TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Evaluation of biomarkers of cell cycle arrest and inflammation in prediction of dialysis or recovery after kidney transplantation

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Keywords
cell cycle arrest, delayed graft function, insulin-like growth factor-binding protein 7, tissue inhibitor of metalloproteinases-2, urinary biomarkers, vascular endothelial growth factor.

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Conflicts of interest
The authors have declared no conflicts of interest.

Received: 18 November 2014
Revision requested: 30 December 2014
Accepted: 7 July 2015
Published online: 28 July 2015

doi:10.1111/tri.12636

Summary

Early prediction of delayed graft function (DGF) after kidney transplantation would facilitate patient management. Cell cycle arrest and inflammation are implicated in the pathogenesis of DGF. We assessed the utility of two novel acute kidney injury (AKI) biomarkers, urinary tissue inhibitor of metalloproteinases-2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP7), and five inflammatory markers to predict DGF following deceased-donor kidney transplantation. Serial urine concentrations of TIMP-2 and IGFBP7 were measured immediately after transplantation in 56 recipients along with vascular endothelial growth factor-A (VEGF-A), macrophage migration inhibitory factor (MIF), monocyte chemotactic protein-1 (MCP-1), trefoil factor 3 (TFF3) and chemokine (C-X-C) ligand 16 (CXCL16). Delayed graft function (DGF) was defined as requirement for dialysis within 7 days. Integrated discrimination improvement analysis was used to evaluate whether these biomarkers enhanced prediction of DGF independently of a validated clinical risk prediction model. DGF occurred in 22 patients (39%). At 4 h after kidney reperfusion, the area under the receiver operator characteristic curve (AUC) for VEGF-A was good (AUC $>$ 0.80); for TIMP-2, IGFBP7 and [TIMP-2] $\times$ [IGFBP7] fair (AUCs 0.70–0.79); and for MIF, MCP-1, TFF3 and CXCL16 poor (AUC $<$ 0.70). Only TIMP-2 and VEGF significantly enhanced the DGF prediction at 4 and 12 h. The cell cycle arrest marker urinary TIMP-2 and the inflammatory biomarker VEGF-A are potentially useful adjuncts to clinical data for early prediction of DGF.

Background

Delayed graft function (DGF) is usually defined as the requirement for dialysis within 7 days of kidney transplantation [1,2] and is most commonly a consequence of the ischaemia–reperfusion injury which inevitably accompanies organ recovery [3]. DGF is associated with increased risk of rejection, inferior long-term graft function and increased graft loss [1,2].

Early identification of patients with DGF would facilitate patient management including choice of immunosuppressive regimens. As with other forms of acute kidney injury (AKI), inability to identify DGF rapidly may impair translation of successful preclinical therapies into clinical practice [4]. Triaging of patients for clinical trials of early intervention to minimize DGF or its effects would be facilitated if DGF could be confidently predicted in the immediate postoperative period.

The absolute value or change in serum creatinine (sCr) has proven a poor biomarker of DGF within the first day following transplantation [4,5]. The interpretation of sCr is affected by the timing and adequacy of pre-operative dialysis, fluid administration [6], variations in muscle mass and hence creatinine generation associated with age, race and sex, and the protracted time required for sCr to reach steady state after transplantation [3,7,8].

Several promising urinary protein biomarkers of AKI including neutrophil gelatinase-associate lipocalin, kidney injury molecule-1 and interleukin-18 have been evaluated as alternative biomarkers of DGF [1,2,4,5,9], but there is insufficient evidence to support the clinical use of these markers [4,5,10].

Several other proteins that signal cellular injury and inflammation show utility for AKI diagnosis but have not been evaluated as markers of DGF [6,11–19]. The markers of cell cycle arrest, insulin-like growth factor-binding protein 7 (IGFBP7) and tissue inhibitor of metalloproteinases-2 (TIMP-2), and the product [TIMP-2] × [IGFBP7], are newly reported biomarkers of renal recovery that detect AKI with improved sensitivity [11,12]. Specifically, a commercial assay for [TIMP-2] × [IGFBP7] has recently been granted US regulatory approval to aid risk assessment for moderate or severe AKI in patients admitted to intensive care units [11,20]. Both vascular endothelial growth factor (VEGF) and monocyte chemotactic protein-1 (MCP-1) predict AKI [13,14] and a negative outcome following rejection [15,16]. Macrophage migration inhibitory factor (MIF) has been reported as a negative prognostic marker in AKI [17], while elevated urinary concentrations of chemokine (C-X-C) ligand 16 (CXCL16) are associated with acute tubular damage in renal allograft biopsies [18]. Expression of trefoil factor 3 (TFF3) in ischaemic kidney allografts is associated with inflammation [19], suggesting a role as a marker of DGF.

Our primary aim was to evaluate the diagnostic utility of urinary cell cycle arrest markers within the first postoperative day in recipients of deceased-donor transplants for prediction of DGF. Secondary aims were to evaluate the inflammatory biomarkers for prediction of DGF, all biomarkers for the prediction of reduced (delayed and slow) graft function, and the association of peak biomarker concentrations with outcome at 1 year.

Patients and methods

This study utilized urine and serum samples collected prospectively from a patient group that has been previously described [5]. Briefly, consecutive patients aged 18 years or older undergoing deceased-donor kidney transplantation at Prince of Wales Hospital, Sydney, were recruited. The study was conducted under the approval of the Institutional Human Ethics Committee (EC00134:10/113). Exclusion criteria were pre-emptive transplantation and inability to provide informed consent. Clinical decisions regarding patient treatment, including dialysis were made independently of researchers by treating physicians. Within the first postoperative week, all patients received a uniform protocol of corticosteroids, basiliximab and mycophenolate sodium and received either thymoglobulin induction followed by calcineurin inhibitor, or calcineurin inhibitor alone at the treating physicians' discretion.

Creatinine reduction ratio (CRR) at any time was defined as sCr immediately after reperfusion (baseline) minus sCr at the timepoint of interest divided by baseline sCr.

