Nephrol Dial Transplant (2020) 35: 1320–1327
doi: 10.1093/ndt/gfy372
Advance Access publication 24 December 2018

ndt
Nephrology Dialysis Transplantation

ORIGINAL ARTICLE

Urinary mitochondrial DNA associates with delayed graft function following renal transplantation

Marcel P.B. Jansen[1], Wilco P.C. Pulskens[1], Melissa Uil[1], Nike Claessen[1], Gerrie Nieuwenhuizen[2], Dorien Standaar[2], Chi M. Hau[3], Rienk Nieuwland[3], Sandrine Florquin[1], Frederike J. Bemelman[2], Jaklien C. Leemans[1] and Joris J.T.H. Roelofs[1]

[1]Department of Pathology, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands, [2]Department of Nephrology, Renal Transplant Unit, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands and [3]Laboratory of Experimental Clinical Chemistry, Amsterdam Cardiovascular Sciences, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands

Correspondence and offprint requests to: Joris J.T.H. Roelofs; E-mail: j.j.roelofs@amc.nl

ABSTRACT

Background. Ischaemia-reperfusion (IR) injury is an important determinant of delayed graft function (DGF) affecting allograft function. Mitochondrial DNA (mtDNA) is released upon cell death and platelet activation into the extracellular environment and has been suggested to be a biomarker in several diseases. Whether extracellular mtDNA accumulates in plasma and/or urine upon renal IR and predisposes DGF is unknown.

Methods. C57BL/6J wild-type mice were subjected to renal IR. In addition, an observational case–control study was set up enrolling 43 patients who underwent kidney transplantation. One day post-IR in mice and a few days following renal transplantation in human, blood and urine were collected. Patients were stratified into DGF and non-DGF groups.

Results. mtDNA-encoded genes accumulate in urine and plasma in both mice subjected to renal IR injury and in humans following renal transplantation. In human renal transplant recipients, cold ischaemia time and renal function correlate with urinary mtDNA levels. Urinary mtDNA levels but not urinary nuclear DNA levels were significantly higher in the DGF group compared with the non-DGF group. Multiple receiver operating characteristic curves revealed significant diagnostic performance for mtDNA-encoded genes cytochrome c oxidase III (COXIII); nicotinamide adenine dinucleotide hydrogen subunit 1 (NADH-deh); mitochondrially encoded, mitochondrially encoded nicotinamide adenine dinucleotide dehydrogenase 2 (MT-ND2) with an area under the curve of, respectively, 0.71 [P = 0.03; 95% confidence interval (CI) 0.54–0.89], 0.75 (P = 0.01; 95% CI 0.58–0.91) and 0.74 (P = 0.02; 95% CI 0.58–0.89).

Conclusions. These data suggest that renal ischaemia time determines the level of mtDNA accumulation in urine, which associates with renal allograft function and the diagnosis of DGF following renal transplantation.

Keywords: AKI, delayed graft function, graft failure, ischaemia reperfusion injury, kidney transplantation

INTRODUCTION

Renal ischaemia-reperfusion (IR) injury is an inevitable event in kidney transplantation [1]. Ischaemia results in cell stress and/or damage, which is paradoxically amplified when blood supply is restored. The injuries generate inflammatory and oxidative damage that together form an important risk factor for the development of delayed graft function (DGF) [2]. DGF is considered to be a manifestation of IR-induced acute kidney injury (AKI) and is associated with adverse allograft and patient outcomes [3–7] and significant health care expenses due to prolonged hospitalization and costly patient management. In concordance with the use of expanded criteria donors, the incidence of DGF has increased in recent years, with an incidence rate ranging between 20% and 45% [3, 8, 9]. To date, there is no effective treatment for DGF; however, early diagnosis and therapeutic intervention (e.g. change of immunosuppression) may improve graft and patient survival. Therefore markers for diagnosing early acute IR-induced renal injury are required.

Circulating cell-free mitochondrial DNA (mtDNA) has been detected and used as a potential marker in various human diseases, in particular in conditions with acute tissue injury such as trauma [10, 11] and acute single-organ injury [12]. Major cellular stress, mitochondrial dysfunction and uncontrolled cell death are key determinants in the release of mtDNA. In addition, during inflammation and upon stimulation, activated platelets have been shown to release mtDNA [13, 14].

Outside of the mitochondrial matrix, mtDNA acts as a damage-associated molecular pattern that can trigger inflammatory responses through Toll-like receptors, nucleotide binding domain and leucine-rich repeat protein 3 and the cyclic guanosine monophosphate–adenosine monophosphate synthase–stimulator of interferon genes signal pathway leading to inflammation [11] and even organ dysfunction [15]. To date, it is unknown whether extracellular mtDNA levels can be used as

early markers for diagnosing DGF. Hence a key goal of the present study was to explore the role of renal IR on mtDNA accumulation and examine the efficacy of plasma and urine mtDNA as a diagnostic marker of DGF.

MATERIALS AND METHODS

Patient inclusion and sample preparation

We enrolled 43 patients who underwent kidney transplantation at the Academic Medical Center in Amsterdam, The Netherlands, between January 2013 and December 2015. In addition, we included 10 healthy volunteers. DGF was defined as the requirement for dialysis during the first week after transplantation. The institutional ethical review board of the University of Amsterdam approved the study (METc 2012_162). Written informed consent was obtained from all volunteers and patients. The study was conducted according to the principles of the Declaration of Helsinki. Blood ($n = 51$) and urine ($n = 53$) samples were collected within 14 days following renal transplantation. Urine was collected and 10 mL of peripheral blood was drawn and collected using an open system in tubes containing 1 mL citrate. Both were processed immediately. In order to obtain platelet-free plasma, blood/plasma was centrifuged twice for 20 min at 1550 *g* at room temperature. To obtain sediment-free urine, urine samples were centrifuged for 10 min at 180 *g* at 4°C, followed by two times for 20 min at 1550 *g* at 4°C. Aliquotes were stored at −80°C until use.

