http://www.kidney-international.org

OPEN

see commentary on page 1072

Serum aminoacylase-1 is a novel biomarker with potential prognostic utility for long-term outcome in patients with delayed graft function following renal transplantation

Matthew P. Welberry Smith[1,2], Alexandre Zougman[1], David A. Cairns[1], Michelle Wilson[1], Tobias Wind[1], Steven L. Wood[1], Douglas Thompson[1,3], Michael P. Messenger[1], Andrew Mooney[2], Peter J. Selby[1], Andrew J.P. Lewington[2] and Rosamonde E. Banks[1]

[1]Clinical and Biomedical Proteomics Group, Cancer Research UK Centre, Leeds Institute of Molecular Medicine, St James's University Hospital, Leeds, UK; [2]Department of Renal Medicine, Lincoln Wing, St James's University Hospital, Leeds, UK and [3]Department of Blood Sciences, St James's University Hospital, Leeds, UK

Early identification and prognostic stratification of delayed graft function following renal transplantation has significant potential to improve outcome. Mass spectrometry analysis of serum samples, before and on day 2 post transplant from five patients with delayed graft function and five with an uncomplicated transplant, identified aminoacylase-1 (ACY-1) as a potential outcome biomarker. Following assay development, analysis of longitudinal samples from an initial validation cohort of 55 patients confirmed that the ACY-1 level on day 1 or 2 was a moderate predictor of delayed graft function, similar to serum creatinine, complementing the strongest predictor cystatin C. A further validation cohort of 194 patients confirmed this association with area under ROC curves (95% CI) for day 1 serum (138 patients) of 0.74 (0.67–0.85) for ACY-1, 0.9 (0.84–0.95) for cystatin C, and 0.93 (0.88–0.97) for both combined. Significant differences in serum ACY-1 levels were apparent between delayed, slow, and immediate graft function. Analysis of long-term follow-up for 54 patients with delayed graft function showed a highly significant association between day 1 or 3 serum ACY-1 and dialysis-free survival, mainly associated with the donor–brain–dead transplant type. Thus, proteomic analysis provides novel insights into the potential clinical utility of serum ACY-1 levels immediately post transplantation, enabling subdivision of patients with delayed graft function in terms of long-term outcome. Our study requires independent confirmation.

Kidney International (2013) **84,** 1214–1225; doi:10.1038/ki.2013.200; published online 5 June 2013

Correspondence: *Rosamonde E. Banks, Clinical and Biomedical Proteomics Group, Cancer Research UK Centre, Leeds Institute of Molecular Medicine, St. James's University Hospital, Beckett Street, Leeds LS9 7TF, UK. E-mail: r.banks@leeds.ac.uk*

Received 30 September 2012; revised 14 March 2013; accepted 21 March 2013; published online 5 June 2013

KEYWORDS: delayed graft function; diagnosis; kidney transplantation; outcomes; renal transplantation

Renal transplantation provides clear benefits for patients with end-stage kidney disease,[1,2] and significant cost savings compared with dialysis.[3,4] In 2010, 16,151 renal transplants were performed in the United States (http://optn.transplant.hrsa.gov), and 2687 in the United Kingdom (http://www.uktransplant.org.uk). However, early complications can significantly impact clinical and economic outcomes, such as delayed graft function (DGF) that affects ~20% of patients in the United States.[5] A number of definitions of DGF have been proposed[6–8] with one commonly used being the need for dialysis in the first week after renal transplantation, other than for isolated hyperkalemia. Although there are parallels with acute kidney injury, the pathology underlying DGF is complex with contributions from donor-derived factors, such as donor age and duration of ischemia, and recipient factors such as ischemia–reperfusion injury (IRI), immunological responses, and immunosuppressant medications.[9] Acute tubular necrosis secondary to IRI is the predominant histological finding but acute cellular or humoral rejection may occur concurrently, and other pathologies are sometimes apparent histologically, e.g., calcineurin inhibitor toxicity. Increasing use of organs donated after circulatory death (DCD) and from extended criteria donors[10] has corresponded with an increase in the incidence of DGF. DGF increases the risk of graft failure, patient death, and death-censored graft failure by two- to three-fold,[11,12] and is associated with a number of complications that contribute to reduced longer-term graft survival, such as a poor transplant function at 1 year, arterial hypertension, and acute rejection.[13] Overall, DGF has been associated with a 41% increased risk of graft loss at just over 3 years.[14]

Early identification, stratification, and increased understanding of DGF has significant potential to improve patient management and outcomes,[15] allowing fluid volume status optimization, timely appropriate dialysis, tailoring of therapies, and avoidance of unnecessary investigation and treatment. There is increasing excitement about the potential of clinical proteomics in identifying new biomarkers with clinical impact,[16] complementing promising markers emerging from genomic-based studies. Urinary markers currently under investigation in renal transplantation include interleukin 18 and neutrophil gelatinase lipocalin,[17] with tissue-associated markers including ICAM-1 and VCAM.[15,18] Unfortunately, in the majority of cases of DGF, urine is not produced or may be mixed with residual native renal output confounding analysis of any results, and biopsied tissue is often only available once DGF is established. Although serum neutrophil gelatinase lipocalin and interleukin 18 have not shown promise,[19] blood-borne biomarkers would be ideal being readily accessible and routinely used in hospital laboratories. However, biomarker discovery with serum or plasma is challenging with only 22 proteins comprising ~99% of the total protein mass, and the wide dynamic range of protein abundances spanning >10 orders of magnitude.[20]

In this study (Figure 1), we have compared serum proteins pre- and postoperatively from patients undergoing renal transplantation, with and without DGF, using our previously optimized immunodepletion followed by label-free single-dimensional liquid chromatography-tandem mass spectrometry analysis strategy.[21] A key candidate marker of DGF was identified as aminoacylase-1 (ACY-1). Following assay development and validation, allowing the measurement of ACY-1 in serum for the first time, the predictive use of ACY-1 for DGF as early as day 1 post transplant was confirmed. Using follow-up data from a cohort of 194 transplant patients where as expected DGF was associated with poor prognosis, serum ACY-1 day 1 post transplant was shown to further subdivide the 54 patients with DGF in terms of their long-term outcome.

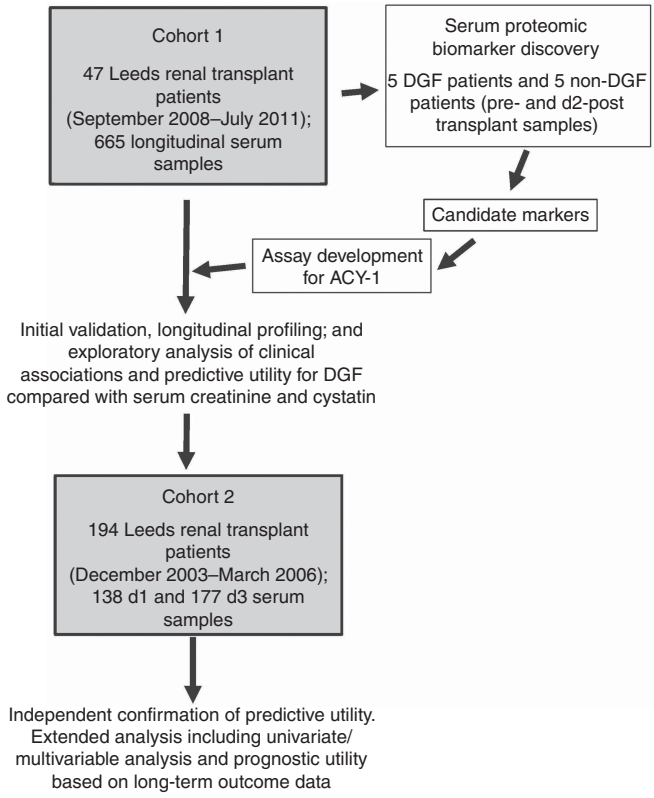

Figure 1 | Schematic showing the study design using samples undergoing renal transplantation at the St James's University Hospital in Leeds. Following initial discovery of aminoacylase-1 (ACY-1) as a novel potential serum marker for delayed graft function (DGF) using mass spectrometry, this was validated using an immunoassay for ACY-1, and the performance as a predictive marker assessed. Using serum samples from a further independent cohort of patients, these results were confirmed and additionally the association with long-term outcome in those patients with DGF was examined.

