



Universiteit
Leiden
The Netherlands

Ischemia/reperfusion injury : a metabolic meltdown

Wijermars, L.G.M.

Citation

Wijermars, L. G. M. (2018, March 21). *Ischemia/reperfusion injury : a metabolic meltdown*. Retrieved from <https://hdl.handle.net/1887/61202>

Version: Not Applicable (or Unknown)

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/61202>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/61202> holds various files of this Leiden University dissertation.

Author: Wijermars, L.G.M.

Title: Ischemia/reperfusion injury : a metabolic meltdown

Issue Date: 2018-03-21

VIII

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

Article under peer review

Leonie G.M. Wijermars, Alexander F. Schaapherder,
Thomas George, Carl M. Hurt, Eric R. Gross

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, $37 \pm 12^*$ 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 $36.5 \pm 13.0^*$, between 7-14 days $44.2 \pm 13.0^*$, 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.

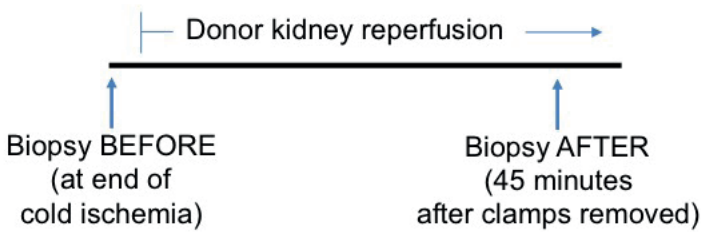
Paired renal cortical biopsies were obtained at the end of the cold ischemic period (prior to implantation) and 45 min after reperfusion of the kidney in the recipient (Figure 1A). Kidney biopsies from an initial 18 donor kidneys were used to conduct the whole genome array portion of the study. In this 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).

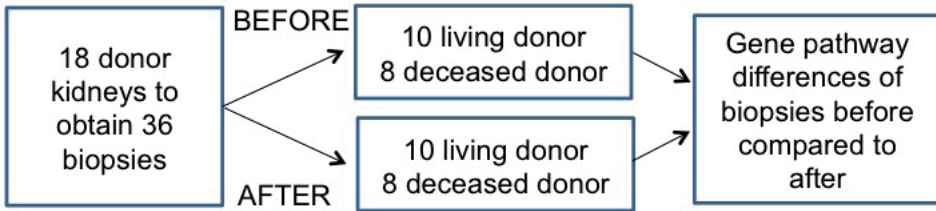
Renal allograft transplantations were perfused and stored with either University of Wisconsin solution or Custodiol® HTK (histidine-tryptophan-ketoglutarate) solution. None of the grafts were machine perfused. For renal transplantation, all patients were induced by propofol, sufentanil and atracurium. 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.



A. Biopsy Timeline



B. Gene Array Studies



C. Validation Studies

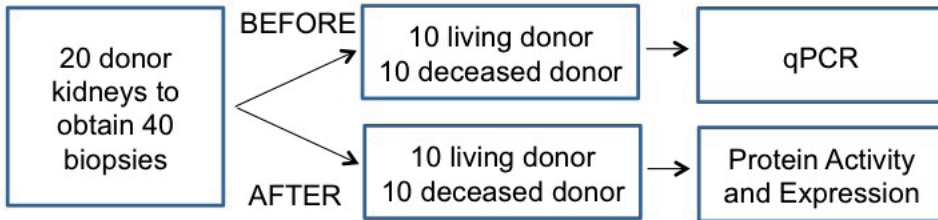


Figure 1. Timeline and allocation of renal biopsies obtained for the study. A. Two biopsies were taken for each transplanted kidney. One biopsy was obtained prior to reperfusion of the kidney at the end of the cold ischemia period. The second biopsy was obtained 45 minutes after the kidney was reperfused. 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 RNeasy (Qiagen, Crawley, 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 (Ambion 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 µl that contained 15 µl of Fast SYBR® Green Master Mix (Life Technologies), 1000nM primer (ALDH₂, ALDH_{7A1} or GAPDH) or 500nM primer (ALDH_{4A1}), 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), ALDH_{4A1} (Abcam), ALDH_{7A1} (Abcam) and ALDH_{1A1} (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 pathways¹⁵ 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