Delayed graft function was defined as the requirement for dialysis within 7 days of transplantation [1]. Slow graft function was defined by a CRR < 0.70 at 7 postoperative days [1] and was considered an intermediate state between immediate and slow graft function. Immediate graft function was defined by a CRR ≥ 0.70 at 7 postoperative days. Consistent with previous authors, we defined reduced graft function (RGF) as either DGF or slow graft function [1].

Samples

sCr was measured immediately after organ reperfusion as part of routine clinical care. Additional blood and urine samples were collected at 4, 8 and 12 h, and 1, 2, 3 and 7 days after organ reperfusion. Blood was immediately centrifuged after clotting and serum refrigerated. Urine was combined (2:1) with protease inhibitor (Complete; Roche, Mannheim, Germany) and refrigerated prior to centrifugation to remove sediment. Serum and urine supernatant was then aliquoted and stored at –80 °C prior to batched assay.

Assays

IGFBP7 was measured using the Milliplex MAP Kit Human IGFBP Magnetic Bead Panel 2 (EMD Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions using the Bio-Plex 200 system (Bio-Rad Laboratories, Berkeley, CA, USA) and commercial software (Bio-Plex Manager 6.0). TIMP-2, VEGF-A ($VEGF_{165}$), MIF, MCP-1, CXCL16 and TFF3 were measured via ELISA using R&D DuoSets (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions utilizing an automated ELISA platform (DSR e1000, Diagnostic Solutions, Preston, Australia). The intra-assay and interassay variability for all assays was <10%.

Urinary biomarker concentrations were normalized to urinary creatinine to account for variations in urine flow rate [21]. Serum and urine creatinine concentrations were measured using enzymatic methods on an automated

chemical analyser (Konelab 20XT, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations.

Statistical analyses

The primary analysis was assessment of the biomarker performance 4 h following reperfusion to predict DGF due to ischaemia/reperfusion injury. Patients diagnosed with rejection as the cause of DGF were excluded from the primary analysis. Further analysis considered changes in biomarker performance over the first day following kidney reperfusion, and the added value of the biomarkers to a clinical model at 4 and 12 h.

Analyses were conducted with SPSS Statistics (IBM, Armonk, NY) and MATLAB 2013b (Mathworks, Natick, MA, USA) and presented using Prism v6.0 (GraphPad, La Jolla, CA, USA). All test were two-tailed. Because dialysis defines DGF absolutely and modifies sCr, patients were excluded from analysis at timepoints after the initiation of dialysis. We have previously demonstrated that anuria was a specific marker of DGF, and therefore, anuric patients were also excluded from analyses at each timepoint [5]. Continuous data were analysed by Mann–Whitney *U*-tests. Categorical variables were analysed by Fisher's exact test. Log-transformed biomarker concentrations were initially analysed over the first postoperative day using a linear mixed-model analysis examining DGF as a predictor of biomarker concentration. ROC analyses were performed without adjustment for multiple testing to assess the predictive performance of each biomarker at each timepoint and categorized as poor to excellent as previously described [22]. Sensitivity analyses were subsequently performed to consider the performance of biomarkers for diagnosis of reduced graft function.

The base model used a published [23] and validated [24] risk prediction model for DGF. Individual DGF risk was calculated online (http://www.transplantcalculator.com) after entering nine recipient-, eight donor- and three transplantation-related factors as described [24] (Table 1). Multivariable logistic regression with forward entry was used to construct new models by adding log-transformed variables to the reference model and calculating the probability of DGF for each patient. Integrated discrimination improvement (IDI) metrics were used to calculate the mean increase in risk for those who developed DGF (IDI-DGF), and reduction in those who did not (IDI-non-DGF), following the addition of each biomarker [25]. This method assesses the ability of a new model to predict the outcome of interest, here DGF, considering the change in the estimation prediction probabilities as a continuous variable [26]. Unlike for DGF, the base model has not been validated for prediction of reduced graft function, so IDI was not evaluated in sensitivity analyses.

The association of variables with eGFR was examined 1 year post-transplantation. Patients with graft loss were assigned a GFR of zero. Variables examined were pre-operative risk factors (listed in Table 1), CRR at D7 (patients being dialysed were assigned a CRR of zero), and the log-transformed maximal biomarker concentration over the first postoperative week.

Results

We recruited 56 deceased-donor kidney transplant recipients. Baseline characteristics of recipients and deceased donors are shown in Table 1. A total of 19 patients (34%) had immediate graft function (IGF), 15 (27%) had slow graft function (SGF), and 22 (39%) had DGF. In no patient was dialysis initiated for isolated hyperkalemia or acidosis; all patients were dialysed for azotaemia with (19 patients) or without (three patients) concurrent fluid overload (summarized Table 1; additional information Table S1). All postoperative dialysis was haemodialysis: four patients were dialysed between 4 and 8 h, the other 18 patients after 12 h. Allograft biopsies were performed within 7 days in 28 patients. All biopsies had features of acute tubular injury, including those with IGF. None were diagnostic of acute rejection. A total of 12 of 22 (55%) patients with DGF and nine of 34 (26%) patients with non-DGF received thymoglobulin prior to calcineurin inhibitor. All 56 patients had commenced a calcineurin inhibitor at the end of 1 week.

DGF prediction at 4 h

The overall time course of each biomarker over the first postoperative day is shown in Fig. 1. Over the first day concentrations of TIMP-2, VEGF-A and [TIMP-2] × [IGFBP7], and CXCL16 were greater in DGF patients than non-DGF patients ($P < 0.05$ for each). Concentrations of IGFBP7, MIF, TFF3 and MCP-1 were not significantly different between the two groups over the first postoperative day.

The area under the receiver operator characteristic curve (AUC) for each urinary biomarker is shown as a function of time in Fig. 2. At 4 h, increased urinary VEGF-A [AUC: 0.81 (95% confidence interval 0.66–0.95)] appeared to best predict DGF, followed by [TIMP-2] × [IGFBP7] [0.76 (0.59–0.93)], TIMP-2 [AUC: 0.73 (0.55–0.91)] and IGFBP7 [AUC: 0.71 (0.54–0.87)]. For reference, performance of previously published biomarkers clusterin, IL-18, neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule (KIM)-1 in the

Table 1. Baseline characteristics of transplant recipients and deceased donors.