RESULTS

Patient groups

Examination of the patient groups (Figure 1, Table 1) shows similar characteristics of cohorts 1 (the discovery and initial validation group) and 2 (the larger validation group with long-term outcome data), with the exceptions of proportion of DCD transplants and the induction regimen, reflecting changing clinical practice. DGF was diagnosed in 31.9% of patients in cohort 1 and 28.4% of patients in cohort 2. Mean age and cold ischemic time (CIT) were significantly higher in the DGF groups in each cohort, as was warm ischemic time (WIT) in cohort 2. The five DGF and five non-DGF patients used for the initial proteomic discovery had no evidence of calcineurin inhibitor toxicity or acute rejection and were matched as closely as possible in terms of mean age, ethnicity mix, CIT, WIT, and mean HLA mismatches at the A, B, and DR loci, and immunosuppression regimens, differing slightly in donor type (five DCD in the DGF group compared with three DCD/two donation after brain death (DBD) in the non-DGF group).

Mass spectrometry and candidate biomarker selection

Across all 20 samples (10 patients, two time points) used for the initial mass spectrometry screen, 553 proteins with at least two peptides (at least one of which was unique) were identified and relative quantification determined (all proteins listed and quantification data shown for selected proteins in Supplementary Data—Proteins 1 and 2). On the basis of the statistical significance ($P<0.05$), 34 candidates differentiated between DGF and non-DGF groups either preoperatively, postoperatively, or by pattern of change. These included cystatin C demonstrating proof-of-principle. ACY-1 was prioritized for further investigation, being undetectable preoperatively but increasing markedly postoperatively, particularly in the DGF group (Figure 2a).

Table 1 | Demographic and clinical details for the DGF and non-DGF patients in cohorts 1 and 2, following renal transplantation at the St James's University Hospital in Leeds

	Cohort 1			Cohort 2		
	DGF	Non-DGF		DGF	Non-DGF	
	$n = 15$ total	$n = 32$ total		$n = 55$ total	$n = 139$	
	6 Biopsied: 4 acute rejection and 2 ATN	8 Biopsied: 6 acute rejection and 2 mild tubular injury	*P*-value	40 Biopsied: 26 ATN, 8 rejection, and 6 both ATN and acute rejection	16 Biopsied: 11 ATN, and 5 ATN and acute rejection	*P*-value
Number of patients, and subgroups	9 Not biopsied: 5 calcineurin inhibitor toxicity and 4 assumed ATN	24 Not biopsied: 12 uncomplicated, 4 post transplant UTIs, and 8 calcineurin inhibitor toxicity	NA	15 Not biopsied: all assumed ATN	123 Not biopsied: uncomplicated transplants	NA
Cause of ESRD (%)						
Chronic pyelonephritis	0.0	18.8	0.573	14.5	12.2	0.719
Diabetes	6.7	3.1		12.7	10.1	
Glomerulonephritis	33.3	31.3		32.7	30.9	
Hypertension	6.7	12.5		5.5	10.8	
Inherited	26.7	12.5		14.5	10.8	
Other	20.0	15.6		7.3	5.0	
Unknown	6.7	6.3		12.7	20.1	
Ethnicity (%)						
Asian	6.7	6.3	0.997	20.0	8.6	0.113
Black/African/Caribbean	6.7	6.3		1.8	1.4	
Caucasian	86.7	87.5		58.2	61.9	
Unknown	0.0	0.0		18.2	28.1	
Transplant number (%)						
1	86.7	78.1	0.807	80.0	90.6	0.247
2–4	13.3	21.9		18.2	7.9	
Unknown	0	0		1.8	1.4	
Pre-emptive (%)						
No	100.0	78.1	0.128	96.4	92.8	0.338
Yes	0.0	21.9		1.8	6.5	
Unknown	0	0		1.8	0.7	
Mean time (months) on dialysis at transplantation ± s.d.[a]	32.8 ± 20.7	32.2 ± 25.0	0.941	54.6 ± 40.4	36.5 ± 36.4	<0.001
Mean age (years) ± s.d.	52.4 ± 12.1	40.6 ± 14.1	0.007	50 ± 13.9	46.2 ± 14.9	0.046
Gender						
% Male	73.3	59.3	0.547	37.0	62.6	0.002
Transplant types						
% DBD	20.0	28.1	0.006 (0.123)[#]	57.4	61.9	<0.001
% DCD	80.0	34.3		38.9	13.7	
% LD	0.0	37.5		3.7	24.5	
Induction (%)						
Alemtuzumab	26.7	31.2	0.983	0	0	NA
Basiliximab	73.3	68.7		100	100	
Maintenance steroids used (%)	26.7	21.9	0.994	14.8	10.1	0.527
Mean ischemic time ± s.d.						
Cold (h:min)	16:06 ± 03:11	10:24 ± 06:08	0.002	16:42 ± 04:48	13:48 ± 08:18	0.019
Warm (min)	40 ± 13	39 ± 9	0.782	49.2 ± 19.1	36.9 ± 13.6	<0.001
Mean total HLA mismatch ± sd	3.3 ± 1.0	2.6 ± 1.8	0.127	2.4 ± 1.3	2.0 ± 1.4	0.096

Abbreviations: ATN, acute tubular necrosis; DBD, donations after brain death; DCD, donations after circulatory death; DGF, delayed graft function; ESRD, end-stage renal disease; HLA, human leukocyte antigen; LD, live donor; NA, not applicable; UTI, urinary tract infection.

P-values are provided for differences between the DGF and non-DGF groups in each cohort.

Details of biopsies performed, and other postoperative events (e.g., UTIs) were collected along with serum creatinine, tacrolimus concentrations, C-reactive protein concentrations, and urinary protein/creatinine ratios. In addition, eight live donors were included in the study to check for the effects of surgery on marker levels (mean age 44.0 ± 12.3, 37.5% male).

[a]Excludes pre-emptive transplants, for second and subsequent transplants, total time on dialysis aggregated; [#]*P*-value if LD excluded as rare in DGF.

Serum concentrations of ACY-1 in cohort 1 and relationship to clinical events

Using our in-house ELISA, data for serum ACY-1 broadly supported the mass spectrometry data for the discovery samples (Figure 2). ACY-1 was undetectable (<15.6 ng/ml) in all pre-transplant samples in this set, and in cohort 1 as a whole 43/47 patients had undetectable ACY-1 pre-transplant with the remainder being <50 ng/ml and with no significant pre-transplant difference between DGF and non-DGF groups. Of the 636 longitudinal samples analyzed, ACY-1 was detected in only 230 samples, predominantly in the 5 days post transplant. Similarly live donors exhibited no ACY-1 concentrations (>50 ng/ml) post surgery with 10/12 having undetectable ACY-1 concentrations, indicating that serum ACY-1 concentrations were not affected by surgery *per se*.

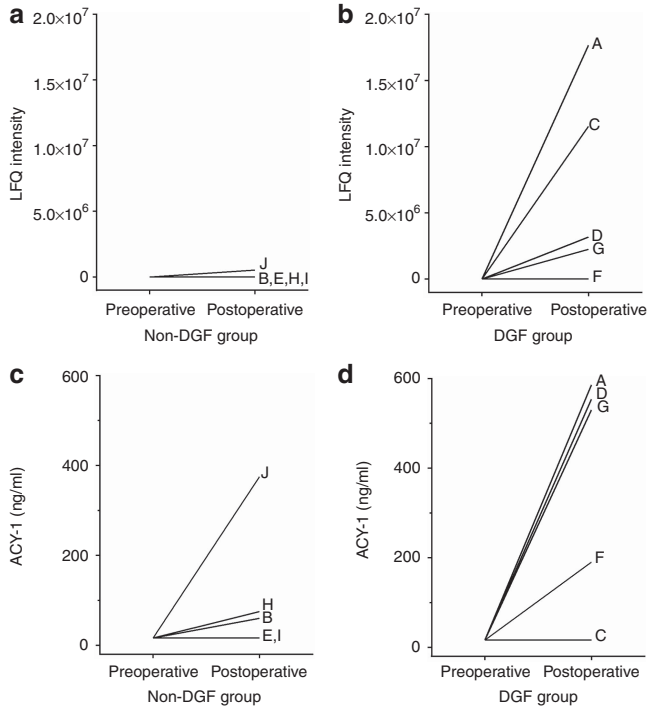

Figure 2 | Serum aminoacylase-1 (ACY-1) concentrations preoperatively and on day 2 postoperatively in the delayed graft function (DGF) and non-DGF groups used for initial biomarker discovery (five patients per group). ACY-1 as measured by (**a**) mass spectrometry label-free intensity and (**b**) subsequent enzyme-linked immunosorbent assay for the same samples. LFQ, label-free quantification. A–J indicate different patients.