<i>PATHWAY</i>	<i>Difference in value</i>	<i>Genes only upregulated in living donors</i>	<i>Genes only upregulated in deceased donors +DGF</i>	<i>Common</i>
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
Glutathione-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 $1314.5 \pm 76.6^*$, ALDH7A1: 1739.9 ± 65.5 versus $1230.4 \pm 83.1^*$, $*p=0.001$) 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).

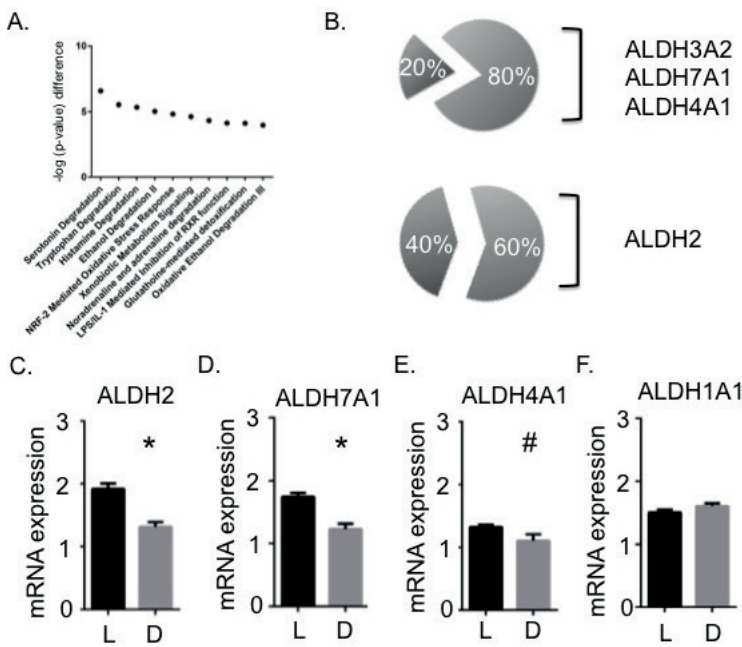
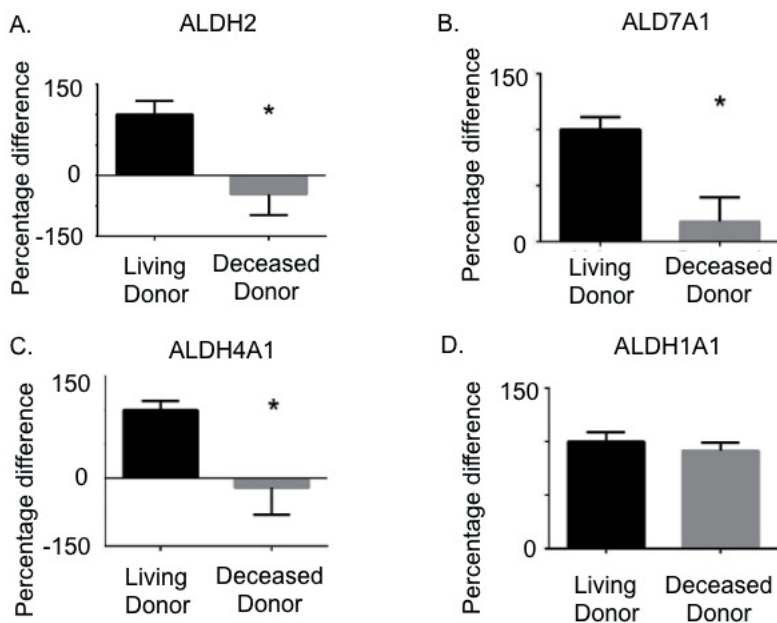