Induction of murine renal I/R

Pathogen-free 9- to 12-week-old male C57BL/6 wild-type (WT) mice were purchased from Charles River Laboratories (Wilmington, MA, USA). Age- and sex-matched WT mice were used in all experiments as a control. The Animal Care and Use Committee of the University of Amsterdam approved all experiments. The renal IR procedure was performed under 2% isoflurane and subcutaneous 0.1 mg/kg Temgesic (Schering-Plough, Kenilworth, NJ, USA) as described previously [16]. To induce renal IR injury, renal pedicles were clamped using non-traumatic vascular clips. For the clamping time titration experiment, mice were subjected to bilateral renal ischaemia for 0 (sham), 15, 20 or 25 min (respectively, $n = 6$, $n = 4$, $n = 6$, $n = 6$), followed by a reperfusion phase of 1 day. For determining urinary platelet factor 4 (PF4) following renal IR, mice ($n = 8$) were subjected to bilateral renal ischaemia for 45 min and urine was collected before renal ischaemia and after 24 h of ischaemia. For determining extracellular DNA in plasma and urine and evaluating platelet thrombus formation in renal tissue following renal IR, mice ($n = 8$) were subjected to bilateral renal ischaemia for 45 min, and sacrificed after 24 h. Sham-operated mice ($n = 8$) underwent the same procedure without clamping of the renal pedicles.

Isolation of free extracellular DNA

Human and murine plasma and urine were processed using the QIAamp DNA mini kit (Qiagen, Venlo, The Netherlands) to isolate free extracellular DNA, which was subsequently stored at −80 C.

Real-time quantitative polymerase chain reaction (qPCR)

All DNA samples were extracted from 250 µL plasma or urine. Real-time qPCR was performed on a Lightcycler 480 Real-time PCR system (Roche Diagnostics, Almere, The Netherlands) using SYBR Green PCR master mix (Thermo Fisher, Uden, The Netherlands). SYBR green dye intensity was analysed with linear regression analysis using LinRegPCR v12.4. To detect and amplify murine mtDNA, the following primers were used: 12S (12S ribosomal RNA; forward: 5′-CTAGCCACACCCCCACGGGA-3′, reverse: 5′-CGTATGACC GCGGTGGCTGG-3′), ND1 [nicotinamide adenine dinucleotide hydrogen subunit 1 (NADH1); forward: 5′-CAAACCGGG CCCCCTTCGAC-3′, reverse: 5′-CGAATGGGCCGGCTGCGT AT-3′] and cytochrome c oxidase subunit 1 (COX1; forward: 5′-CCAGTGCTAGCCGCAGGCAT-3′, reverse: 5′-TTGGGTCC CCTCCTCCAGCG-3′). To detect and amplify human mtDNA, the following primers were used: cytochrome c oxidase III (COXIII; forward: 5′-ATGACCCACCAATCACATG C-3′, reverse: 5′-ATCACATGGCTAGGCCGGAG-3′), NADH-deh (forward: 5′-ATACCCATGGCCAACCTCCT-3′, reverse: 5′-GGGCCTTTGCGTAGTTGTAT-3′) and MT-ND2 (forward: 5′-CTCACATGACAAAAACTAGCCCCCA-3′, reverse: 5′-TCCACCTCAACTGCCTGCTATGA-3′). To detect and amplify human non-coding DNA (ncDNA), the following primers were used: glyceraldehyde-3-phosphate dehydrogenase (GAPDH; forward: 5′-AGGGCCCTGACAACTCTTTT-3′, reverse: 5′-TTACTCCTTGGAGGCCATGT-3′) [11]. PCRs were run in the same analysis and analysed in duplicates and a no-template control was included in the analysis. Since cell-free plasma and urine samples were used to approximate extracellular DNA levels, a correction for cell count was not needed and therefore the transcript levels were not corrected for cell count by normalizing with a reference gene such as a housekeeping gene. All primers were ordered from and synthesized by Invitrogen (Thermo Fisher, Uden, The Netherlands). To confirm the specificity of PCR amplification products, melting curve analysis was used.

Enzyme-linked immunosorbent assay (ELISA)

Platelet activation marker human CXCL4/PF4 or murine CXCL4/PF4 were measured in plasma and urine using specific ELISAs (R&D Systems, Oxon, UK) according to the manufacturer's protocol.

Histopathology

Murine renal tissue was fixed and processed as previously described [17]. Paraffin-embedded sections were used for periodic acid–Schiff diastase (PAS-D) staining. The degree of tubular damage was assessed on PAS-D-stained 4-µm-thick sections by scoring tubular cell necrosis in 10 non-overlapping high-power fields (magnification ×40) in the corticomedullary junction. The degree of injury was scored by a pathologist in a blinded fashion on a 5-point scale: 0, no damage; 1, 10% of the

corticomedullary junction injured; 2, 10–25% injured; 3, 25–50% injured; 4, 50–75% injured; 5 = >75% injured. In order to determine platelet thrombus formation in renal tissue, Glycoprotein 1 beta alpha (GP1ba), clone SP219 (dilution 1: 200, Spring Bioscience, Pleasanton, CA, USA) was used and visualized with 3,3-diaminobenzidine (DAB) or Vector Blue. The percentage of positive GP1ba staining in 10 non-overlapping high-power fields was quantified using FIJI image analysis software.

Statistical analysis

All data sets were tested for their distribution prior to analyses. Differences between experimental groups were determined using Mann–Whitney U test. Statistical analysis on human data was performed using Kruskal–Wallis with Dunn's *post hoc* testing. Correlations were performed using Spearman's test. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software, La Jolla, CA, USA). All data are presented as mean ± standard error of the mean (SEM). A P-value <0.05 was considered statistically significant.