Examples of serum ACY-1 profiles with different clinical courses are shown in Figure 3 (full data for all patients including additional clinical events and measurements is in Supplementary Data—Profiles). A peak in ACY-1 concentration (>200 ng/ml) was observed at ⩽4 days post transplantation in 10/15 patients with DGF (66.7%) but in only 6/32 (18.8%) non-DGF transplants, three of whom had an uncomplicated clinical course immediately post transplant. Longitudinal profiles of ACY-1 concentration did not follow the trends seen in any of serum creatinine, cystatin C, C-reactive protein, tacrolimus, or urinary protein/creatinine ratio, with the exception of a peak in ACY-1 in one patient suffering from tacrolimus toxicity with an extremely high concentration of tacrolimus (trough level 33 ng/ml, intended range 9–14 ng/ml). Importantly, there was no distinct peak in serum ACY-1 in patients at the time of positive mid-stream urinary cultures ($n=19$; ACY-1 < 15.6–88.3 ng/ml), episodes of postoperative dialysis, or at the time of biopsy-proven rejection, with the exception of one patient where ACY-1 concentration peaked at 260 ng/ml 4 days before biopsy, becoming undetectable by the time of the biopsy.

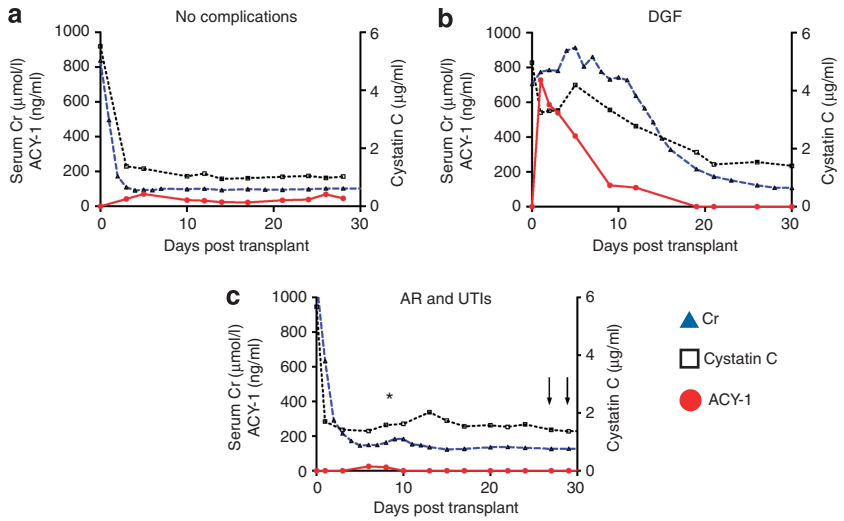

Figure 3 | Examples of profiles for serum aminoacylase-1 (ACY-1), creatinine (Cr), and cystatin C concentrations longitudinally following renal transplantation in three patients with different clinical courses. (**a**) Uncomplicated transplant, (**b**) delayed graft function (a marked peak in ACY-1 concentration >200 ng/ml was seen in 10/15 patients with DGF (66.7%) but only 6/32 (18.8%) non-DGF patients), (**c**) acute rejection (AR; *) followed by two urinary tract infections (UTIs; ↓). The pre-transplant concentrations are shown as day 0. Full profiles for all cohort 1 patients and associated clinical events and measurements are provided in Supplementary Data—Profiles.

Serum concentrations of ACY-1 post transplant in cohort 2 compared with cohort 1

In cohort 2 (the larger final validation set), of the 138 day 1 samples, 30 patients had undetectable serum ACY-1 concentrations (4/42 (9.5%) DGF patients, 26/96 (27.1%) non-DGF), with a similar pattern being seen (18.8% vs. 42.6%, respectively) for day 3 values also (data not shown). In both cohorts, day 1 concentrations of ACY-1 were significantly different between the DGF and non-DGF groups although overlapping (Figure 4a and b). When non-DGF patients from the larger cohort 2 were categorized on the basis of creatinine reduction ratio (CRR = (day 0 creatinine minus day 7 creatinine)/day 0 creatinine),[17,22] significant differences in day 1 serum ACY-1 (Figure 4c) between patients categorized as slow graft function (CRR < 0.7) and immediate graft function (CRR $\geqslant 0.7$) were seen with an increasing trend from immediate graft function to slow graft function to DGF, demonstrating a relationship between the rate of graft function improvement and ACY-1. Similar results were also seen for day 3 serum ACY-1 concentrations (data not shown).

Serum ACY-1 associations and predictive utility for DGF

For initial exploratory examination of the predictive utility in cohort 1, ACY-1 and cystatin C concentrations for days 1 or 2 were combined as one time point ($n = 35$). The area under the receiver operating characteristic (ROC) curve (AUC) for day 1/2 ACY-1 predicting DGF was 0.74, with corresponding figures for creatinine and cystatin C of 0.79 and 0.92, respectively. ACY-1 and cystatin C combined improved the AUC to 0.94 (Figure 5a). In cohort 2, the AUC for day 1 ACY-1 was 0.77 with corresponding figures for creatinine and cystatin C of 0.75 and 0.9, respectively, with the latter improving to 0.93 if combined with ACY-1 (Figure 5b). Data-derived optimum cut-points for day 1 serum ACY-1 and cystatin C in cohort 2 (Table 2) show high specificity and sensitivity, respectively. The optimum cut-point from the combination of both through a logistic regression model provide higher sensitivity and similar specificity, as is apparent through the increased Youden index[23] (0.71 vs. 0.43 and 0.64; Table 2).

Using the larger final validation cohort 2, significant associations were observed between serum ACY-1 concentration and biopsy-proven acute tubular necrosis, transplant type, age at transplantation, CIT, total WIT, and day 1 serum creatinine and cystatin C (Table 3). On univariate analysis, day 1 serum ACY-1, cystatin C and creatinine concentrations, and transplant type, WIT and total HLA mismatch were all significantly associated with the development of DGF (Table 4). A multivariable logistic regression model for the prediction of DGF was developed incorporating day 1 serum ACY-1, creatinine and cystatin C concentrations, patient age and gender, transplant type (DBD, DCD), HLA mismatch, CIT, WIT, and initial steroid use. Day 1 serum ACY-1 was significant using the likelihood ratio test ($P = 0.013$; Table 4) and maintained a high, if reduced, odds ratio compared with univariate analysis. Cystatin C was the only other independent predictor of DGF in this model ($P < 0.0001$).

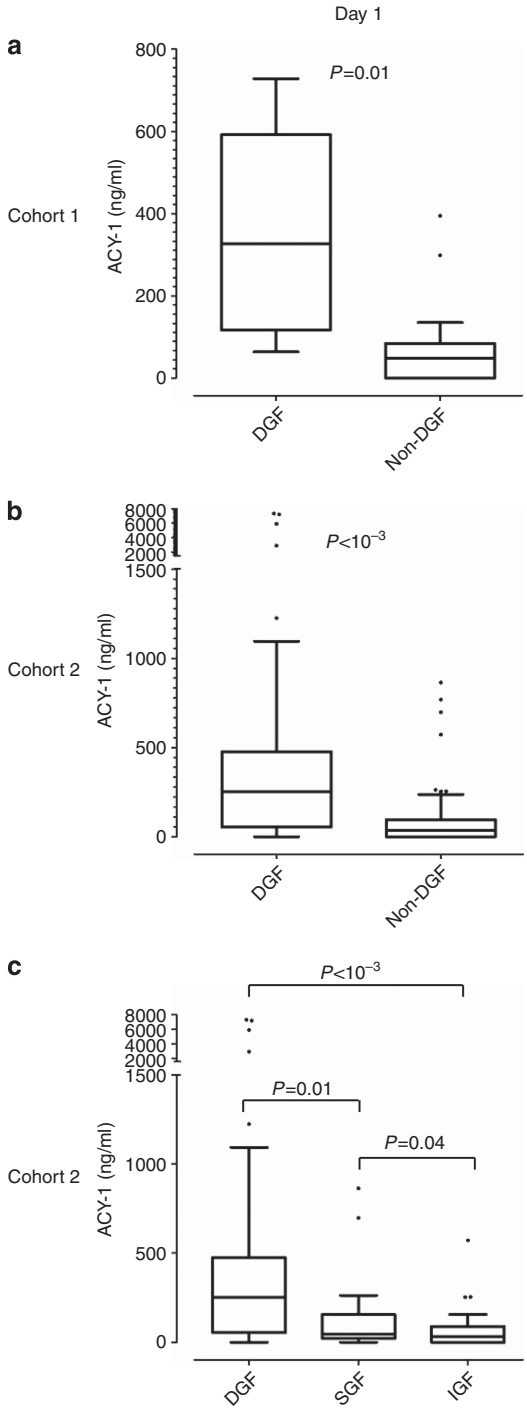

Figure 4 | Serum aminoacylase-1 (ACY-1) concentrations on day 1 post transplant in the delayed graft function (DGF) and non-delayed graft function groups. (**a**) Cohort 1 and (**b**) cohort 2, and (**c**) on day 1 post renal transplant in patients in cohort 2 with DGF, slow graft function (SGF), and immediate graft function (IGF). Tukey's box plots show median values and interquartile ranges with significant differences between the groups indicated as determined by the Mann–Whitney test.