Total, $n = 56$	Non-DGF, $n = 34$	DGF, $n = 22$	*P*
Recipient characteristics			
Male*, *n* (%)	21 (62)	15	0.78
Age*, years	56 (49–62)	50 (47–62)	0.76
Ethnicity, *n* (%)			
Black African*	0	0	
Caucasian	22 (65)	17 (77)	0.38
Non-Caucasian	12 (35)	5 (23)	
Asian	10 (29)	2 (9)	
Pacific Islander	2 (6)	1 (4)	
Other	0	2 (9)	
Body mass index (kg/m^2)*, median (IQR)	25 (23–28)	30 (25–33)	0.05
% Peak PRA*, median (IQR)	3 (1–11)	10 (3–39)	0.12
Duration of dialysis* (months), median (IQR)	64 (31–84)	75 (35–91)	0.46
Previous transplant* (yes), *n* (%)	3 (8)	5 (23)	0.24
Pretransplant transfusion* (yes), *n* (%)	7 (21)	6 (27)	0.74
Diabetes mellitus*, *n* (%)	5 (14)	6 (27)	0.31
Extrarenal transplant†, *n*	0	0	
Hemodialysis‡, *n* (%)	28 (82)	20 (91)	0.46
Tacrolimus, *n* (%)	26 (76)	17 (77)	1.00
Pre-operative urine output mL/day; median (range)	0 (0-2000)	0 (0-1000)	0.27
Deceased-donor characteristics	$n = 34$§	$n = 22$§	
Male, n (%)	18 (53)	11 (50)	1.00
Age*, years (IQR)	54 (41–65)	56 (43–61)	0.78
Cardiac death*, *n* (%)	2 (6)	9 (41)	0.002
ECD, *n (%)*	8 (23)	3 (14)	0.50
Terminal sCr*, μmol/L (IQR)	81 (60–95)	61 (56–82)	0.10
Cause of death, *n* (%)			
Stroke*	14 (41)	10 (45)	0.79
Anoxia*	3 (9)	2 (9)	1.00
Transplant characteristics			
HLA mismatches*, median (IQR)	4 (2–5)	4 (2–6)	0.54
Cold ischaemia time (min), median (IQR)*	531 (408–787)	708 (514–997)	0.04
Warm ischaemia time (min), median (IQR)*	43 (34–52)	47 (32–67)	0.68
Machine Perfusion†, *n*	0	0	
Postoperative dialysis¶			
Dialysis sessions (*n*), median (range)	0	3 (1–9)	N/A
Indications			
Azotaemia alone, *n* (%)	0	3 (14)	N/A
Azotaemia and fluid overload, *n* (%)	0	19 (86)	N/A

DGF, Delayed graft function; non-DGF, nondelayed graft function; ESKD, end-stage kidney disease; ECD, expanded criteria donor; sCr, serum creatinine.

*Variable in risk prediction model (reference [23]).

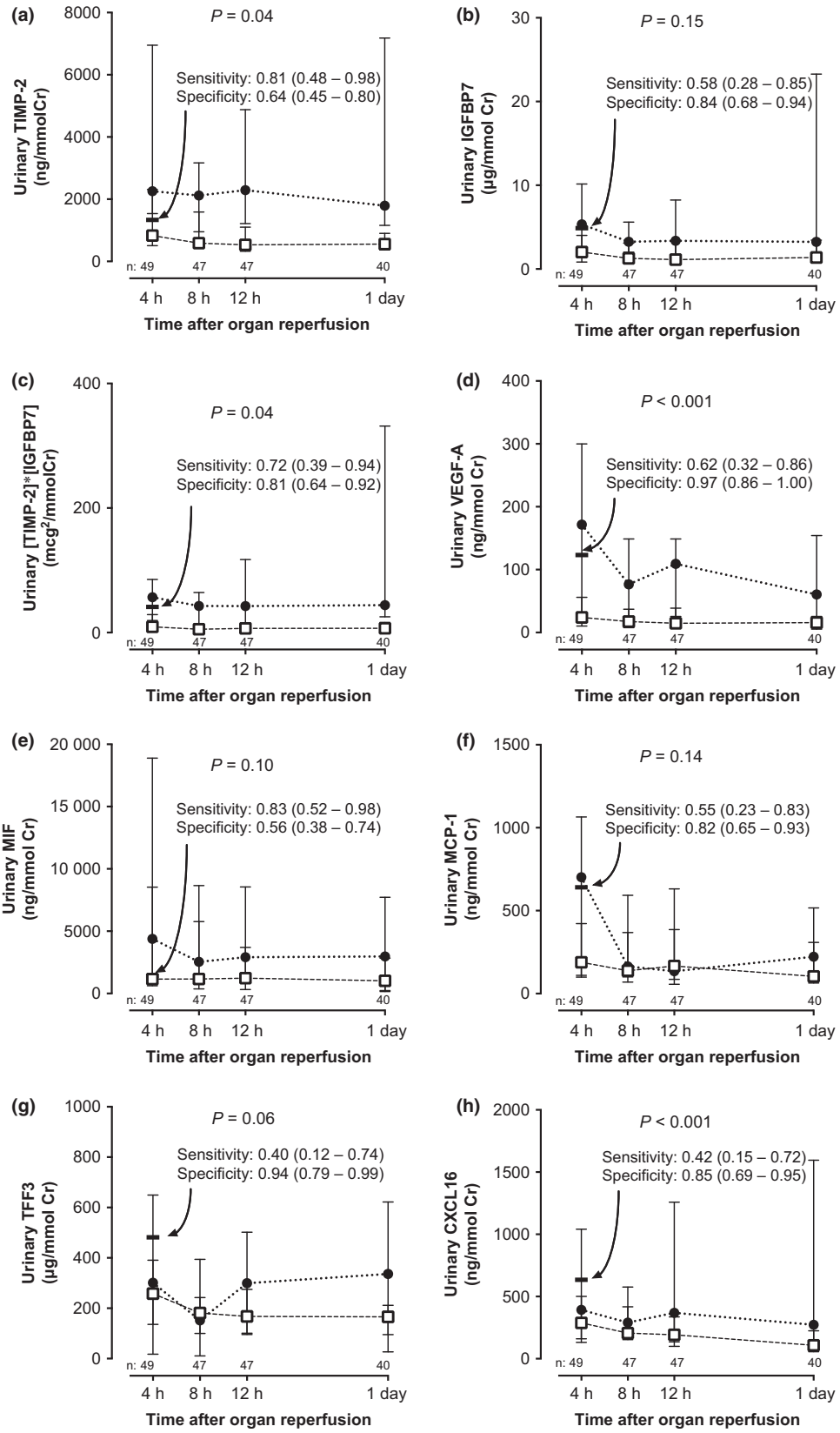
†Exclusion criterion for risk prediction model.