RESULTS

Renal IR results in mtDNA accumulation that associates with renal injury and intrarenal platelet activation

To analyse whether renal IR leads to enhanced exposure of extracellular mtDNA levels *in vivo*, renal IR was performed in mice and blood and urine samples were collected to measure the levels of mtDNA-encoded genes NADH, 12S, COXI. Both plasma and urine mtDNA levels were increased following ischaemia AKI compared with sham controls, demonstrating that renal IR injury leads to elevated levels of mtDNA accumulation *in vivo* (Figure 1A and B). As a consequence of renal IR, renal function decreased, as shown by increases in creatinine and urea in plasma (Figure 1C and D). Extracellular mtDNA can be liberated upon cell death and platelet activation [10, 14]. In line with these findings, we observed platelet microthrombi in renal peritubular vessels located in the corticomedullary regions (Figure 1E–G) upon renal IR. In addition, platelet activation marker PF4 in urine was significantly elevated (Figure 1H) following renal IR. PAS-D and platelet double staining of renal sections following renal ischaemia time titration revealed an ischaemia time-dependent increase of platelet microthrombi concomitant with acute renal necrosis (Figure 1I). Correlation analysis revealed that ischaemia time significantly correlates with both platelet microthrombi formation ($P < 0.0001$, $r = 0.77$) and renal injury ($P < 0.0001$, $r = 0.97$). These results demonstrate that renal IR injury results in the liberation of mtDNA, which associates with intrarenal platelet activation and renal cell death.

Renal transplantation results in increased plasma and urinary mtDNA

IR leading to renal injury is an unavoidable consequence of renal transplantation [1]. As shown in our mouse model, renal IR increases mtDNA levels in urine and plasma. To analyse whether renal IR leads to enhanced extracellular mtDNA accumulation in humans in the context of the renal transplantation setting, an observational case–control study was performed in which we enrolled 43 cases that received a kidney transplant and 10 healthy controls between January 2013 and January 2015. Blood samples and urine were collected within 14 days following transplantation. The demographic and clinical characteristics of the subjects are shown in Table 1. The level of circulating total extracellular DNA, measured as mtDNA-encoded genes COXIII, NADH-deh, mitochondrially encoded NADH dehydrogenase 2 (MT-ND2) and ncDNA-encoded gene GAPDH, was determined in cell-free plasma and sediment-free urine. Both plasma and urine mtDNA were increased following transplantation compared with healthy controls. Likewise, ncDNA was increased (Figure 2A and B). In addition, in urine both renal platelet activation marker PF4 (Figure 2C) and injury marker kidney injury molecule-1 (KIM-1) (Figure 2D) were increased, indicating that renal transplantation results in renal injury and intrarenal platelet activation. To investigate renal platelet thrombi formation, we stained allograft biopsies for platelets. However, platelet microthrombi formation could be detected in one patient only (data not shown). Consistent with results obtained in the *in vivo* renal IR model, these findings indicate that renal IR as a consequence of renal transplantation results in mtDNA accumulation, renal injury and local renal platelet activation.

Cold ischaemia time in a renal transplantation setting correlates with urinary mtDNA release, which associates with DGF development following renal transplantation

Ischaemia impacts renal transplant function and negatively affects renal transplant survival [18]. Also in our study, graft function was inversely correlated with the cold ischaemia time ($P < 0.0001$, $r = 0.69$). To evaluate whether ischaemia associates with the release of extracellular DNA in renal transplant recipients, we performed correlation analysis between the patients' cold ischaemia time and plasma and urine ncDNA and mtDNA levels. No correlations were found between cold ischaemia time and plasma ncDNA, plasma mtDNA levels (Table 2) or urinary ncDNA levels (Table 3). In addition, in contrast to our *in vivo* mouse model, no correlation was found between ischaemia time and intrarenal platelet activation and renal injury in human (Table 3). Correlation was found between cold ischaemia time and urinary mtDNA levels (Table 3). To investigate whether DNA release is associated with renal function, we performed correlation analysis between patients' plasma and urinary ncDNA and mtDNA levels and plasma creatinine concentration. This revealed no correlation between plasma ncDNA and mtDNA concentrations and plasma creatinine levels (Table 2); however, a significant correlation was found between urinary ncDNA and mtDNA and plasma creatinine levels (Table 3). Correlation was detected between urine PF4 and urinary ncDNA and mtDNA levels, but no correlation was found between urinary KIM-1 and urinary ncDNA and mtDNA levels (Table 3). In addition, no correlation was found between urinary PF4 and KIM-1 (Table 3). Immunosuppressive therapy with tacrolimus has been shown to enhance platelet aggregation and secretion [19] and causes