A risk assessment plot for this full multivariable model (new model) and the model without ACY-1

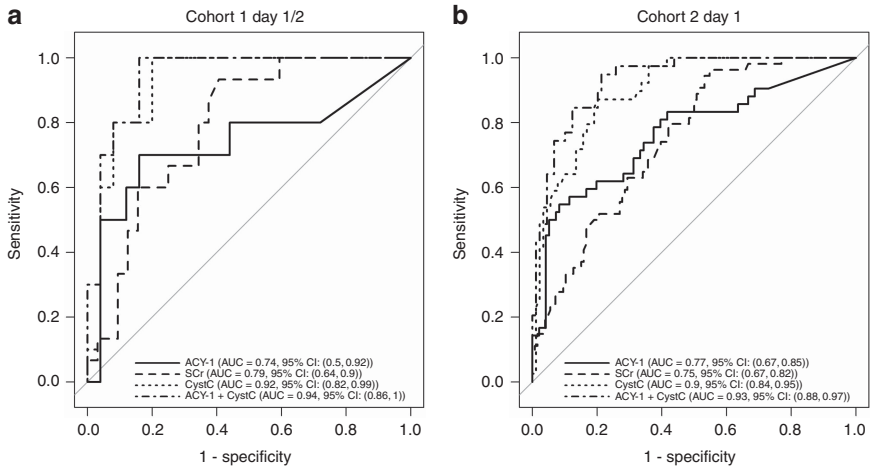

Figure 5 | Receiver operating characteristic curves for the prediction of delayed graft function, each showing serum aminoacylase-1 (ACY-1), creatinine (SCr), cystatin C (CystC), and ACY-1 combined with cystatin C. (**a**) Cohort 1 results—days 1/2 post transplant ($n = 47$ but $n = 35$ for ACY-1 and CystC); (**b**) Cohort 2 results—day 1 post transplant ($n = 194$ but $n = 138$ for ACY-1 and 128 for CystC). AUC, area under the curve; CI, confidence interval.

Table 2 | Estimates and 95% confidence intervals for measures of diagnostic accuracy for ACY-1, cystatin C, serum creatinine, and combined serum ACY-1 and cystatin C for optimal cut-offs as determined in cohort 2 data for day 1 measurements (for serum Cr the additional condition that specificity was >50% was applied)

	Estimate	LCI	UCI
ACY-1 (cut-off 200 ng/ml)			
Sensitivity	54.76	39.71	69.81
Specificity	88.54	82.17	94.91
PPV	67.65	51.92	83.37
NPV	81.73	74.30	89.16
OR	9.35	3.90	22.40
Youden index	0.43		
Cystatin C (cut-off 3.3 μg/ml)			
Sensitivity	87.18	76.69	97.67
Specificity	76.40	67.58	85.23
PPV	61.82	48.98	74.66
NPV	93.15	87.36	98.95
OR	22.02	7.64	63.47
Youden index	0.64		
Serum creatinine (cut-off 550 μmol/l)			
Sensitivity	79.63	68.89	90.37
Specificity	55.56	46.88	64.23
PPV	43.43	33.67	53.20
NPV	86.42	78.96	93.88
OR	4.89	2.31	10.35
Youden index	0.35		
ACY-1 + cystatin C (cut-off probability 0.2)			
Sensitivity	92.31	83.94	100
Specificity	78.65	70.14	87.16
PPV	65.45	52.89	78.02
NPV	95.89	91.34	100
OR	44.21	12.26	159.37
Youden index	0.71		

Abbreviations: ACY-1, aminoacylase-1; LCI, lower confidence interval; NPV, negative predictive value; OR, odds ratio relative to diagnostic cut-off; PPV, positive predictive value; UCI, upper confidence interval.

(reference model) are shown in Supplementary Figure S1 online for patients with and without DGF. Neither model showed evidence of a lack of goodness of fit (Supplementary Table S1 online) nor were the AUCs significantly different as could be anticipated from bootstrap confidence intervals (CIs; $P = 0.345$). The greatest predictive benefit was shown to be for those patients who did not have DGF where 47.4% (95% CI 24.5–70.2%) of patients have a reduced risk according to the new model (in agreement with the high NPV in Table 2). There was also a reduction in the risk for those patients with DGF and this resulted in an overall net reclassification index with a CI that contains zero. The integrated discrimination index for those without DGF was 0.0196 (95% CI 0.004–0.036), further demonstrating the increase in negative predictive value of ACY-1.

Serum ACY-1 and outcome

Serum ACY-1 concentrations on day 1 post transplant showed no correlation with length of DGF, serum creatinine, estimated glomerular filtration rate (modified MDRD), and urinary protein/creatinine ratio at 1 year (cohorts 1 and 2). Similarly analysis of longer-term follow-up data in cohort 2 showed no significant association between ACY-1 concentration on day 1 post transplant and overall or dialysis-free survival.

Given the significant association of ACY-1 with DGF and the significantly increased serum concentrations seen in both DBD and DCD transplant types with DGF (Figure 6), a subgroup analysis using the DGF group only ($n = 54$) was undertaken and showed an association between day 1/3 (day 3 used if day 1 values unavailable) serum ACY-1 and dialysis-free survival in DGF patients (day 1 hazard ratio = 0.993, 95% CI (0.988, 0.999), $P = 0.0174$). Reflecting hazard ratios < 1, survival curves showed a significant

Table 3 | Associations between day 1 serum ACY-1 concentration and relevant variables in cohort 2 ($n = 138$ with day 1 samples)

Characteristic	Subgroup	N	Median (range)	P-value*
Gender	M	90	43.4 (15.6, 7324.5)	
	F	48	74.5 (15.6, 7207.2)	0.1277
Age at transplantation (years)	—	138	0.30	0.0003
Transplant type	DBD	79	48.3 (15.6, 5905.3)	
	DCD	29	365.6 (29.3, 7324.5)	
	LD	30	15.6 (15.6, 156.1)	<0.0001
CIT (min)	—	138	0.25	0.0033
Total WIT (min)	—	138	0.53	<0.0001
Total HLA mismatch	0, 1, and 2	82	50.7 (15.6, 5905.3)	
	3, 4, 5, and 6	56	76.2 (15.6, 7324.5)	0.1261
Initial steroid use	Y	15	72.3 (15.6, 1094.2)	
	N	123	53.2 (15.6, 7324.5)	0.9397
DGF	Y	42	253.7 (15.6, 7324.5)	
	N	96	33.6 (15.6, 864.1)	<0.0001
Biopsy proven AR	Y	11	66.3 (15.6, 1094.2)	
	N	127	53.4 (15.6, 7324.5)	0.6354
Biopsy proven ATN	Y	29	143.9 (15.6, 7324.5)	
	N	109	44.0 (15.6, 1225.1)	0.0007
Both ATN and AR on biopsy	Y	4	140.8 (18.8, 511.1)	
	N	134	53.7 (15.6, 7324.5)	0.5965
Serum creatinine day 1 (μmol/l)	—	130	0.25	0.0050
Serum cystatin C day 1 (μg/ml)	—	128	0.41	<0.0001

Abbreviations: ACY-1, aminoacylase-1; AR, acute rejection; ATN, acute tubular necrosis; CIT, cold ischemic time; DBD, donation after brain death; DCD, donation after cardiac death; DGF, delayed graft function; F, female; HLA, human leukocyte antigen; LD, live donor; M, male; N, no; WIT, warm ischemic time; Y, yes.
*Spearman's rank correlation coefficient and *P*-value if a single continuous variable, or median (range) and *P*-value from Wilcoxon–Mann–Whitney test or Kruskal–Wallis test if comparing two or three subgroups.
'Initial steroid use' refers to whether patients received maintenance oral prednisolone as part of their initial daily immunosuppression regime. Biopsy results—a total of 32 of these 138 patients had biopsies, and therefore the N category for the biopsy results includes those who did not undergo a biopsy.