‡All others peritoneal dialysis.

§No donor contributed a kidney to more than one recipient within the cohort.

¶All postoperative dialysis was haemodialysis.

Figure 1 Urinary biomarker concentrations stratified by allograft function on the first day after renal transplantation. (a) Tissue inhibitor of metalloproteinase-2 (TIMP-2), (b) insulin-like growth factor-binding protein 7 (IGFBP7), (c) the product of TIMP-2 and IGFBP7 [TIMP-2] × [IGFBP7], (d) vascular endothelial growth factor (VEGF-A), (e) macrophage migration inhibitory factor (MIF), (f) monocyte chemotactic protein-1 (MCP-1). (g) trefoil factor 3 (TFF3), (h) chemokine (C-X-C motif) ligand 16 (CXCL16). Delayed graft function (closed circles, dots) and non-delayed graft function (DGF) (open squares, dashes) are shown. Data represent group median concentrations (interquartile range). Sensitivity (95% CI) and specificity (95% CI) for optimized cut-off using Youden method[50]. *P* value: log-transformed values analysed using a linear mixed-model analysis examining DGF as a predictor of biomarker concentration; n: number of patients available for analysis after exclusions for DGF (dialysis) and anuria at each time.



same population is presented in Table S2. There was no statistical difference between AUCs for clusterin or IL-18 and VEGF, [TIMP-2] × [IGFBP7], TIMP-2 and IGFBP7 at any time.

CXCL16 [AUC: 0.61 (0.42–0.81)] did not predict DGF, and neither did MCP-1 [AUC: 0.60 (0.37–0.82)], MIF [AUC: 0.65 (0.47–0.84), nor TFF3 [AUC: 0.60 (0.42–0.89)]. Excluding patients with anuria – which was highly specific for DGF[5] – the presence of oliguria (UO < 0.5 ml/kg/h) was not predictive of DGF (Table S3).

Neither sCr [AUC: 0.56 (0.41–0.71)], nor the CRR [AUC: 0.64 (0.49–0.79)], nor urinary creatinine concentration alone [AUC 0.59 (0.39–0.79)] predicted DGF at 4 h.

Added value for prediction of DGF at 4 h

The added value of biomarkers at 4 h was assessed by integrated discrimination improvement analysis (IDI) after each variable was added to the baseline model (see Table 2 and Fig. 3).

The addition of TIMP-2 to the baseline model at 4 h increased the average calculated risk for DGF patients [average improvement (IDI-DGF) by 0.11 (95% CI: 0.004–0.33)] and decreased the average calculated risk for non-DGF patients [average reduction (IDI-non-DGF) by 0.05 (0.003–0.16)] with the final model yielding an AUC of 0.81 (0.65–0.99) (Fig. 3, Table 2).

The addition of VEGF-A to the base model at 4 h maximally improved risk prediction, [IDI-DGF: 0.19 (0.03–0.45); IDI-non-DGF: 0.08 (0.02–0.20)] with a final AUC of 0.85 (0.72–0.99).

By contrast, the addition of IGFBP7 alone to the base model at 4 h did not significantly improve the prediction of DGF [IDI-DGF: 0.01 (−0.003 to 0.09)] and non-DGF [IDI-non-DGF: 0.00 (-2×10^{-4} to 0.11)]. Similarly, [TIMP-2] × [IGFBP7] did not enhance the clinical model (Table 3).

Once 4 h VEGF-A was included, the inclusion of TIMP-2 did not further improve the model (data not shown).

DGF prediction at 12 h

The AUC of several biomarkers was greater at 12 h than at 4 h, including TIMP-2 and [TIMP-2] × [IGFBP7] (Fig. 2), although a significant difference between the AUCs at 4 and 12 h was not demonstrated for any marker. At 12 h, IGFBP7 and TFF3 were fair, while MIF, CXCL16 and MCP-1 remained poor biomarkers of DGF (Fig. 2).

At 12 h, sCr was a statistically significant, but 'poor' predictor of DGF (AUC: 0.68 (0.53–0.84), while the performance of CRR was 'fair' [AUC: 0.71 (0.56–0.86)] and urinary creatinine did not predict DGF at all [0.67 (0.45–0.89)].

Added value for prediction of DGF at 12 h

At 12, TIMP-2 and VEGF-A added value for the prediction of DGF although the enhancement provided by VEGF-A was modest. Despite univariate analysis suggesting utility, IDI analysis showed that IGFBP7, [TIMP-2] × [IGFBP7], TFF3, sCr and CRR did not significantly enhance the prediction of DGF at this time (Table 2).

Sensitivity analyses

The performance of each biomarker for identifying RGF (*i.e.* either delayed or slow graft function) was also analysed. Both VEGF-A and TIMP-2 performed less well for distinguishing RGF from immediate graft function than each did for distinguishing DGF from non-DGF. At 4 h, the AUC for VEGF-A for predicting RGF was 0.67 (0.51–0.82) and for TIMP-2, the AUC was 0.66 (0.82–0.50). Conversely, the performance of IGFBP7 was better for prediction of RGF than for DGF [AUC for RGF: 0.70 (0.55–0.84)]. The performance of [TIMP-2] × [IGFBP7] was similar for DGF and RGF [AUC for RGF: 0.76 (0.62–0.91)]. MCP-1, MIF, CXCL16 and MCP-1 remained poor markers of RGF. The diagnostic performance for predicting RGF at other timepoints is presented in Fig. S1, and urinary biomarker concentrations stratified for DGF, SGF, and IGF are presented in Fig. S2.

A residual urine output was present in only 10 patients, which did not allow adequate comparison of pre-operative and postoperative biomarker values.