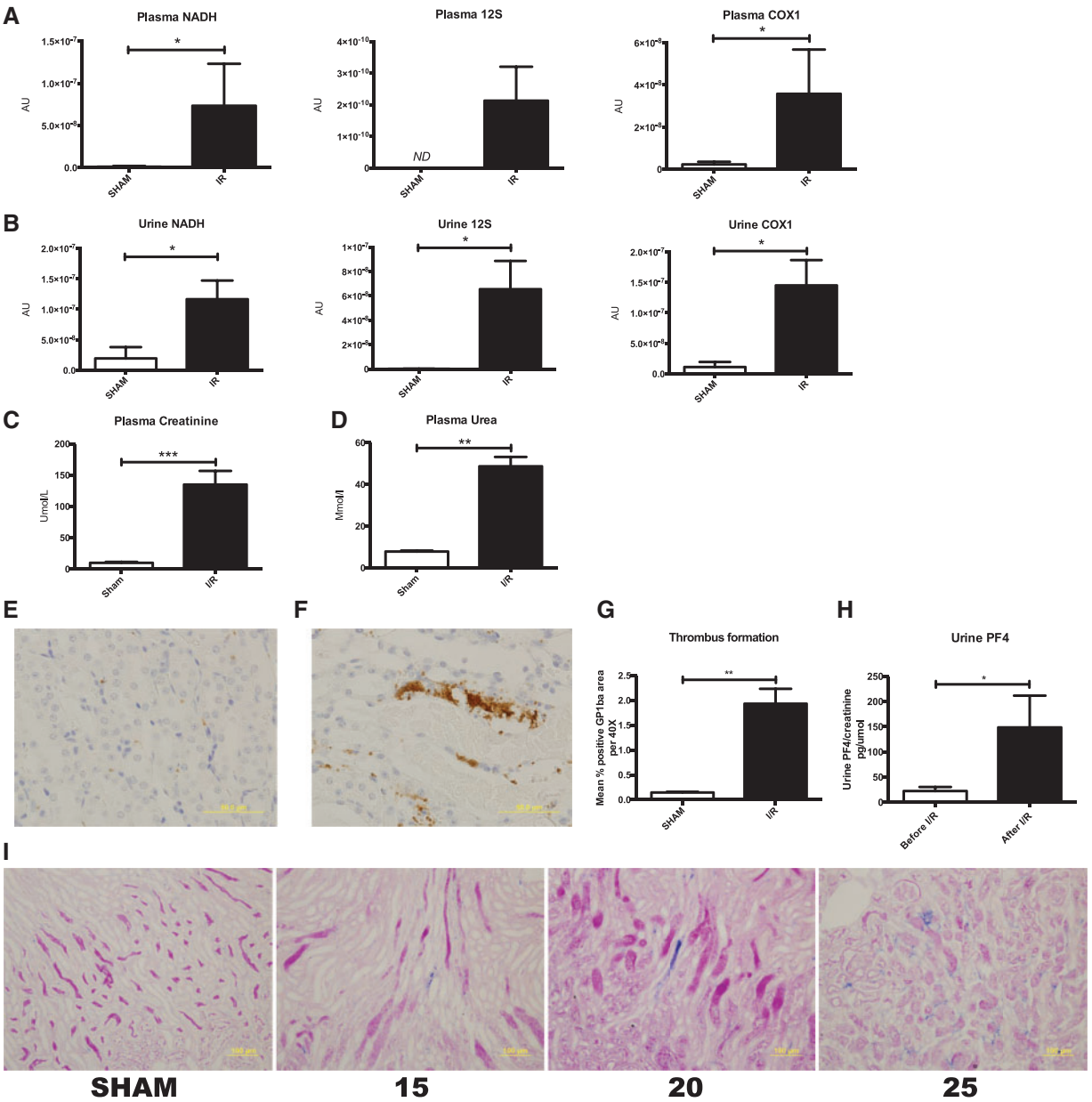

FIGURE 1: Renal IR results in mtDNA accumulation that associates with renal injury and intrarenal platelet activation. The level of free circulating mtDNA as reflected by mtDNA-encoded genes NADH, 12S and COX1 is enhanced in plasma and urine of mice subjected to renal IR-induced AKI ($n = 7$) compared with sham-operated mice ($n = 6$), as reflected by (**A**) plasma and (**B**) urine levels. Renal dysfunction and platelet activation as reflected by levels of (**C**) plasma creatinine and (**D**) plasma urea. Representative pictures of GP1ba stained renal tissue sections of SHAM-operated mice ($n = 8$), 40× magnification, scale bar = 50μm, (**E**) and mice subjected to renal I/R with 1 day of reperfusion ($n = 8$), 40× magnification, scale bar = 50μm (**F**). Percentage of positive GP1ba staining in 10 non-overlapping fields was quantified using image analysis software FUJI (**G**). Intra-renal platelet activation as reflected by urinary platelet factor 4 (PF4) was measured before and 1 day after renal I/R (**H**). Representative pictures of GPIba stained pictures of PAS-D (pink) / GP1ba (blue) double stained renal tissue sections 20× magnification, scale bar=100μm of renal ischemia time experiment with 0 ($n = 6$), 15 ($n = 4$), 20 ($n = 6$), 25 ($n = 6$) minutes of renal artery clamping, and 24 hours of reperfusion (**I**). AU: arbitrary units. Data are mean ± SEM. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$.

nephrotoxicity [20]. However, no correlation could be detected when performing correlation analysis between patients' plasma tacrolimus levels and urinary mtDNA, ncDNA, PF4 and KIM-1 levels (Table 3). To explore whether local renal DNA release is associated with DGF, we compared urinary ncDNA and mtDNA expression levels in renal transplant recipients that developed DGF with renal transplant recipients that did not develop DGF. This uncovered a significantly higher expression of urinary mtDNA, but not urinary ncDNA, in renal transplant recipients that developed DGF (Figure 3A). Urinary PF4 and KIM-1 did not differ between the DGF and non-DGF group (Figure 3B and C). In addition, no difference was seen in plasma mtDNA and plasma ncDNA between the DGF and non-DGF group (Supplementary data, Figure S1). Multiple receiver

operating characteristics (ROC) curves were used to determine the diagnostic performance of urinary ncDNA and mtDNA for DGF diagnosis. The area under the curve (AUC) for mtDNA-encoded genes COXIII, NADH-deh and MT-ND2 showed significant diagnostic performance, with an AUC of, respectively, 0.71 [P = 0.03, 95% confidence interval (CI) 0.54–0.89], 0.75 (P = 0.01, 95% CI 0.58–0.91) and 0.74 (P = 0.02, 95% CI 0.58–0.89) (Figure 3D). In contrast, the ROC curve of ncDNA-encoded gene GAPDH and urinary PF4 and KIM-1 showed poor diagnostic performance, with an AUCs of 0.62 (P = 0.22, 95% CI 0.43–0.80), 0.55 (P = 0.60, 95% CI 0.36–0.74) and 0.57 (P = 0.50, 95% CI 0.36–0.77), respectively (Figure 3E; Supplementary data, Figure 2A and B). Together, these findings indicate a renal ischaemia-dependent increase of extracellular mtDNA in urine upon renal transplantation, which may function as a prognostic marker for DGF.