Table 4 | Univariate and multivariable analysis of factors associated with delayed graft function in cohort 2 as determined by logistic regression

		Univariate			Multivariable		
Factor	Level	Odds ratio	95% CI	P-value	Odds ratio	95% CI	P-value
Gender	Male	1			1		
	Female	0.901	(0.456, 1.780)	0.7645	0.888	(0.223, 3.377)	0.8612
Age at transplantation (years)	—	1.015	(0.992, 1.039)	0.2085	1.000	(0.959, 1.043)	0.9871
Transplant type	DBD	1			1		
	DCD	3.066	(1.457, 6.453)	0.0032	0.730	(0.086, 6.181)	0.7724
CIT (min)	—	1	(0.998, 1.001)	0.5720	1.000	(0.997, 1.003)	0.8496
Total WIT (min)	—	1.040	(1.017, 1.064)	0.0007	1.043	(0.991, 1.097)	0.1040
Total HLA mismatch	0, 1, and 2	1			1		
	3, 4, 5, and 6	2.500	(1.255, 4.979)	0.0091	1.679	(0.415, 6.798)	0.4676
Initial steroid use	N	1			1		
	Y	1.239	(0.424, 3.618)	0.6949	2.465	(0.290, 20.99)	0.4089
Serum ACY-1 day 1 (per increase 1 μg/ml)	—	31.17	(3.400, 285.7)	0.0023	13.82	(1.321, 434.9)	0.0115
Serum creatinine day 1 (per increase 1 μmol/l)	—	1.004	(1.002, 1.006)	<0.0001	1.001	(0.997, 1.004)	0.7526
Serum cystatin C day 1 (per increase 1 μg/ml)	—	5.536	(2.775, 11.05)	<0.0001	6.865	(2.678, 17.60)	<0.0001

Abbreviations: ACY-1, aminoacylase-1; CI, confidence interval; CIT, cold ischemic time; DBD, donation after brain death; DCD, donation after cardiac death; HLA, human leukocyte antigen; N, no; WIT, warm ischemic time; Y, yes.
'Initial steroid use' refers to whether patients received maintenance oral prednisolone as part of their initial daily immunosuppression regime. *P*-values from Wald test for all variables other than multivariable ACY-1 day 1, where likelihood ratio test *P*-value is more appropriate because of larger relative odds ratio.

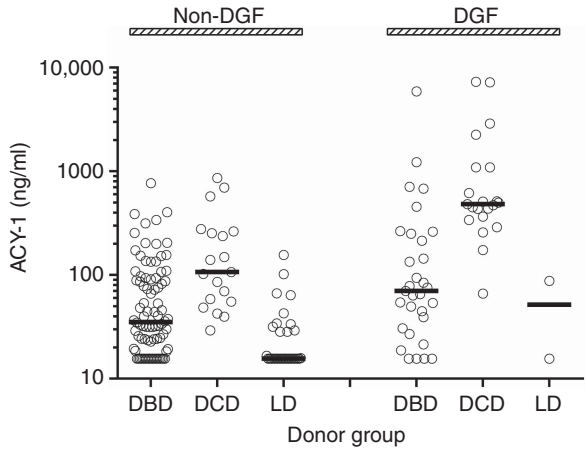

Figure 6 | Serum aminoacylase-1 (ACY-1) concentrations (log scale) on day 1 or 3 post transplant for delayed graft function (DGF) and non-DGF patients in cohort 2, separated by donor type. Medians are indicated by the horizontal bar. Within the non-DGF group, significant differences were seen between each donor type ($P<0.001$) with median values of 35.2, 107, and 15.6 ng/ml for donation after brain death (DBD), donation after cardiac death (DCD), and live donor (LD), respectively, and similarly within the DGF group between DBD and DCD groups with median values of 70.3 and 483.2 ng/ml. Comparing DGF and non-DGF groups, significantly higher ACY-1 concentrations were seen in patients receiving transplants from both DBD ($P=0.023$) and DCD ($P<0.001$).

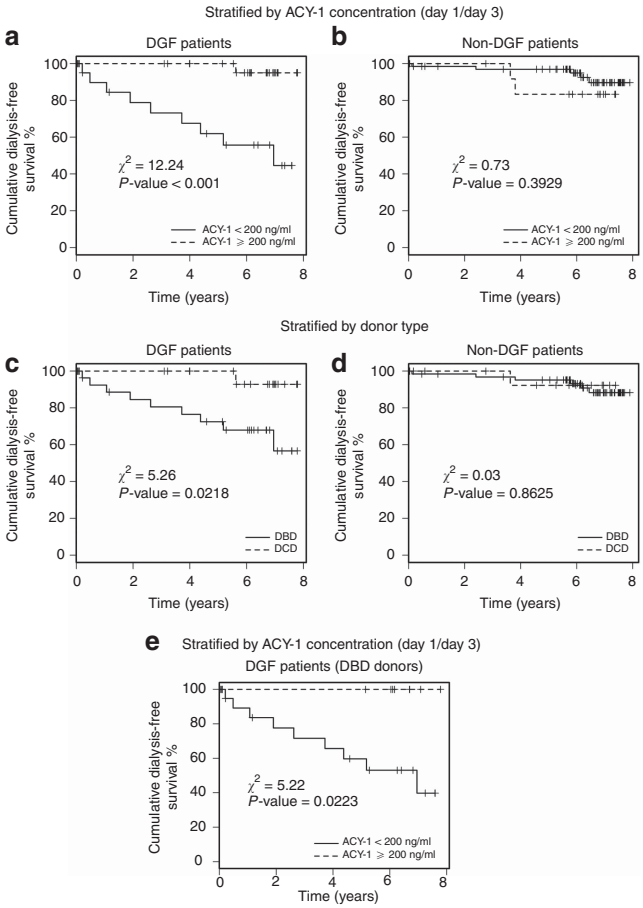

Figure 7 | Kaplan–Meier estimates of survival function for dialysis-free survival post renal transplant in cohort 2 separated by salient characteristics, where median follow-up was 5.93 years with a range of 0.02–7.90 years. (**a**) Delayed graft function (DGF) and (**b**) non-DGF patients separated by serum concentrations of aminoacylase-1 (ACY-1) on day 1 post transplant (or day 3 if no day 1 measurement was available). Numbers of events were: 1/28 (3.6%) with ACY-1 ⩾ 200 and 9/24 (37.5%) with ACY-1 < 200 for DGF patients, and 2/16 (12.5%) with ACY-1 ⩾ 200 and 5/89 (5.6%) with ACY-1 < 200 for non-DGF patients. (**c**) DGF and (**d**) non-DGF patients separated by donor type (donation after cardiac death (DCD) and donation after brain death (DBD)). Numbers of events were: 1/21 (4.8%) for DCD and 9/31 (29%) for DBD within the DGF patients, and 1/19 (5.3%) for DCD and 6/86 (7%) for DBD in the non-DGF group. (**e**) DGF patients with donor type DBD separated by ACY-1 concentration (as above). Numbers of events were: 0/9 (0%) for DBD/ACY-1 ⩾ 200 and 9/22 (40.9%) for DBD/ACY-1 < 200. Reasons for return to dialysis included recurrent focal segmental glomerular fibrosis, vascular rejection, and chronic scarring on biopsy.

negative association ($P<0.001$) between serum ACY-1 day 1/3 post transplant in patients with DGF and risk of returning to dialysis within 5 years from transplantation, which was not seen in the non-DGF group (Figure 7a and b). The subdivision of patients with DGF into two groups based on serum ACY-1 concentration on day 1 post transplant produces groups of similar size and although the number of events ($n=10$ returning to dialysis) is small, the differences remain striking. No similar associations with outcome were observed for serum cystatin C or creatinine in patients following DGF. Formal multivariable analysis was not statistically valid given the low number of events and numbers of variables, but extensive examination of the clinical data detected no factors that differed between the high and low-ACY-1 subgroups of DGF patients, which could be linked to the differences in outcome with the possible exception of donor type. Within the DGF patients only, a marked difference in dialysis-free survival was seen with DBD type transplants accounting for 9/10 events (Figure 7c and d) and within the DBD type transplant patients in the DGF group, a clear difference in outcome based on ACY-1 serum concentrations days 1/3 post transplant was seen (Figure 7e).

Retrospective analysis of the 15 patients with DGF in cohort 1 also suggested an association of ACY-1 with outcome. Although no graft failure and return to dialysis events have yet occurred, three patients appear to have failing transplants with estimated glomerular filtration rates of 10.8, 16.1, and 13.6. Defining these as a poor outcome group and generating a contingency table classifying patients by outcome and ACY-1 (Supplementary Table S2 online), no evidence of a significant association ($P=0.506$) is seen. However, for ACY-1 ⩾ 200 the odds of having a poor outcome are 1:8, whereas if ACY-1 < 200 the odds of a poor outcome are 2:3. A conditional maximum likelihood estimate of the odds ratio is calculated to be 0.215 (95% CI 0.003–5.545), suggesting an association with outcome analogous to that seen in cohort 2, and the lack of a significant test result is probably due to a combination of low

power in a small sample and the more limited amount of follow-up time.