Biomarkers and 1-year outcome

One-year follow-up was available in 50 patients. Compared with non-DGF, DGF was associated with lower eGFR at 1 year 33 (IQR: 24–51) vs. 45 (35–55) mL/

Figure 2 Biomarker performance in prediction of delayed graft function. The areas under the receiver operator characteristic curve (AUCs) for each urinary biomarker are shown: Panel (a): tissue inhibitor of metalloproteinase-2 (TIMP-2), (b): insulin-like growth factor-binding protein 7 (IGFBP7), (c): the factor of TIMP-2 and IGFBP7, [TIMP-2] × [IGFBP7], (d): vascular endothelial growth factor (VEGF-A), (e) macrophage migration inhibitory factor (MIF), (f) monocyte chemotactic protein-1 (MCP-1). (g) trefoil factor 3 (TFF3), (h) chemokine (C-X-C motif) ligand 16 (CXCL16). Data points represent the AUCs. Shaded areas represent 95% confidence intervals (CI). $P < 0.05$ when shaded areas do not overlap the line of identity (AUC = 0.50).

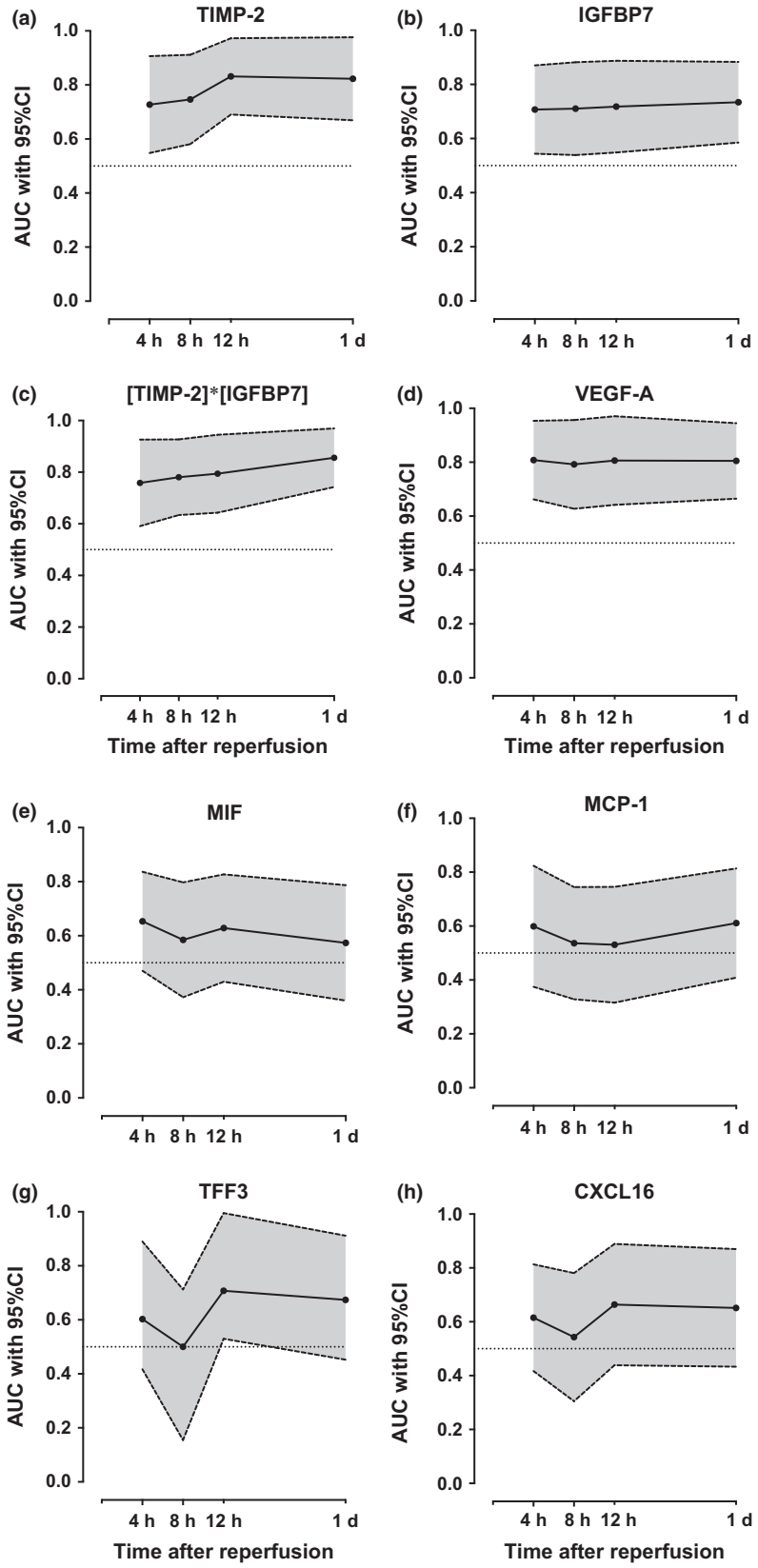


Table 2. Prediction of DGF using urinary biomarkers versus the clinical model alone.