Table 1. Patient characteristics

Patient demographics	Healthy controls	IR non-DGF	DGF
Total subjects	10	30	12
Male/female	5/5	23/7	7/5
Age (years)	46 ± 3.2	62 ± 22[a]	60 ± 4
Non-living/living donor (%)	NA	53/47	100/0[b]
Cold ischaemia time (min)	NA	390 ± 67	1047 ± 94[c]
Plasma creatinine (μmol/L)	NA	419 ± 51	335 ± 63
Sampling time after transplantation (days)	NA	8 ± 0.4	9 ± 0.9
Tacrolimus (μg/L)	NA	12.75 ± 1.2[d]	9.5 ± 1.4
Prednison	NA	21/30	11/12
Mycophenolate mofetil	NA	21/30	7/12

Data are presented as mean ± SEM, absolute numbers or percentages. NA indicates not applicable.
[a]The mean age in this group was significantly higher when compared with the healthy control group. P < 0.05 determined by analysis of variance followed by a Bonferroni *post hoc* test.
[b]Percentage of non-living donor recipients was significantly higher compared with the non-DGF group. P < 0.001 determined by Mann–Whitney U test.
[c]Mean cold ischaemia time was significantly higher when compared with the non-DGF group. P < 0.0001 determined by Mann–Whitney U test.
[d]Mean plasma tacrolimus was significantly higher when compared with the DGF group. P < 0.05 determined by Mann–Whitney U test.

DISCUSSION

The development of DGF is considered to be a manifestation of IR-induced AKI and is associated with adverse allograft and patient outcome [3–7]. Early diagnosis of renal IR injury and subsequent therapeutic intervention may prevent or ameliorate DGF and improve graft and patient survival. Therefore markers for diagnosing early acute renal injury that can be used to determine and perhaps target the development of DGF are needed. Recent studies have shown that extracellular mtDNA can function as a predictive marker in several diseases, including AKI following cardiac surgery [21] and AKI upon sepsis [22]. However, to date, it is unknown whether extracellular mtDNA can function as a marker for DGF diagnosis. Therefore, in the current study, the aim was to explore the role of extracellular

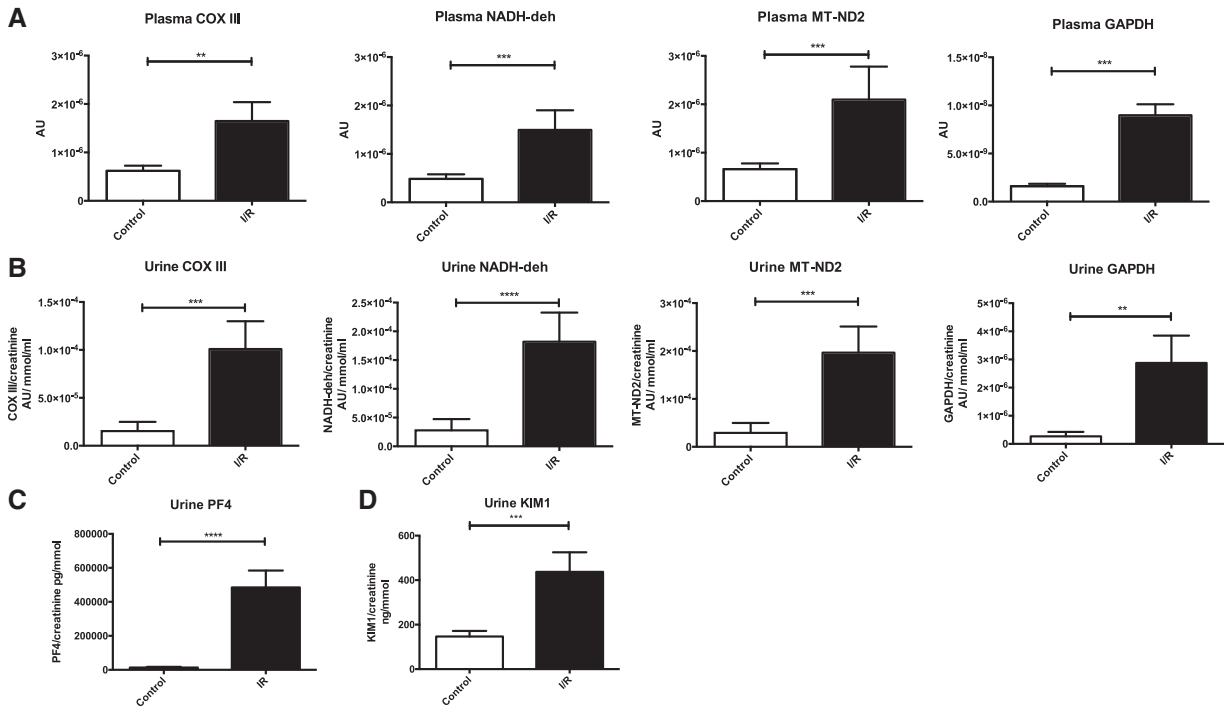

FIGURE 2: Renal transplantation results in increased plasma and urinary mtDNA and an increased local platelet activation. (**A**) Plasma and (**B**) urinary mtDNA levels as reflected by the mtDNA-encoded genes COXIII, NADH-deh and MT-ND2 and ncDNA levels as reflected by the housekeeping gene GAPDH in healthy controls ($n = 10$) and renal transplant recipients ($n = 43$). (**C**) Local renal platelet activation as reflected by urinary PF4 in healthy controls when compared with renal transplant recipients. (**D**) Renal tubular cell injury as reflected by urinary KIM-1 in healthy controls compared with renal transplant recipients. AU, arbitrary units. Data are mean ± SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001.

mtDNA as marker of renal IR injury in the context of renal transplantation and allograft function.

In the current study, we demonstrate significantly increased urinary mtDNA and plasma levels following renal IR injury *in vivo*. To explore whether mtDNA is also increased upon renal IR in the context of renal transplantation, we set up an observational case–control study including renal transplant recipients and examined the accumulation of extracellular mtDNA and ncDNA in plasma and urine. Since renal IR is an inevitable event in kidney transplantation [1], the allograft kidney serves as a model for studying renal IR injury. In accordance with our *in vivo* renal IR mouse model, we found increased levels of mtDNA but also ncDNA in plasma and urine of renal transplant recipients compared with healthy controls, indicating that renal transplantation in itself results in increased extracellular DNA accumulation. These findings are in line with previous studies demonstrating increased levels of extracellular DNA in urine and blood upon organ transplantation [23, 24].