Figure 2. Summary of gene array studies. A. Whole genome array changes before and after transplantation were compared from each living donor and +DGF deceased donor kidney using Ingenuity pathway analysis. The difference in P-values between living donor kidneys and deceased donor kidneys gene arrays 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 ALDH7A1 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. $5.7 \pm 0.8^*$, ALDH7A1 3.7 ± 0.4 vs. $5.6 \pm 0.6^*$ and ALDH4A1: 2.5 ± 0.3 vs. $4.6 \pm 0.6^{**}$, *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: $1.2 \pm 0.1^*$ vs. 0.7 ± 0.07 , *p<0.001, ALDH4A1: $1.7 \pm 0.3^*$ 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. ALDH_{7A1} C. ALDH_{4A1} D. ALDH_{1A1} (used for reference). ALDH₂, ALDH_{7A1} and ALDH_{4A1} 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.*



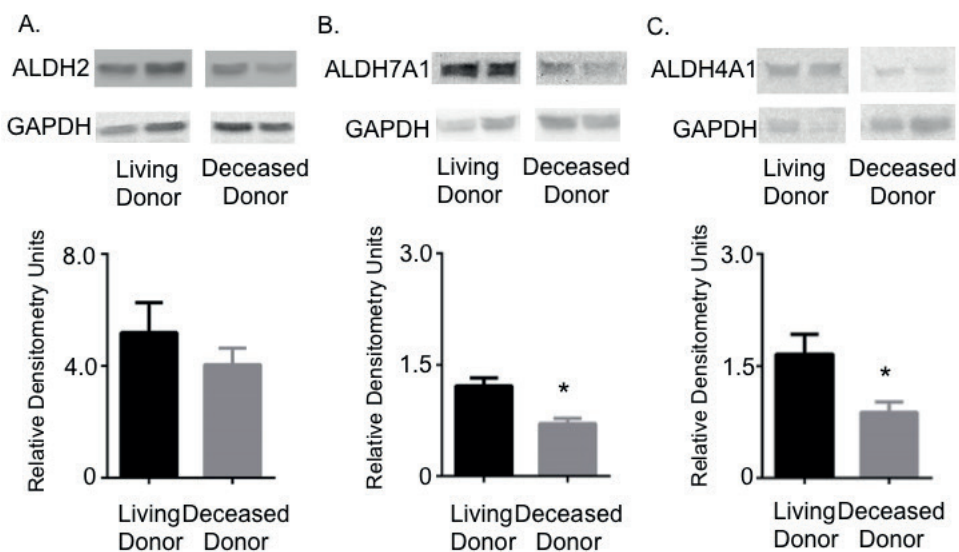
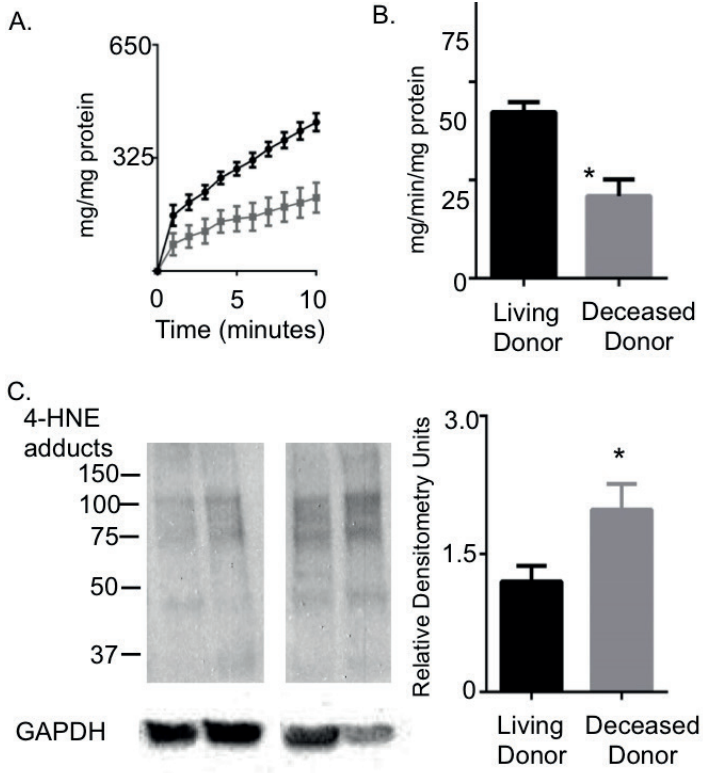


