ali, sorry voor alle avonden en weekenden die ik in m'n eentje achter mijn computer heb doorgebracht. Dank voor je begrip wanneer ik weer eens in mijn promotie-tijdmachine zat en voor je standvastigheid mij daar af en toe uit te slepen! Zonder jouw oneindige geduld, relativeringsvermogen, grappen, liefde, onuitputtelijke energie, vrolijkheid en steun was dit alles nooit gelukt! Wat heerlijk dat het erop zit, echt een prestatie van ons samen!