Renal ischaemia time is a major determinant of renal IR injury and subsequent renal function, predisposing DGF [25]. Recently, increased accumulation of mtDNA in urine measured in non-diabetic CKD patients [26] and patients with diabetic nephropathy [27] has been associated with renal tissue scarring and function decline. In order to explore whether increased extracellular mtDNA and ncDNA levels are linked to renal ischaemia and renal function in renal transplant recipients, we performed a correlation analysis between urinary and plasma mtDNA and ncDNA levels and cold ischaemia time and plasma creatinine levels. This revealed that urinary mtDNA but not plasma mtDNA levels correlate with the degree of cold ischaemia time. In accordance, Whitaker *et al.* [21] demonstrated, in a mouse model of renal IR injury, a renal ischaemia time-dependent increase of urinary mtDNA levels. In addition, we showed that urinary mtDNA but not plasma mtDNA levels correlate with plasma creatinine levels, thus inversely correlating with renal function. Together, these data suggest that the release of intrarenal extracellular mtDNA, which is filtered by the kidney transplant, is dependent on renal ischaemia time and associates with renal allograft function.

Both cell injury and platelet activation have been associated with increased extracellular mtDNA accumulation [10, 13, 28]. In addition, in a previous study we demonstrated that platelets release mtDNA upon stimulation with thrombin [14]. In line with our *in vivo* data, showing increased intrarenal platelet activation and renal injury upon renal IR, we found that renal transplant recipients when compared with healthy controls had increased urinary levels of platelet activation marker PF4 as well as renal injury marker KIM-1. However, in contrast to our findings in mouse kidneys, human transplant biopsies did not show an accumulation of activated platelets, reflected by platelet microthrombi. This discrepancy may result from sampling bias, since the corticomedullary region of the kidney was rarely present in human transplant biopsies, while this region contained most of the accumulated platelets in mice. In addition, kinetics of platelet microthrombi formation may play a role since mouse kidneys were harvested 1 day after IR, while the human samples were taken 1–8 days after transplantation on average.

Taken together, these data indicate that intrarenal platelet activation and renal injury increased following renal IR in mice and, judging from the increased urinary PF4 levels and KIM-1 in human, also upon renal transplantation in human.

To evaluate the contribution of intrarenal platelet activation and renal cell injury to the release of extracellular mtDNA, we performed a correlation analysis between urinary PF4 and KIM-1 marker and mtDNA levels. This revealed a correlation between urinary PF4 and urinary mtDNA but not between urinary KIM-1 and mtDNA, suggesting that intrarenal platelet activation is more likely to be a source of extracellular mtDNA than renal tubular cell injury following renal transplantation. In contrast to our *in vivo* data, showing correlations between renal ischaemia and platelet activation and renal cell necrosis, no correlation was found between cold ischaemia time and urinary

Table 2. Correlation analysis with plasma mtDNA

	Cold ischaemia time			Plasma creatinine		
	r_s	P-value	*n*	r_s	P-value	*n*
Plasma GAPDH	0.12	0.51	32	0.04	0.83	41
Plasma COX III	0.06	0.74	32	0.11	0.49	41
Plasma NADH-deh	0.05	0.79	32	0.09	0.56	41
Plasma MT-ND2	0.02	0.93	32	0.12	0.46	41

Plasma ncDNA levels as reflected by the housekeeping gene GAPDH and plasma mtDNA levels as reflected by COXIII, NADH-deh and MT-ND2 were correlated with cold ischaemia time and plasma creatinine. Linear regression was performed and Spearman's rank order coefficients were calculated. Data are mean ± SEM.

Table 3. Correlation analysis with urinary mtDNA

	Cold ischaemia time			Plasma creatinine			Plasma tacrolimus			Urine KIM-1			Urine PF4		
	r_s	P-value	*n*	r_s	P-value	*n*	r_s	P-value	*n*	r_s	P-value	*n*	r_s	P-value	*n*
Urinary GAPDH	0.30	0.09	34	0.31	<0.01**	43	0.13	0.41	43	0.12	0.44	43	0.35	0.03*	43
Urinary COX III	0.57	<0.001***	34	0.48	<0.01**	43	0.09	0.56	43	0.17	0.29	43	0.38	0.01*	43
Urinary NADH-deh	0.55	<0.001***	34	0.49	<0.001***	43	0.02	0.89	43	0.21	0.17	43	0.36	0.02*	43
Urinary MT-ND2	0.53	<0.01**	34	0.48	<0.001***	43	0.01	0.93	43	0.17	0.28	43	0.33	0.03*	43
Urinary PF4	−0.02	0.90	34	0.32	<0.05*	43	0.16	0.32	43	0.06	0.73	43	–	–	43
Urinary KIM-1	0.19	0.28	34	0.18	0.24	43	−0.03	0.83	43	–	–	–	0.06	0.28	43

Urinary ncDNA levels as reflected by the housekeeping gene GAPDH and urinary mtDNA levels as reflected by COXIII, NADH-deh and MT-ND2 were correlated with cold ischaemia time, plasma creatinine, plasma tacrolimus, urine PF4 and urine KIM-1. Linear regression was performed and Spearman's rank order coefficients were calculated. Data are mean ± SEM.

*P < 0.05, **P < 0.01, ***P < 0.001.