Figure 4. Western blot from kidney biopsies taken after reperfusion of transplanted kidneys. A. ALDH2 B. ALDH7A1 C. ALDH4A1. All western blots were normalized to GAPDH. Both ALDH7A1 and ALDH4A1 were significantly different in kidneys of living donors compared to kidneys of deceased donors that developed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

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. $36.9 \pm 11.5^*$ 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, $n=6/\text{group}$, 4-HNE adducts: 1.2 ± 0.2 vs. $1.9 \pm 0.3^*$ $p < 0.028$).

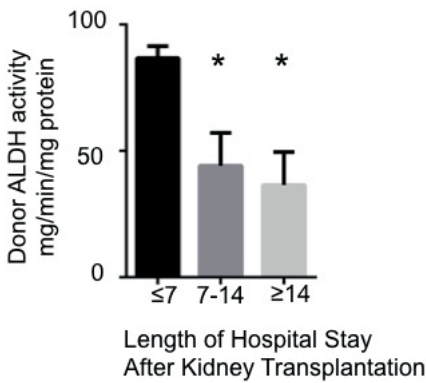




*Figure 5. ALDH activity and reactive aldehyde adducts from kidney biopsies taken after reperfusion of transplanted kidneys. A. ALDH enzymatic activity when challenged with acetaldehyde. B. Cumulative ALDH enzymatic activity. C. 4-hydroxynonenal-induced protein adducts Western blots were normalized to 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. $44.2 \pm 13.0^*$ for length of stay between 7 and 14 days, vs. $36.5 \pm 13.0^*$ 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 following kidney transplantation and preserving ALDH enzymes or increasing ALDH activity may potentially decrease the incidence of DGF by increasing reactive aldehyde metabolism.