	Variables	IDI-DGF (95% BI)	IDI-non-DGF (95% BI)	AUB (95% BI)
Base Model		*	*	0.70 (0.52 to 0.88)
4 h (n = 49)†				
Base Model +	TIMP-2	0.11 (0.004 to 0.33)‡	0.05 (0.003 to 0.16)‡	0.81 (0.66 to 0.97)
Base Model +	IGFBP7	0.01 (−0.003 to 0.09)	0.00 (−0.001 to 0.05)	0.75 (0.59 to 0.92)
Base Model +	[TIMP-2] × [IGFBP7]	0.05 (-6×10^{-4} to 0.21)	0.02 (-2×10^{-4} to 0.11)	0.80 (0.64 to 0.96)
Base Model +	VEGF-A	0.19 (0.03 to 0.45)‡	0.08 (0.02 to 0.20)‡	0.85 (0.72 to 0.99)
Base Model +	MIF	0.02 (-5×10^{-4} to 0.15)	0.01 (-2×10^{-4} to 0.07)	0.73 (0.56 to 0.90)
Base Model +	MCP-1	0.00 (−0.01 to 0.02)	0.00 (−0.003 to 0.01)	0.69 (0.51 to 0.88)
Base Model +	TFF3	0.01 (−0.002 to 0.08)	0.00 (-6×10^{-4} to 0.03)	0.71 (0.53 to 0.89)
Base Model +	CXCL16	0.01 (−0.004 to 0.10)	0.01 (-9×10^{-4} to 0.05)	0.70 (0.53 to 0.87)
8 h (n = 47)†				
Base Model +	TIMP-2	0.13 (7×10^{-4} to 0.39)‡	0.04 (7×10^{-4} to 0.15)‡	0.84 (0.68 to 1.00)
Base Model +	IGFBP7	0.02 (−0.004 to 0.16)	0.01 (−0.001 to 0.07)	0.78 (0.61 to 0.95)
Base Model +	[TIMP-2] × [IGFBP7]	0.05 (−0.01 to 0.29)	0.02 (−0.002 to 0.11)	0.82 (0.64 to 1.00)
Base Model +	VEGF-A	0.11 (0.001 to 0.43)‡	0.04 (7×10^{-4} to 0.15)‡	0.81 (0.64 to 0.98)
Base Model +	MIF	0.02 (−0.001 to 0.14)	0.01 (-4×10^{-4} to 0.06)	0.69 (0.50 to 0.88)
Base Model +	MCP-1	0.02 (−0.0008 to 0.15)	0.01 (-2×10^{-4} to 0.06)	0.70 (0.51 to 0.90)
Base Model +	TFF3	0.02 (−0.001 to 0.19)	0.01 (-3×10^{-4} to 0.07)	0.65 (0.45 to 0.85)
Base Model +	CXCL16	0.00 (−0.006 to 0.02)	0.00 (−0.002 to 0.009)	0.66 (0.47 to 0.85)
12 h (n = 47)†				
Base Model +	TIMP-2	0.23 (0.03 to 0.46)‡	0.09 (0.02 to 0.21)‡	0.90 (0.78 to 1.00)
Base Model +	IGFBP7	0.03 (−0.002 to 0.19)	0.01 (-8×10^{-4} to 0.08)	0.83 (0.67 to 0.98)
Base Model +	[TIMP-2] × [IGFBP7]	0.07 (−0.007 to 0.31)	0.02 (−0.002 to 0.12)	0.88 (0.74 to 1.00)
Base Model +	VEGF-A	0.06 (2×10^{-5} to 0.26)‡	0.02 (4×10^{-5} to 0.09)‡	0.77 (0.59 to 0.94)
Base Model +	MIF	0.04 (-3×10^{-4} to 0.20)	0.02 (-1×10^{-5} to 0.09)	0.69 (0.51 to 0.88)
Base Model +	MCP-1	0.01 (−0.004 to 0.11)	0.00 (-9×10^{-4} to 0.04)	0.73 (0.55 to 0.91)
Base Model +	TFF3	0.00 (−0.002 to 0.01)	0.00 (-9×10^{-4} to 0.004)	0.67 (0.48 to 0.86)
Base Model +	CXCL16	0.03 (−0.002 to 0.19)	0.01 (-3×10^{-4} to 0.07)	0.72 (0.54 to 0.89)

Model enhancement was analysed by calculation of the IDI. The clinical base model was derived from recipient-, donor- and transplant-related factors (reference [23]).

IDI, integrated discrimination improvement; DGF, Delayed graft function; non-DGF, nondelayed graft function; VEGF-A, vascular endothelial growth factor-A; MIF, macrophage migration inhibitory factor.

*Metrics not calculated for baseline model.

†Patients excluded if anuric or already dialysed; 4 patients were dialysed between 4 and 8 h and were excluded from subsequent analysis.

‡$P < 0.05$ vs. Base Model.

min/1.73 m^2, $P = 0.04$; two patients with DGF had subsequent graft loss. One-year eGFR was independently associated with both donor age and CRR at D7 (Table 3). The maximal concentrations of urinary biomarkers over the first postoperative week were not associated with eGFR at 1 year.

Discussion

This study demonstrated that 4 h after renal transplantation, the urinary biomarker VEGF-A was a good predictor of DGF. TIMP-2, [TIMP-2] × [IGFBP7] and IGFBP7 were fair predictors, while MIF, MCP-1, TFF3 and CXCL16 were poor. As comparison of AUCs is of limited utility in assessing the added value of a biomarker over clinical variables [25], we used IDI analysis to determine the added value provided by these urinary biomarkers to a clinical risk prediction model of DGF.

Vascular endothelial growth factor-A and TIMP-2 added value to the model, with VEGF-A having the highest IDI ($IDI_{total} = 0.18 + 0.08 = 0.27$). The revised model incorporating VEGF-A and the base model predicted DGF with a final AUC of 0.85, suggesting good clinical utility. At 4 h, TIMP-2 also demonstrated clinical model enhancement. These biomarkers continued to be useful at 12 h. The other biomarkers evaluated added no value for the prediction of DGF at 4 or 12 h. Although VEGF-A was the best-performed biomarker at 4 h, the median concentration of VEGF-A declined quite rapidly in patients with DGF, suggesting further evaluation of the diagnostic utility of VEGF should focus on the early 'window of opportunity'. By contrast, the performance of TIMP-2 improved over the first 12 h. Although there is evidence for combining TIMP-2 and IGFBP7 in AKI [11,12,27], the composite marker [TIMP-2] × [IGFBP7] did not perform better than TIMP-2 alone in this study.