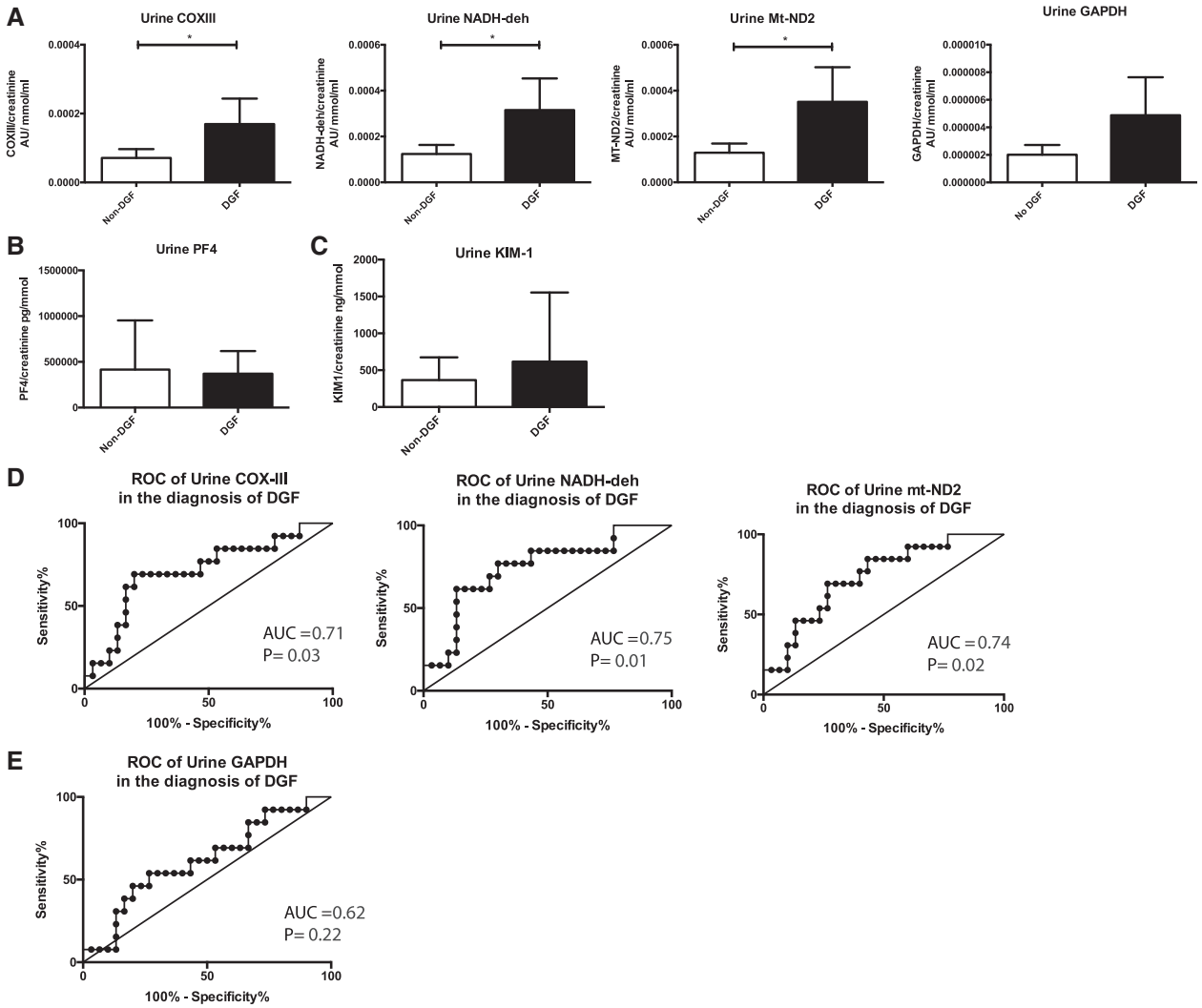

FIGURE 3: Cold ischaemia time in renal transplantation setting correlates with urinary mtDNA release, which is a predictor of DGF development following renal transplantation. (**A**) The levels of urinary mtDNA as reflected by COXIII, NADH-deh and MT-ND2 but not ncDNA, as reflected by the housekeeping gene GAPDH, measured in renal transplant recipients that developed DGF is significantly enhanced compared with renal transplant recipients without DGF (non-DGF). (**B**) Local renal platelet activation as reflected by urinary PF4 and (**C**) renal tubular cell injury as reflected by urinary KIM-1) do not differ between the DGF and non-DGF group. (**D**) Following construction of multiple ROC curves, the AUC for mtDNA-encoded genes COXIII, NADH-deh and MT-ND2 showed diagnostic performance, with an AUC of, respectively, 0.71 ($P = 0.03$, 95% CI 0.54–0.89), 0.75 ($P = 0.01$, 95% CI 0.58–0.91), 0.74 ($P = 0.02$, 95% CI 0.58–0.89) (**D**). (**E**) ROC curve of ncDNA-encoded gene GAPDH showed diagnostic performance with an AUC of 0.62 ($P = 0.22$, 95% CI 0.43–0.80) (**E**). Data are mean ± SEM. *$P < 0.05$.

PF4 and KIM-1 in the context of renal transplantation. These results indicate that renal ischaemia-induced mtDNA release is likely not solely dependent on intrarenal platelet activation and renal injury.

In order to evaluate the diagnostic value of accumulating mtDNA and ncDNA levels for DGF, we stratified renal transplant recipients into a DGF and a non-DGF group and assessed mtDNA and ncDNA levels in plasma and urine. We found that only urinary mtDNA significantly increased in the DGF group as opposed to the non-DGF group. These findings indicate that urinary mtDNA is likely suitable as a marker for DGF. Neither urinary PF4 nor KIM-1 differed between the DGF group and the non-DGF group, implying that both intrarenal platelet activation and renal tubular injury are not increased in DGF. Analysis of the ROC curve was performed to evaluate the diagnostic effectiveness of urinary mtDNA and ncDNA for determining the occurrence of DGF versus no DGF following renal transplantation. Urinary mtDNA COXIII, NADH-deh and MT-ND2 were significantly predictive of DGF development versus no DGF development in renal transplant recipients, whereas ncDNA was not.

In conclusion, the research presented here demonstrates that renal ischaemia time correlates with extracellular mtDNA in urine following renal transplantation, which, likely in part derived from intrarenal platelet activation, may serve as a prognostic non-invasive marker for determining DGF following renal transplantation. Future studies will be required to evaluate the origin of the released mtDNA. Thus the contribution of

platelet-derived mtDNA release in the context of AKI can be explored, for example, by performing cohort studies that enable assessment of the effect of antiplatelet drugs on mitochondrial release and disease severity in the context of AKI. A better understanding of the origin and effect of mtDNA accumulation during DGF may provide strategies to improve allograft survival.

SUPPLEMENTARY DATA

Supplementary data are available at ndt online.