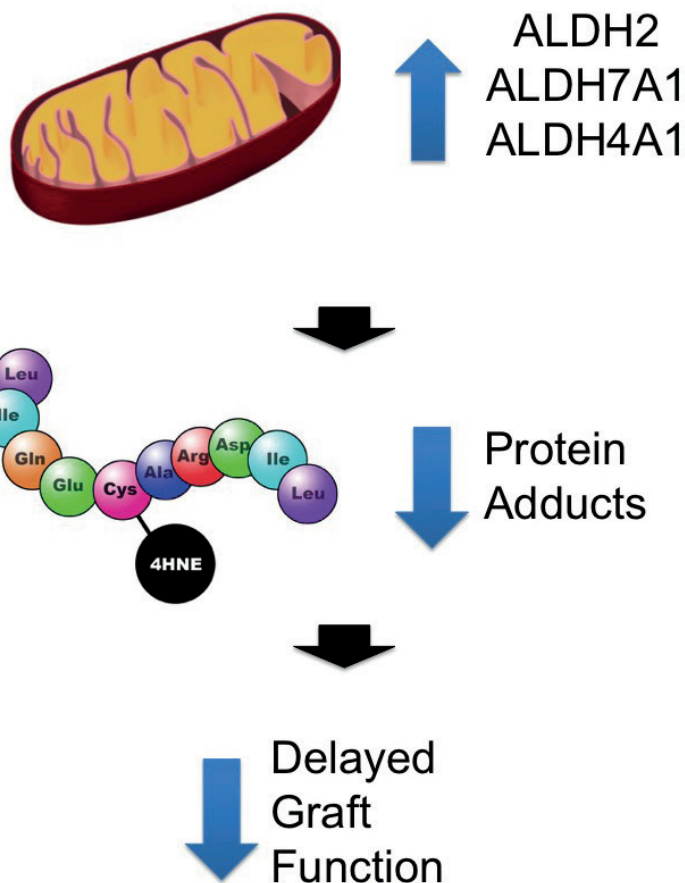
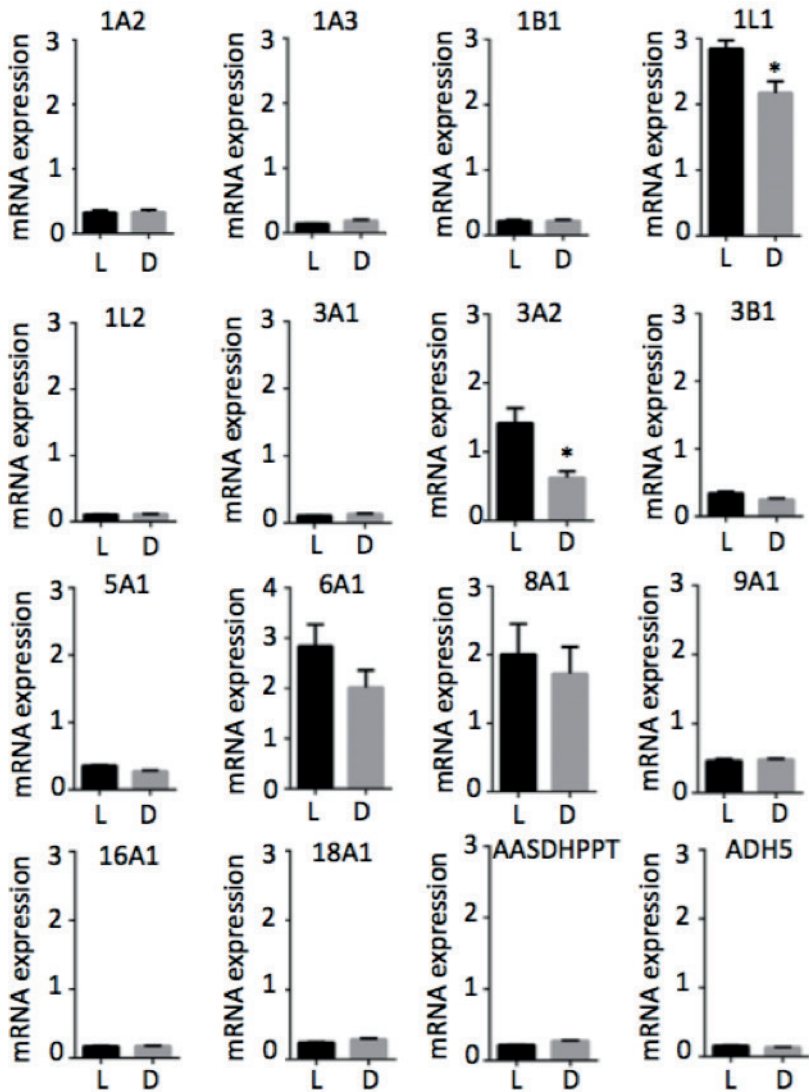


Figure 7. Summary figure. ALDH enzymes are important in clearing toxic reactive aldehydes including 4-HNE in transplanted kidneys. The ALDH enzymes decrease 4-HNE-induced protein adduct formation, and overall reduce the amount of reactive aldehydes that are associated with DGF.