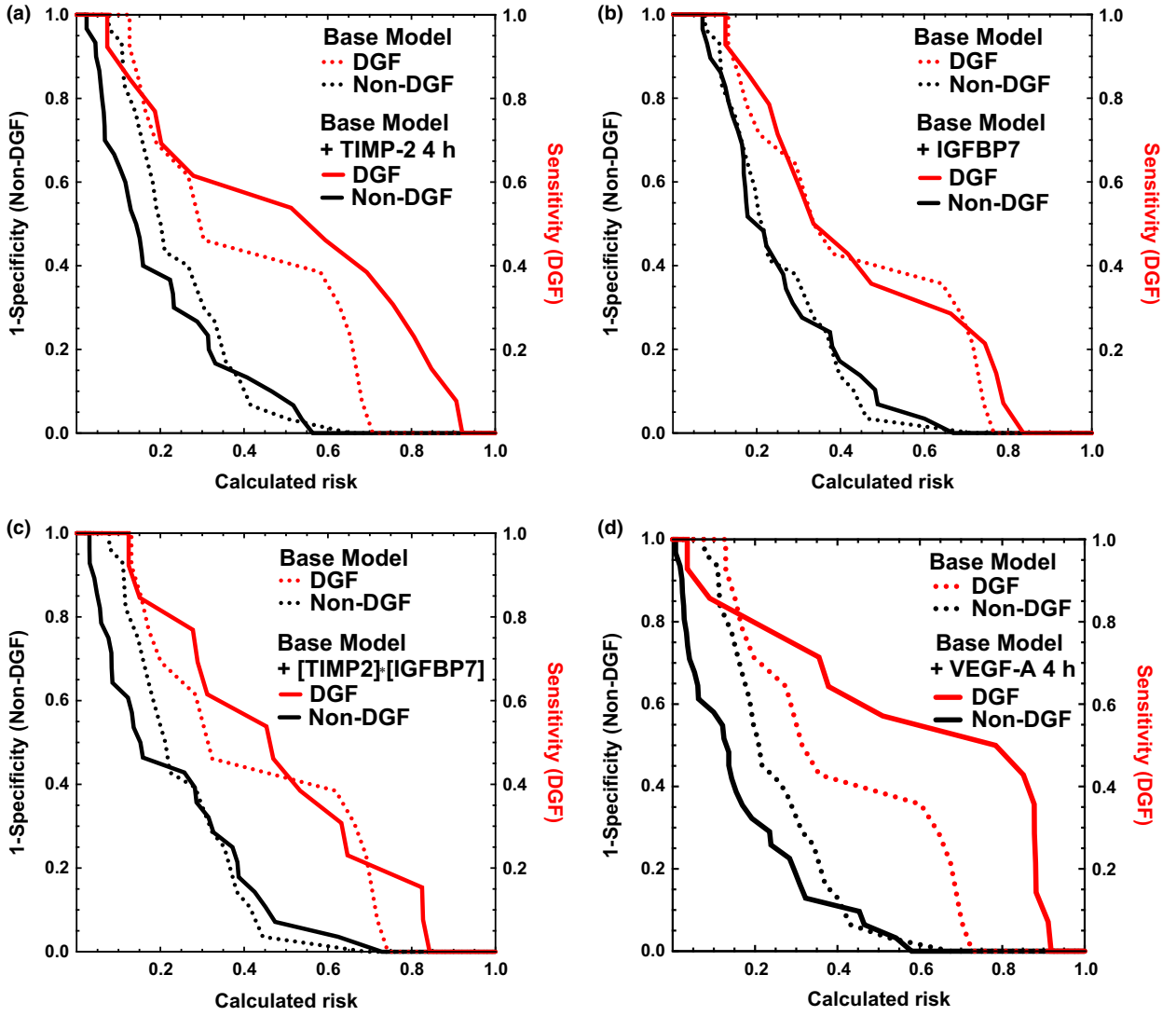


Figure 3 Clinical model enhancement in predicting delayed graft function at 4 h by adding urinary biomarkers to the base model. Panel (a) tissue inhibitor of metalloproteinase-2 (TIMP-2), (b) insulin-like growth factor-binding protein 7 (IGFBP7), (c) the factor of TIMP-2 and IGFBP7, [TIMP-2] × [IGFBP7], (d) vascular endothelial growth factor (VEGF-A). The risk assessment plots for the base model (dotted lines) and models after addition of variables (solid lines) are shown. Red lines represent sensitivity versus the calculated risk for patients who developed delayed graft function. Black lines represent 1-specificity versus the calculated risk for those who did not have delayed graft function. Improved risk assessment is demonstrated by movement of the red curve to the top-right corner and of the black curve to the bottom left corner after addition of a biomarker (reference [25]). The base model was derived from donor-, recipient- and transplant-related factors as previously reported (reference [23]).

As previously reported, the diagnostic utility of standard markers of creatinine clearance, sCr, or the creatinine reduction ratio is only poor to fair over the first 12 h [5,28,29] and urinary creatinine is nondiagnostic [5], and in the absence of absolute anuria, urine output was also nondiagnostic at 4 h. After accounting for donor-, recipient- and transplantation-related factors that influence the base model, neither sCr nor CRR improved the prediction of DGF within the first 12 h, although sCr or its change potentially influenced the decision to dialyse four patients within the first 8 h.

The elevated urinary concentration of TIMP-2 in patients with DGF suggests that DGF is associated with tubular epithelial G1 cell cycle arrest. Urinary concentrations of IGFBP7 in patients with slow graft function were elevated to intermediate levels compared with patients with immediate or delayed graft function. Consequently, IGFBP7 poorly discriminated patients with DGF and non-DGF but helped discriminate immediate from reduced graft function. Although TIMP-2 and IGFBP7 have been mainly examined as inhibitors of tumour growth [30,31], these molecules appear to induce cell cycle arrest in AKI

Table 3. Predictive variables for eGFR at 1 year after transplantation.

Variable		β	(95% CI)	*P*	Model	Model *r*
A	Donor Age (per decade)	−6.2	(−9.4 to −3.0)	<0.001	A	0.51
B	Creatinine reduction ratio (per 10% fall at D7)	1.5	(0.19 to 2.9)	0.03	A + B	0.58*

eGFR at 1 year was not associated with the maximal concentrations of urinary biomarkers within the first postoperative week.
*For model $eGFR_{1y}$ (ml/min/1.73 m^2) = 67 − [6.2 × donor age (decades)] + [15 × CRR_{D7}].