FUNDING

This work was supported by the Dutch Kidney Foundation IP10.20 (to W.P.C.P. and J.C.L.) and KJP10.017 (to J.J.T.H.R.) and by the Netherlands Organization for Health Research and Development Clinical Fellowship grant 40-00703-97-12480 (to J.J.T.H.R.)

CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests. The results presented in this article have not been published previously in whole or part, except in abstract format.

REFERENCES

1. Salvadori M, Rosso G, Bertoni E. Update on ischemia-reperfusion injury in kidney transplantation: pathogenesis and treatment. *World J Transplant* 2015; 5: 52–67
2. Perico N, Cattaneo D, Sayegh MH *et al.* Delayed graft function in kidney transplantation. *Lancet* 2004; 364: 1814–1827
3. Yarlagadda SG, Coca SG, Formica RN Jr *et al.* Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. *Nephrol Dial Transplant* 2008; 24: 1039–1047
4. Grosso G, Corona D, Mistretta A *et al.* Delayed graft function and long-term outcome in kidney transplantation. *Transplant Proc* 2012; 44: 1879–1883
5. Narayanan R, Cardella CJ, Cattran DC *et al.* Delayed graft function and the risk of death with graft function in living donor kidney transplant recipients. *Am J Kidney Dis* 2010; 56: 961–970
6. Ojo AO, Wolfe RA, Held PJ *et al.* Delayed graft function: risk factors and implications for renal allograft survival. *Transplantation* 1997; 63: 968–974
7. Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. *Am J Transplant* 2011; 11: 2279–2296
8. Matas AJ, Smith JM, Skeans MA *et al.* OPTN SRTR 2011annual data report: kidney. *Am J Transplant* 2013; 13: 11–46
9. Willicombe M, Rizzello A, Goodall D *et al.* Risk factors and outcomes of delayed graft function in renal transplant recipients receiving a steroid sparing immunosuppression protocol. *World J Transplant* 2017; 7: 34–42
10. Zhang Q, Raoof M, Chen Y *et al.* Circulating mitochondrial DAMPs cause inflammatory responses to injury. *Nature* 2010; 464: 104–107
11. Zhang Q, Itagaki K, Hauser CJ. Mitochondrial DNA is released by shock and activates neutrophils via p38 map kinase. *Shock* 2010; 34: 55–59
12. Bliksøen M, Mariero LH, Ohm IK *et al.* Increased circulating mitochondrial DNA after myocardial infarction. *Int J Cardiol* 2012; 158: 132–134
13. Boudreau LH, Duchez AC, Cloutier N *et al.* Platelets release mitochondria serving as substrate for bactericidal group IIA-secreted phospholipase A2 to promote inflammation. *Blood* 2014; 124: 2173–2183
14. Jansen MPB, Pulskens WP, Butter LM *et al.* Mitochondrial DNA is released in urine of SIRS patients with acute kidney injury and correlates with severity of renal dysfunction. *Shock* 2018; 49: 301–310
15. Aswani A, Manson J, Itagaki K *et al.* Scavenging circulating mitochondrial DNA as a potential therapeutic option for multiple organ dysfunction in trauma hemorrhage. *Front Immunol* 2018; 9: 891
16. Jansen MP, Emal D, Teske GJ *et al.* Release of extracellular DNA influences renal ischemia reperfusion injury by platelet activation and formation of neutrophil extracellular traps. *Kidney Int* 2017; 91: 352–364
17. Roelofs JJ, Rouschop KM, Leemans JC *et al.* Tissue-type plasminogen activator modulates inflammatory responses and renal function in ischemia reperfusion injury. *J Am Soc Nephrol* 2005; 17: 131–140
18. Zhao H, Alam A, Soo AP *et al.* Ischemia-reperfusion injury reduces long term renal graft survival: mechanism and beyond. *EBioMedicine* 2018; 28: 31–42
19. Fernandes JB, Naik UP, Markell MS *et al.* Comparative investigation of the effects of the immunosuppressants cyclosporine A, cyclosporine G, and FK-506 on platelet activation. *Cell Mol Biol Res* 1993; 39: 265–274
20. Hošková L, Málek I, Kopkan L *et al.* Pathophysiological mechanisms of calcineurin inhibitor-induced nephrotoxicity and arterial hypertension. *Physiol Res* 2017; 66: 167–180
21. Whitaker RM, Stallons LJ, Kneff JE *et al.* Urinary mitochondrial DNA is a biomarker of mitochondrial disruption and renal dysfunction in acute kidney injury. *Kidney Int* 2015; 88: 1336–1344
22. Hu Q, Ren J, Ren H *et al.* Urinary mitochondrial DNA identifies renal dysfunction and mitochondrial damage in sepsis-induced acute kidney injury. *Oxid Med Cell Longev* 2018; 2018: 8074936
23. Lo YM, Tein MS, Pang CC *et al.* Presence of donor-specific DNA in plasma of kidney and livertransplant recipients. *Lancet* 1998; 351: 1329–1330
24. Zhang J, Tong KL, Li PK *et al.* Presence of donor- and recipient-derived DNA in cell-free urine samples of renal transplantation recipients: urinary DNA chimerism. *Clin Chem* 1999; 45: 1741–1746
25. Mikhalski D, Wissing KM, Ghisdal L *et al.* Cold ischemia is a major determinant of acute rejection and renal graft survival in the modern era of immunosuppression. *Transplantation* 2008; 85: S3–S9
26. Wei PZ, Kwan BC, Chow KM *et al.* Urinary mitochondrial DNA level in non-diabetic chronic kidney diseases. *Clin Chim Acta* 2018; 484: 36–39
27. Wei PZ, Kwan BC, Chow KM *et al.* Urinary mitochondrial DNA level is an indicator of intra-renal mitochondrial depletion and renal scarring in diabetic nephropathy. *Nephrol Dial Transplant* 2018; 33: 784–788
28. Qin C, Gu J, Hu J *et al.* Platelets activation is associated with elevated plasma mitochondrial DNA during cardiopulmonary bypass. *J Cardiothorac Surg* 2016; 11: 90

Received: 9.2.2018; Editorial decision: 22.10.2018