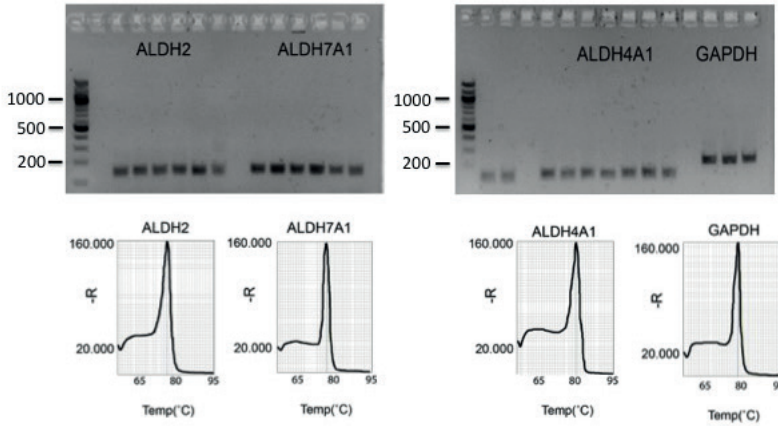


SUPPLEMENTARY FIGURES

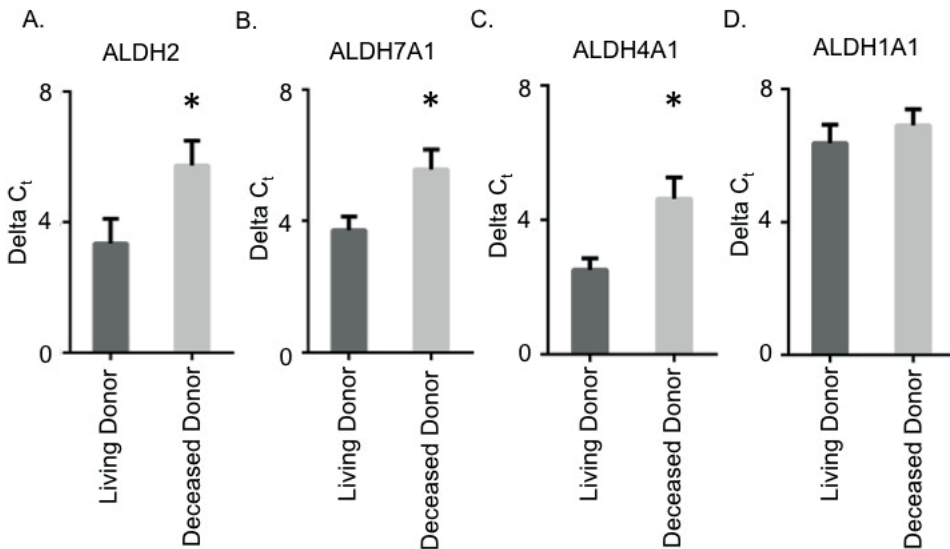


*Supplemental Figure 1. ALDH family of enzymes gene array results. 16 additional ALDH enzyme family genes. Of the 16 genes, only ALDH1L1 and ALDH3A2 showed significant differences between the living donor kidneys compared to the deceased donor kidneys. *P<0.01.*





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. ALDH_{7A1} C. ALDH_{4A1} D. ALDH_{1A1}. 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 ALDH_{7A1} and ALDH_{4A1} (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	GAGAAGGCTGGGGCTCATT	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*
<i>Recipient – cause of renal failure</i>		
*Glomerulonefritis	40%	12.5%
*Polycystic kidney disease	20%	25%
*DM type 2	10%	12.5%
*Obstructive uropathy	10%	0%
*Maligne hypertension	0%	0%
*Renal failure e.c.i.	20%	50%
<i>Donor cause of death</i>		
*Living donor	100%	
*CVA		0%
*SAB		25%
*TRAUMA		37.5%
*CA-OHCA-AMI		25%
*Suicide		0%
*Miscellaneous		12.5%



<i>Histocompatibility (HLA mismatches, %)</i>		
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, significant 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*
<i>Recipient – cause of renal failure</i>		
*Glomerulonephritis	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%
<i>Donor cause of death</i>		
*Living donor	100%	
*CVA		20%
*SAB		20%
*TRAUMA		20%
*CA-OHCA-AMI		30%
*Suicide		10%
*Miscellaneous		0%



<i>Histocompatibility (HLA mismatches, %)</i>		
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, significant 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, ALDH_{4A1}, ALDH_{7A1} and ALDH_{1A1} 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).