[11,12]. It has been proposed that cell cycle arrest occurs transiently during ischaemia/reperfusion injury, possibly to prevent damaged cells from dividing [11], and is a potential therapeutic target for facilitating recovery from ischaemia–reperfusion injury [32]. We examined the individual concentrations of [TIMP-2] and [IGFBP7] and the product [TIMP-2] × [IGFBP7] as all three metrics have been reported recently as potential biomarkers of AKI in heterogeneous ICU patients [11] and after cardiac surgery [12]. A device which measures the combination biomarker [TIMP-2] × [IGFBP7] is now commercially available and has recently been granted US regulatory approval to aid risk assessment for moderate or severe AKI in patients admitted to intensive care units [11,20] although these proteins are potentially affected by factors other than AKI [33]. Of the two molecules, TIMP-2 appears to date to be the better individual marker of AKI [11], and in the present study, the combination did not improve diagnosis. We did not evaluate other combinations of biomarkers or biomarker ratios, as there is little physiological or empirical support for such an approach.

Increased VEGF expression may be protective or deleterious in renal injury depending on the timing and circumstances of induction [34]. On the one hand, VEGF-induced renal neoangiogenesis appears to protect against tubular atrophy and interstitial fibrosis [35,36]. Conversely, VEGF is pro-inflammatory [37] and can produce pathological glomerular hypertrophy [38]. The present study suggests an association between VEGF-A and DGF, particularly in the immediate hours after transplantation but does not resolve whether VEGF expression is protective or deleterious. Clinically, VEGF is a marker of AKI [13] and increased urinary VEGF concentrations are associated with graft loss after acute rejection [16].

The other proteins that were examined as biomarkers of inflammation were poor markers of DGF. Despite the documented expression of MIF [15,39–41], MCP-1 [15,39,41], CXCL16 [18] and TFF3 [19,42] by kidney epithelium and association with inflammation in native kidneys and allografts, this study suggests that urinary concentrations of these proteins are not good early diagnostic markers of DGF.

The AUCs for VEGF-A, [TIMP-2] × [IGFBP7], TIMP-2 and IGFBP7 at 4 h are similar to clusterin and IL-18 as previously reported[5]. They are similar to the data of Hall *et al.*[4] who reported an AUC of 0.81 (0.70–0.92) for NGAL and of 0.76 (0.64–0.88) for IL-18 at 6 h, although comparison between studies is fraught.

Donor age is a known risk factor for poor long-term graft function [43], and we observed that donor age was inversely related to eGFR at 1 year. Not surprisingly, the fractional fall in sCr over the first postoperative week, the CRR, was weakly correlated with 1-year eGFR. There was no association between 1-year eGFR and peak values of VEGF-A, TIMP-2 or any other biomarker evaluated. Several factors potentially affect graft function over the first year such as acute rejection [44], infections including BK virus [45], and prescription of and adherence to immunosuppressive medication [46], and these can probably never be anticipated by a single urinary biomarker. Some authors have reported that long-term outcomes are associated with the trajectory of biomarker concentrations during or following kidney injury [12,47] rather than peak concentration. Nevertheless, this study suggests that while VEGF-A, TIMP and [TIMP-2] × [IGFBP7] are more useful than sCr for early DGF prediction, graft function at 12 months is better explained by alternative factors.

This study has strengths and limitations. Limitations include the use of research assays, the lack of standardized criteria for initiating dialysis and an sCr-based definition of SGF. Definitions of graft function based on changes in sCr have well documented limitations as study endpoints [29]. Similarly, defining DGF by dialysis requirement is arguably subjective. However, this definition is simple, is associated with increased rejection and graft loss [48], underlies the clinical risk prediction model used here [23] and is the most common method. There is no consensus on normalization of urinary biomarkers to creatinine in AKI or DGF [21,49]. Although creatinine clearance is in nonsteady state following kidney transplantation, fluid administration and variable urine output may alter absolute biomarker concentration independently of GFR. As normalization improves the prediction of incipient AKI [21], we adopted this practice for the diagnosis of DGF.

The concentration of urinary proteins examined did not correlate to the number of dialysis sessions required, or the duration of postoperative dialysis required; however, it is not established that these parameters are related to long-term morbidity [48]. This analysis suggests that the role of urinary biomarkers is in predicting DGF in patients with some urine output. Firstly, while anuria precludes analysis

of urinary biomarkers, anuria is a specific, albeit insensitive marker of DGF. In patients with at least some urine output, the presence or absence of oliguria was not diagnostic of DGF (see Table S2 and reference [5]). While 'anuria or biomarker positive' might prove a very useful diagnostic combination, the emphasis of this analysis is determining which biomarker, if any, might be best suited to purpose.

Because DGF is mainly the consequence of perioperative ischaemia–reperfusion injury, there is also the semantic issue of whether DGF is 'predicted' or simply 'detected' before dialysis is initiated. We have used the term 'predicted' to emphasize that early identification of risk will facilitate early and appropriate triaging of affected patients. Other limitations are modest cohort size, and the loss of eight patients to 12-month follow-up. This study was not an attempt at validation, but explored the utility of the biomarkers evaluated.

This proof-of-concept study is novel and hypothesis forming by demonstrating that measurement of selected biomarkers soon after renal transplantation can aid prediction of outcome. The recently identified biomarker of AKI, TIMP-2 and the vascular marker VEGF-A, both enhanced a well-validated clinical model, similar to clusterin and IL-18 [5]. The study suggests that TIMP-2 and VEGF-A are potentially useful adjuncts to clinical data and warrant further investigation in multicentre studies for the early diagnosis of DGF.

Authorship

TJP, PWP, JWP, NAB and ZHE: designed the research. TJP, PWP and MK: performed the research. TJP, PWP and MK: collected data. TJP, JWP and ZHE: analysed data. TJP, PWP, JWP, MK, NAB and ZHE: wrote the paper.

Funding

Funding for this study included Australian National Health and Medical Research Council project grant 1011772. TJP gratefully acknowledges the financial support of the Jacquot Research Entry Scholarship and a UNSW Australian Postgraduate Award.

Acknowledgements

The authors thank the nursing staff of Prince Of Wales Hospital for their assistance in sample collection.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Biomarker performance in prediction of reduced (delayed or slow) graft function.

Figure S2. Urinary biomarker concentrations after renal transplantation stratified by allograft function in the first day after transplantation.

Table S1. Indications for dialysis and number of dialysis sessions required in 22 patients after kidney transplantation.

Table S2. Biomarker performance in prediction of delayed graft function.

Table S3. Performance of oliguria in prediction of delayed graft function.

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