DISCUSSION

This study clearly illustrates the potential of proteomics in biomarker discovery and providing new insights into underlying pathophysiology. Relatively little is known about ACY-1, a zinc-binding homodimeric cytoplasmic protein with a predicted monomeric mass of 45.8 kDa.[24] In eukaryotic cells, 50–80% of all cellular proteins are acylated at the N terminus, a co- or post-translational modification that can affect protein function and stability. ACY-1 catalyzes the hydrolysis of *N*-acetylated peptides, particularly the *N*-acetylated neutral aliphatic amino acids, releasing free amino acids for protein synthesis.[25] The highest levels of expression and activity are found in the kidney followed by the liver, brain, skeletal muscle and pancreas, and low expression in several other organs.[24,26–29] Pan-tubule and predominantly proximal tubule ACY-1 expression has been shown in pig[26] and human kidneys, respectively,[28] with a proposed role in amino-acid salvage.[30] An inborn error of metabolism with variable neurological features has been associated with ACY-1 mutations, with effects on ACY-1 expression and activity depending on the mutation[31] and increased urinary *N*-acetylated amino acids.[27] Located on chromosome 3p21.1, a role as a tumor suppressor gene has been proposed in both renal and lung cancers.[24,32,33]

The low or undetectable serum ACY-1 concentrations pre-transplantation and the absence of any increase with infection, or postsurgery in live donors, suggests the post transplant elevation of ACY-1 in DGF is not simply due to impaired renal clearance or a consequence of inflammation. DGF is complex with multiple risk factors and underlying mechanisms involving various cells including tubules and the vasculature, from preprocurement to the postoperative period.[9,34] Assuming a renal tubular source, the increased serum ACY-1 in many cases of DGF could reflect the extent of tubular damage and this is supported by the association with acute tubular necrosis (although the number of biopsies was small) and the trend in serum ACY-1 concentrations from uncomplicated transplants through slow graft function to DGF. Conversely, the relationship between higher ACY-1 and better outcome following DGF may indicate increased synthesis and a role in the repair process, potentially via effects on amino-acid availability for protein synthesis. The relatively high specificity but low sensitivity of this marker for DGF may indicate different DGF subgroups with different pathologies and outcomes. ACY-1 was one of 75 urinary proteins changing significantly in rats treated with cyclosporine or sirolimus (but not tacrolimus)-treated rats,[35] and ACY-1 forms adducts with the biologically active metabolite generated in the kidney from mycophenolate mofetil,[36] proposed to be involved in organ toxicity. However, we found no links with calcineurin inhibitor toxicity or mycophenolate mofetil. Interestingly analysis of serum samples from 22 patients with AKI, 10 of whom were within 3 days of diagnosis, showed serum ACY-1 concentrations all <60 ng/ml (data not shown), possibly indicating that whether reflecting damage or repair or any possible underlying IRI, it is very specific to a pathological process encountered primarily in certain transplant situations and/or only occurs under the more extreme and extensive ischemic/hypoxic conditions at that time. This is also supported by the marked differences in serum ACY-1 between the DBD, DCD, and LD transplants.

In terms of markers for DGF, combining gene expression, particularly in chemokines CCL19 and 21 and proteasome subunits PSMB8 and 10, in zero-hour biopsies from deceased and live donor grafts, with relevant clinical factors, has resulted in AUC values of 0.74 for DGF and 0.93 for acute rejection.[37] Expression of RANTES and CCR1/CCR2 in biopsies from patients with DGF has also been reported to be associated with graft function at 1 year post transplantation and later.[38] Several existing markers of use in AKI have also been examined[39,40] but tissue KIM-1, for example, doesn't correlate with DGF,[41] whereas urinary neutrophil gelatinase lipocalin and interleukin 18 have some predictive value for DGF[17,42] but no correlation with long-term function.[43] Day 1 serum neutrophil gelatinase lipocalin concentrations had no predictive value for DGF but similar to our study, serum cystatin C had an AUC of 0.83.[17] Our specificity (88.5%) and negative predictive values (81.7%) for ACY-1 in relation to DGF are high enough to potentially contribute to biomarker panels. Clearly serum cystatin C, which directly reflects renal function, is superior to ACY-1 in predicting DGF early with AUC similar to the 0.96 seen in a meta-analysis of its use in predicting AKI in various clinical settings.[44] However, using both ACY-1 and cystatin C is slightly better on day 1 with ACY-1 having higher specificity and if a more sensitive ACY-1 assay was developed, further discrimination by ACY-1 may be possible. However, the major benefit of ACY-1 may be its apparent prognostic utility within the DGF patient group, although yet to be confirmed in further independent studies.

Further mechanistic insight may be apparent from gene expression studies in mouse models of IRI.[45] Sphingosine kinase-1 catalyzes the formation of sphingosine-1-phosphate that has been implicated in protection/repair in renal IRI[46–48], differs between grafts classified by function,[49] and is reported to interact with ACY-1 (refs 32,50). Inflammatory and immune-response genes in donor biopsies have been predominantly associated with IRI and DGF, with integrated systems biology analysis approaches being proposed to provide further insight.[15] A recent comparison of DGF and non-DGF pre-implantation biopsies found no significant clustering of pathways until the DGF group was subclassified on the basis of renal function during the first year, when genes implicated in T-cell activation, antigen presentation, and cell adhesion were associated with subsequent poorer function.[49] This subclassification of DGF patients is analogous to the situation with ACY-1 in our study, where clear elevations in serum ACY-1 post transplant are

seen in only about two-thirds of patients with DGF. This may imply different underlying pathophysiological subgroups and also demonstrates the prognostic value of ACY-1 within the DGF patient group. Although several studies report a higher frequency of DGF with DCD donor type, importantly our examination of donor type confirms the lack of effect of DGF on outcomes in the DCD transplant recipient group despite prolonged periods of warm ischemia[51–53] but the detrimental effect of DGF on outcome in DBD patients.[53,54] From animal models, major systemic effects of brain death such as catecholamine release and hypotension and subsequent activation of proinflammatory mediators/cytokine response in donor organs which then provokes further host responses following transplantation have been proposed.[55] Different underlying pathologies responsible for the DGF with the different donor types have been discussed with the possibility of pre-existing or pre-terminal conditions, resulting in less reversible changes with DBD organs than the acute tubular necrosis encountered following terminal warm ischemia in the DCD donors.[53] This would align with one of our hypotheses that ACY-1 is essentially a marker of repair/response to damage with those patients showing lower response having poorer outcome, as can be seen in a subgroup of DBD donor type transplant patients with lower ACY-1 concentrations immediately post transplant.

This study illustrates the significant potential that clinical proteomics can have in biomarker discovery. The identification of serum ACY-1 with fair predictive value in the immediate postoperative period, and, most importantly, the potential ability to stratify patients within the DGF group, in particular in the DBD transplant, in terms of long-term outcome, may contribute to the development of outcome signatures, enabling better patient stratification and post transplant management. Further larger studies are needed to confirm these findings together with development of a more sensitive assay and biological studies to understand the source and regulation of ACY-1 expression in DGF and its relationship to specific underlying pathophysiology.

MATERIALS AND METHODS

Patient groups and study design

Patients were consented before undergoing renal transplantation (Figure 1). Venous blood samples were obtained prospectively pre-transplant and longitudinally at least three times a week post transplant (mean 14 samples/patient). Blood was collected and serum obtained according to stringent standardized procedures. Patient cohort 1 included 55 patients (665 samples; 47 renal transplant patients and 8 live donors), 15 of whom had DGF (based on the definition of needing dialysis in the first week after renal transplantation other than for isolated hyperkalemia), and cohort 2 included 194 patients (138 with day 1 and 177 with day 3 samples), 55 of whom had DGF (Figure 1). Initial biomarker discovery was carried out using serum samples from five patients in cohort 1 with DGF and five with no complications, matched clinically as far as possible, comparing samples taken pre-transplant and at day 2 post transplant (20 samples in total). Validation of the initial findings was then undertaken using samples from cohort 1 followed by cohort 2 with numbers being determined based on statistical power (Supplementary Methods online). For example, 10 DGF and 25 non-DGF patients with day 1/2 ACY-1 measurements gave a power of 80% to detect an AUC >0.8 when Bonferroni correcting a 5% significance test for two comparisons.[56]

Serum immunodepletion and sample preparation

The 20 serum samples in the discovery set were subjected to immunodepletion using the Multiple Affinity Reagent System 14 column (Agilent, Stockport, UK) as previously described.[21] Immunodepleted fractions were desalted and concentrated and the resulting material was digested with trypsin using a modification[21] of the filter-assisted sample preparation method (FASP).[57]

Label-free mass spectrometry

After acidification to a final concentration of 0.1% TFA, peptide samples were block randomized by patient and analyzed ($3\times2\,\mu g$ injections per sample) using a Dionex UltiMate 3000 RSLCnano system connected to LTQ Orbitrap Velos mass spectrometer equipped with a Proxeon nanoelectrospray ion source.[58] Samples were injected directly onto an in-house 25-cm capillary emitter column packed with 3.5-μm Kromasil C18 media. The total acquisition time was 300 min, the major part of the gradient being 3–25% ACN in 0.1% formic acid at the flow rate of 0.4 μl/min. Survey MS scans were acquired in the orbitrap with the resolution set to 60,000. Up to the 20 most intense ions per scan were fragmented and analyzed in the linear trap.