REFERENCES

1. Wu WK, Famure O, Li Y, Kim SJ: Delayed graft function and the risk of acute rejection in the modern era of kidney transplantation. *Kidney Int* 2015; 88: 851-8
2. Hassanain M, Tchervenkov JI, Cantarovich M, Metrakos P, Paraskevas S, Keith D, Baran D, Fernandez M, Mangel R, Chaudhury P: Recovery of graft function early posttransplant determines long-term graft survival in deceased donor renal transplants. *Transplant Proc* 2009; 41: 124-6
3. Hassanain M, Tchervenkov J, Cantarovich M, Metrakos P, Paraskevas S, Keith D, Baran D, Fernandez M, Mangel R, Chaudhury P: Delayed graft function has an equally bad impact on deceased donor renal graft survival in both standard criteria donors and expanded criteria donors. *Transplant Proc* 2009; 41: 133-4
4. Sainz MM, Toro JC, Poblete HB, Perez LF, Nicovani VH, Carrera MG: Incidence and factors associated with delayed graft function in renal transplantation at Carlos Van Buren Hospital, January 2000 to June 2008. *Transplant Proc* 2009; 41: 2655-8
5. Schroppe B, Legendre C: Delayed kidney graft function: from mechanism to translation. *Kidney Int* 2014; 86: 251-8
6. Bolli R, Jeroudi MO, Patel BS, DuBose CM, Lai EK, Roberts R, McCay PB: Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proc Natl Acad Sci U S A* 1989; 86: 4695-9
7. Chen CH, Budas GR, Churchill EN, Disatnik MH, Hurley TD, Mochly-Rosen D: Activation of aldehyde dehydrogenase-2 reduces ischemic damage to the heart. *Science* 2008; 321: 1493-5
8. Esterbauer H, Schaur RJ, Zollner H: Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991; 11: 81-128
9. Uchida K, Stadtman ER: Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase. A possible involvement of intra- and intermolecular cross-linking reaction. *J Biol Chem* 1993; 268: 6388-93
10. Bang S, Kim KY, Yoo S, Kim YG, Hwang SW: Transient receptor potential A1 mediates acetaldehyde-evoked pain sensation. *Eur J Neurosci* 2007; 26: 2516-23
11. Hill BG, Dranka BP, Zou L, Chatham JC, Darley-Usmar VM: Importance of the bioenergetic reserve capacity in response to cardiomyocyte stress induced by 4-hydroxynonenal. *Biochem J* 2009; 424: 99-107
12. Zambelli VO, Gross ER, Chen CH, Gutierrez VP, Cury Y, Mochly-Rosen D: Aldehyde dehydrogenase-2 regulates nociception in rodent models of acute inflammatory pain. *Sci Transl Med* 2014; 6: 251ra118
13. de Vries DK, Lindeman JH, Tsikas D, de Heer E, Roos A, de Fijter JW, Baranski AG, van Pelt J, Schaapherder AF: Early renal ischemia-reperfusion injury in humans is dominated by IL-6 release from the allograft. *Am J Transplant* 2009; 9: 1574-84
14. de Vries DK, van der Pol P, van Anken GE, van Gijlswijk DJ, Damman J, Lindeman JH, Reinders ME, Schaapherder AF, Kooten C: Acute but transient release of terminal complement complex after reperfusion in clinical kidney transplantation. *Transplantation* 2013; 95: 816-20
15. Wijermars LG, Schaapherder AF, de Vries DK, Verschuren L, Wust RC, Kostidis S, Mayboroda OA, Prins F, Ringers J, Bierau J, Bakker JA, Kooistra T, Lindeman JH: Defective postreperfusion metabolic recovery directly associates with incident delayed graft function. *Kidney Int* 2016; 90: 181-91
16. Jackson B, Broucker C, Thompson DC, Black W, Vasiliou K, Nebert DW, Vasiliou V: Update on the aldehyde dehydrogenase gene (ALDH) superfamily. *Hum Genomics* 2011; 5: 283-303