Data analysis and statistical methods

Label-free mass spectrometry data analysis was performed using MaxQuant (v1.1.1.25).[59] Proteins were identified using Andromeda[60] and the IPI human database (v3.75, 19/08/2010) with the criteria of $\geqslant2$ peptides (at least one being unique), and proteins identified from the decoy database and known contaminants were removed. Differential expression between and within patient groups was assessed using non-parametric (Wilcoxon) significance tests on the changes in label-free quantification intensity. The false discovery rate was estimated using the q-value method.[61]

In the validation analysis, ROC curves were constructed to assess predictive ability of serum ACY-1, creatinine, and cystatin C.[62] The area under the ROC curve was estimated and 95% CIs estimated from 2000 bootstrap resamples. AUCs were compared using a bootstrap significance test with the significance of differences between bootstrap AUCs assessed using a normal approximation. Risk assessment plots[63] and summary statistics relating to the net reclassification index and integrated discrimination index[64,65] were estimated using bespoke R functions and CIs estimated with 2000 bootstrap resamples. The association of relevant clinical factors known to predict DGF was quantified and tested using univariate logistic regression models, and multivariable logistic regression was used to assess the independent predictive ability of ACY-1 for DGF in models containing other relevant factors.[66] In the multivariable model likelihood ratio tests and Wald tests, and simple and profile likelihood–based confidence intervals were contrasted due to the large relative magnitude of some coefficients.[67] The relationship between ACY-1 and other marker concentrations and dialysis-free survival was assessed using Cox proportional-hazard regression, Kaplan–Meier survival functions, and the log-rank test. All statistical tests were two-sided and all analyses were undertaken in the

R environment for statistical computing (R Development Core Team, Vienna, Austria).

Comparison with other analytes

Serum creatinine, C-reactive protein, tacrolimus, and urinary protein/creatinine ratios were measured as per clinical protocol/indication, and additionally serum creatinine and cystatin C were measured in cohort 1 at the same time points used in the study.

ELISA development

ACY-1 concentrations were determined for the 980 serum samples in both cohorts with samples being block randomized, using a sandwich ELISA developed and validated in-house (Supplementary Methods online) using rat anti-human ACY-1 monoclonal antibody (clone 475626; R&D Systems; Minneapolis, MN) and polyclonal goat anti-ACY-1 antibody (R&D Systems).

DISCLOSURE

The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

ACKNOWLEDGMENTS

We thank the patients for consenting to the use of their samples and data, to the Renal Research Nursing team at St. James's University Hospital, and the Clinical and Biomedical Proteomics Group sample processing team. This paper presents independent research funded by the Medical Research Council, Kidney Research UK, the Yorkshire Kidney Research Fund, the Leeds Teaching Hospitals Charitable Foundation, and the NIHR under its Programme Grants for Applied Research Programme (grant reference number RP-PG-0707-10101).

SUPPLEMENTARY MATERIAL

Figure S1. Risk assessment plot for ACY-1 when added to reference multivariable model.
Table S1. Summary statistics for model improvement metrics when ACY-1 is added to reference logistic regression model.
Table S2. Contingency table classifying graft outcome against day 1 serum ACY-1 concentration in the patients with DGF in cohort 1.
Supplementary Data Profiles—Early Follow-up and events for Cohort 1.
Supplementary Data—Proteins 1: All serum proteins identified with at least 2 peptides at least one of which was unique (full data will be deposited in PRIDE)
Supplementary Data—Proteins 2.
Supplementary Methods: Patient groups and study design.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Port FK, Wolfe RA, Mauger EA *et al.* Comparison of survival probabilities for dialysis patients vs cadaveric renal transplant recipients. *JAMA* 1993; **270**: 1339–1343.
2. Wolfe RA, Ashby VB, Milford EL *et al.* Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. *N Engl J Med* 1999; **341**: 1725–1730.
3. de Wit GA, Ramsteijn PG, de Charro FT. Economic evaluation of end stage renal disease treatment. *Health Policy* 1998; **44**: 215–232.
4. Kontodimopoulos N, Niakas D. An estimate of lifelong costs and QALYs in renal replacement therapy based on patients' life expectancy. *Health Policy* 2008; **86**: 85–96.
5. Tapiawala SN, Tinckam KJ, Cardella CJ *et al.* Delayed graft function and the risk for death with a functioning graft. *J Am Soc Nephrol* 2010; **21**: 153–161.
6. Akkina SK, Connaire JJ, Israni AK *et al.* Similar outcomes with different rates of delayed graft function may reflect center practice, not center performance. *Am J Transplant* 2009; **9**: 1460–1466.
7. Roels L, Coosemans W, Donck J *et al.* Inferior outcome of cadaveric kidneys preserved for more than 24 h in histidine-tryptophan-ketoglutarate solution. Leuven Collaborative Group for Transplantation. *Transplantation* 1998; **66**: 1660–1664.
8. Chang SH, Russ GR, Chadban SJ *et al.* Trends in kidney transplantation in Australia and New Zealand, 1993-2004. *Transplantation* 2007; **84**: 611–618.
9. Siedlecki A, Irish W, Brennan DC. Delayed graft function in the kidney transplant. *Am J Transplant* 2011; **11**: 2279–2296.
10. Keitel E, Michelon T, dos Santos AF *et al.* Renal transplants using expanded cadaver donor criteria. *Ann Transplant* 2004; **9**: 23–24.
11. Halloran PF, Aprile MA, Farewell V *et al.* Early function as the principal correlate of graft survival. A multivariate analysis of 200 cadaveric renal transplants treated with a protocol incorporating antilymphocyte globulin and cyclosporine. *Transplantation* 1988; **46**: 223–228.
12. Snyder JJ, Kasiske BL, Gilbertson DT *et al.* A comparison of transplant outcomes in peritoneal and hemodialysis patients. *Kidney Int* 2002; **62**: 1423–1430.
13. Sola R, Alarcon A, Jimenez C *et al.* The influence of delayed graft function. *Nephrol Dial Transplant* 2004; **19**(Suppl 3): iii32–iii37.
14. 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* 2009; **24**: 1039–1047.
15. Muhlberger I, Perco P, Fechete R *et al.* Biomarkers in renal transplantation ischemia reperfusion injury. *Transplantation* 2009; **88**: S14–S19.
16. Smith MP, Banks RE, Wood SL *et al.* Application of proteomic analysis to the study of renal diseases. *Nat Rev Nephrol* 2009; **5**: 701–712.
17. Hall IE, Yarlagadda SG, Coca SG *et al.* IL-18 and urinary NGAL predict dialysis and graft recovery after kidney transplantation. *J Am Soc Nephrol* 2010; **21**: 189–197.
18. Schwarz C, Regele H, Steininger R *et al.* The contribution of adhesion molecule expression in donor kidney biopsies to early allograft dysfunction. *Transplantation* 2001; **71**: 1666–1670.
19. Hall IE, Doshi MD, Poggio ED *et al.* A comparison of alternative serum biomarkers with creatinine for predicting allograft function after kidney transplantation. *Transplantation* 2011; **91**: 48–56.
20. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. *Mol Cell Proteomics* 2002; **1**: 845–867.
21. Smith MP, Wood SL, Zougman A *et al.* A systematic analysis of the effects of increasing degrees of serum immunodepletion in terms of depth of coverage and other key aspects in top-down and bottom-up proteomic analyses. *Proteomics Clin Appl* 2011; **5**: 561.
22. Johnston O, O'Kelly P, Spencer S *et al.* Reduced graft function (with or without dialysis) vs immediate graft function—a comparison of long-term renal allograft survival. *Nephrol Dial Transplant* 2006; **21**: 2270–2274.
23. Youden WJ. Index for rating diagnostic tests. *Cancer* 1950; **3**: 32–35.
24. Cook RM, Burke BJ, Buchhagen DL *et al.* Human aminoacylase-1. Cloning, sequence, and expression analysis of a chromosome 3p21 gene inactivated in small cell lung cancer. *J Biol Chem* 1993; **268**: 17010–17017.
25. Perrier J, Durand A, Giardina T *et al.* Catabolism of intracellular N-terminal acetylated proteins: involvement of acylpeptide hydrolase and acylase. *Biochimie* 2005; **87**: 673–685.
26. Lindner H, Hopfner S, Tafler-Naumann M *et al.* The distribution of aminoacylase I among mammalian species and localization of the enzyme in porcine kidney. *Biochimie* 2000; **82**: 129–137.
27. Sass JO, Mohr V, Olbrich H *et al.* Mutations in ACY1, the gene encoding aminoacylase 1, cause a novel inborn error of metabolism. *Am J Hum Genet* 2006; **78**: 401–409.
28. Yamauchi A, Ueda N, Hanafusa S *et al.* Tissue distribution of and species differences in deacetylation of N-acetyl-L-cysteine and immunohistochemical localization of acylase I in the primate kidney. *J Pharmacy Pharmacol* 2002; **54**: 205–212.
29. Miller YE, Kao B. Monoclonal antibody based immunoassay for human aminoacylase-1. *J Immunoassay* 1989; **10**: 129–152.
30. Lindner HA, Tafler-Naumann M, Rohm KH. N-acetylamino acid utilization by kidney aminoacylase-1. *Biochimie* 2008; **90**: 773–780.
31. Sommer A, Christensen E, Schwenger S *et al.* The molecular basis of aminoacylase 1 deficiency. *Biochim Biophys Acta* 2011; **1812**: 685–690.
32. Zhong Y, Onuki J, Yamasaki T *et al.* Genome-wide analysis identifies a tumor suppressor role for aminoacylase 1 in iron-induced rat renal cell carcinoma. *Carcinogenesis* 2009; **30**: 158–164.