17. Brocker C, Cantore M, Failli P, Vasiliou V: Aldehyde dehydrogenase 7A1 (ALDH7A1) attenuates reactive aldehyde and oxidative stress induced cytotoxicity. *Chem Biol Interact* 2011; 191: 269-77
18. Yoon KA, Nakamura Y, Arakawa H: Identification of ALDH4 as a p53-inducible gene and its protective role in cellular stresses. *J Hum Genet* 2004; 49: 134-40
19. Chen CH, Ferreira JC, Gross ER, Mochly-Rosen D: Targeting aldehyde dehydrogenase 2: new therapeutic opportunities. *Physiol Rev* 2014; 94: 1-34
20. Malyszko J, Lukaszuk E, Glowinska I, Durlik M: Biomarkers of delayed graft function as a form of acute kidney injury in kidney transplantation. *Sci Rep* 2015; 5: 11684
21. Castellano G, Intini A, Stasi A, Divella C, Gigante M, Pontrelli P, Franzin R, Accetturo M, Zito A, Fiorentino M, Montinaro V, Lucarelli G, Ditunno P, Battaglia M, Crovace A, Staffieri F, Oortwijn B, van Amersfoort E, Pertosa G, Grandaliano G, Gesualdo L: Complement Modulation of Anti-Aging Factor Klotho in Ischemia/Reperfusion Injury and Delayed Graft Function. *Am J Transplant* 2015
22. Muller L, Nicolas-Robin A, Bastide S, Martinez O, Louart G, Colavolpe JC, Vachier F, Alonso S, Lefrant JY, Riou B, AzuRea G: Assessment of neutrophil gelatinase-associated lipocalin in the brain-dead organ donor to predict immediate graft function in kidney recipients: a prospective, multicenter study. *Anesthesiology* 2015; 122: 96-105
23. Fonseca I, Reguengo H, Oliveira JC, Martins LS, Malheiro J, Almeida M, Santos J, Dias L, Pedroso S, Lobato L, Henriques AC, Mendonca D: A triple-biomarker approach for the detection of delayed graft function after kidney transplantation using serum creatinine, cystatin C, and malondialdehyde. *Clin Biochem* 2015; 48: 1033-8
24. Gross ER, Zambelli VO, Small BA, Ferreira JC, Chen CH, Mochly-Rosen D: A personalized medicine approach for Asian Americans with the aldehyde dehydrogenase 2*2 variant. *Annu Rev Pharmacol Toxicol* 2015; 55: 107-27
25. Rothman DJ: Ethical and social consequences of selling a kidney. *JAMA* 2002; 288: 1640-1
26. Fischer M, Wetherill LF, Carr LG, You M, Crabb DW: Association of the aldehyde dehydrogenase 2 promoter polymorphism with alcohol consumption and reactions in an American Jewish population. *Alcohol Clin Exp Res* 2007; 31: 1654-9
27. Salomons GS, Bok LA, Struys EA, Pope LL, Darmin PS, Mills PB, Clayton PT, Willemsen MA, Jakobs C: An intriguing "silent" mutation and a founder effect in alantoin (ALDH7A1). *Ann Neurol* 2007; 62: 414-8
28. Brocker C, Lassen N, Estey T, Pappa A, Cantore M, Orlova VV, Chavakis T, Kavanagh KL, Oppermann U, Vasiliou V: Aldehyde dehydrogenase 7A1 (ALDH7A1) is a novel enzyme involved in cellular defense against hyperosmotic stress. *J Biol Chem* 2010; 285: 18452-63
29. Zhong Z, Hu Q, Fu Z, Wang R, Xiong Y, Zhang Y, Liu Z, Wang Y, Ye Q: Increased Expression of Aldehyde Dehydrogenase 2 Reduces Renal Cell Apoptosis During Ischemia/Reperfusion Injury After Hypothermic Machine Perfusion. *Artif Organs* 2016; 40: 596-603