33. Miller YE, Minna JD, Gazdar AF. Lack of expression of aminoacylase-1 in small cell lung cancer. Evidence for inactivation of genes encoded by chromosome 3p. *J Clin Invest* 1989; **83**: 2120–2124.
34. Abu Jawdeh BG, Rabb H. Delayed kidney allograft function—what does it tell us about acute kidney injury? *Contrib Nephrol* 2011; **174**: 173–181.
35. Klawitter J, Kushner E, Jonscher K *et al.* Association of immunosuppressant-induced protein changes in the rat kidney with changes in urine metabolite patterns: a proteo-metabonomic study. *J Proteome Res* 2010; **9**: 865–875.
36. Asif AR, Armstrong VW, Voland A *et al.* Proteins identified as targets of the acyl glucuronide metabolite of mycophenolic acid in kidney tissue from mycophenolate mofetil treated rats. *Biochimie* 2007; **89**: 393–402.
37. Kotsch K, Kunert K, Merk V *et al.* Novel markers in zero-hour kidney biopsies indicate graft quality and clinical outcome. *Transplantation* 2010; **90**: 958–965.
38. Dikow R, Becker LE, Schaier M *et al.* In renal transplants with delayed graft function chemokines and chemokine receptor expression predict long-term allograft function. *Transplantation* 2010; **90**: 771–776.
39. Halawa A. The early diagnosis of acute renal graft dysfunction: a challenge we face. The role of novel biomarkers. *Ann Transplant* 2011; **16**: 90–98.
40. Malyszko J. Biomarkers of acute kidney injury in different clinical settings: a time to change the paradigm? *Kidney Blood Press Res* 2010; **33**: 368–382.
41. Schroppel B, Kruger B, Walsh L *et al.* Tubular expression of KIM-1 does not predict delayed function after transplantation. *J Am Soc Nephrol* 2010; **21**: 536–542.
42. Parikh CR, Jani A, Mishra J *et al.* Urine NGAL and IL-18 are predictive biomarkers for delayed graft function following kidney transplantation. *Am J Transplant* 2006; **6**: 1639–1645.
43. Hollmen ME, Kyllonen LE, Inkinen KA *et al.* Urine neutrophil gelatinase-associated lipocalin is a marker of graft recovery after kidney transplantation. *Kidney Int* 2011; **79**: 89–98.
44. Zhang Z, Lu B, Sheng X *et al.* Cystatin C in prediction of acute kidney injury: a systemic review and meta-analysis. *Am J Kidney Dis* 2011; **58**: 356–365.
45. Supavekin S, Zhang W, Kucherlapati R *et al.* Differential gene expression following early renal ischemia/reperfusion. *Kidney Int* 2003; **63**: 1714–1724.
46. Park SW, Kim M, Kim M *et al.* Sphingosine kinase 1 protects against renal ischaemia-reperfusion injury in mice by sphingosine-1-phosphate1 receptor activation. *Kidney Int* 2011; **2011**: 1315–1327.
47. Bajwa A, Jo SK, Ye H *et al.* Activation of sphingosine-1-phosphate 1 receptor in the proximal tubule protects against ischaemia-reperfusion injury. *J Am Soc Nephrol* 2010; **21**: 955–965.
48. Sola A, Weigert A, Jung M *et al.* Sphingosine-1-phosphate signalling induces the production of Lcn-2 by macrophages to promote kidney regeneration. *J Pathol* 2011; **225**: 597–608.
49. Mas VR, Scian MJ, Archer KJ *et al.* Pretransplant transcriptome profiles identify among kidneys with delayed graft function those with poorer quality and outcome. *Mol Med* 2011; **17**: 1311–1322.
50. Maceyka M, Nava VE, Milstien S *et al.* Aminoacylase 1 is a sphingosine kinase 1-interacting protein. *FEBS Lett* 2004; **568**: 30–34.
51. Le Dinh H, Weekers L, Bonvoisin C *et al.* Delayed graft function does not harm the future of donation-after-cardiac death in kidney transplantation. *Transpl Proc* 2012; **44**: 2795–2802.
52. Nagaraja P, Roberts GW, Stephens M *et al.* Influence of delayed graft function and acute rejection on outcomes after kidney transplantation from donors after cardiac death. *Transplantation* 2012; **94**: 1218–1223.
53. Singh RP, Farney AC, Rogers J *et al.* Kidney transplantation from donation after cardiac death donors: lack of impact of delayed graft function on post-transplant outcomes. *Clin Transplant* 2011; **25**: 255–264.
54. Brook NR, White SA, Waller JR *et al.* Non-heart beating donor kidneys with delayed graft function have superior graft survival compared with conventional heart-beating donor kidneys that develop delayed graft function. *Am J Transplant* 2003; **3**: 614–618.
55. Kusaka M, Pratschke J, Wilhelm MJ *et al.* Activation of inflammatory mediators in rat renal isografts by donor brain death. *Transplantation* 2000; **69**: 405–410.
56. Obuchowski NA, Lieber NL, Wians FHJ. ROC curves in clinical chemistry: uses, misuses, and possible solutions. *Clin Chem* 2004; **50**: 1118–1125.
57. Ziolkowski P, Gamian E, Osiecka B *et al.* Immunohistochemical and proteomic evaluation of nuclear ubiquitous caesein and cyclin-dependent kinases substrate in invasive ductal carcinoma of the breast. *J Biomed Biotechnol* 2009; **2009**: 919645.
58. Zougman A, Ziolkowski P, Mann M *et al.* Evidence for insertional RNA editing in humans. *Curr Biol* 2008; **18**: 1760–1765.
59. Cox J, Matic I, Hilger M *et al.* A practical guide to the MaxQuant computational platform for SILAC-based quantitative proteomics. *Nat Protoc* 2009; **4**: 698–705.
60. Cox J, Neuhauser N, Michalski A *et al.* Andromeda: a peptide search engine integrated into the MaxQuant environment. *J Proteome Res* 2011; **10**: 1794–1805.
61. Storey JD. A direct approach to false discovery rates. *J Royal Stats Soc Series B—Stat Methodol* 2002; **64**: 479–498.
62. Robin X, Turck N, Hainard A *et al.* pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinform* 2011; **12**: 77.
63. Pickering JW, Endre ZH. New metrics for assessing diagnostic potential of candidate biomarkers. *Clin J Am Soc Nephrol* 2012; **7**: 1355–1364.
64. Pencina MJ, D'Agostino RBS, D'Agostino RBJ *et al.* Evaluating the added predictive ability of a new marker: From area under the ROC curve to reclassification and beyond. *Stat Med* 2008; **27**: 157–172.
65. Pencina MJ, D'Agostino RBS, Steyerberg EW. Extensions of net reclassification improvement calculations to measure usefulness of new biomarkers. *Stat Med* 2011; **30**: 11–21.
66. Irish WD, Ilsley JN, Schnitzler MA *et al.* A risk prediction model for delayed graft function in the current era of deceased donor renal transplantation. *Am J Transplant* 2010; **10**: 2279–2286.
67. Hauck WW, Donner A. Wald's test as applied to hypotheses in logit analysis. *J Am Stat Assoc* 1977; **72**: 851–853.