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Use of a Targeted Urine Proteome Assay (TUPA) to Identify Protein Biomarkers of Delayed Recovery After Kidney Transplant

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Abstract

Purpose—Development of delayed graft function (DGF) following kidney transplant is associated with poor outcomes. An ability to rapidly identify patients with DGF versus those with immediate graft function (IGF) may facilitate the treatment of DGF and the research needed to improve prognosis. The purpose of this study was to use a Targeted Urine Proteome Assay (TUPA) to identify protein biomarkers of delayed recovery from kidney transplant.

Experimental Design—Potential biomarkers were identified using the TUPA Multiple Reaction Monitoring (MRM) assay to interrogate the relative DGF/IGF levels of expression of 167 proteins in urine taken 12–18 hours after kidney implantation from 21 DGF, 15 SGF (slow graft function), and 16 IGF patients. An iterative Random Forest analysis approach evaluated the

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Conflict of Interest Statement

The authors of this manuscript have no conflicts of interest to disclose.

Supporting Information:

1. Materials and Methods
2. Supporting Tables 1–15
3. Supporting Figures 1–6

relative importance of each biomarker, which was then used to identify an optimum biomarker panel that provided the maximum sensitivity and specificity with the least number of biomarkers.

Conclusions and clinical relevance—Four proteins were identified that together distinguished DGF with a sensitivity of 77.4%, specificity of 82.6% and AUC of 0.891. This panel represents an important step towards identifying DGF at an early stage so that more effective treatments can be developed to improve long term graft outcomes.

Keywords

Disease Biomarkers; Kidney transplant; Targeted Proteomics; Urine

Introduction

Kidney function during the first week following renal transplant varies tremendously, with some recipients experiencing immediate graft function (IGF, characterized by a rapid fall in serum creatinine), while others exhibit delayed graft function (DGF, requiring at least one treatment of dialysis post-transplant). While DGF occurs infrequently in living donor kidney transplants, its incidence in deceased donor transplants is 20 to 33% [1–4]. Recent strategies for increasing the recipient (e.g., elderly) and donor pools have also increased the risk of sub-optimal allograft function. Hence, both “extended-criteria donor” (ECD) and “donation after circulatory determination of death” (DCD) kidneys are associated with higher rates of DGF as compared with standard-criteria kidneys [5]. The short term negative impact of DGF, which is caused primarily by ischemia-reperfusion injury (IRI) during allograft procurement and transplantation, includes increased lengths of stay and hospital costs primarily because of the need for dialysis [6, 7]. Over the longer term, DGF is associated with a 41% increased risk of graft loss at 3.2 years of follow-up [8]. Even in patients who were not dialyzed after transplant, those with slow rates of decline in serum creatinine (slow graft function, SGF) have poorer long-term outcomes as compared with IGF [3, 9, 10]. While there is general agreement that DGF is associated with worse outcomes, there is less agreement regarding diagnostic criteria given multiple DGF definitions reported in the literature [11–15]. Current approaches for diagnosing DGF or SGF often include need for dialysis, changes in serum creatinine, and urine output. However, all three approaches are retrospective and can be confounded by residual native function [16]. As with other forms of acute kidney injury (AKI) caused by IRI, the delay in diagnosis necessitated by these retrospective approaches greatly impedes efforts to prevent or treat renal injury [17]. Such a delay is particularly pernicious in the setting of transplant as the most common immunosuppression regimens utilize nephrotoxic calcineurin inhibitors. Rapidly distinguishing DGF from IGF post-operatively could allow early tailoring of immunosuppressants, both agents and doses, to renal function.

Multiple investigators are intensely studying novel urinary biomarkers for the early detection of AKI as well as the differential diagnosis and prognosis of kidney injury. Coca et al. [18] evaluated over 30 novel serum and urine biomarkers with three of the most thoroughly studied being neutrophil gelatinase-associated lipocalin (NGAL), IL-18, and kidney injury molecule-1 (KIM-1). Noninvasive measurement of such biomarkers soon after transplant may allow rapid prediction of post-operative renal function with resulting improvements in

outcomes. In addition, identification of markers associated with rapid recovery of function may suggest future targets for therapeutics. Based on urine analyses carried out 6–18 hrs following transplant, Hall et al. [19] reported area under the curve (AUC) values for predicting DGF using NGAL, IL-18 and KIM-1 that ranged from 0.67 for KIM-1 to 0.81 for NGAL. Hollmen et al. [20] demonstrated that post-operative urinary NGAL not only predicted DGF but also distinguished prolonged from short DGF. Once again, however, AUCs were between 0.7–0.8. Although Choi et al. [21] reported a statistically significant increase in urine NGAL on postoperative day 2 between IGF and DGF patients, there was no significant difference on postoperative day 0.

The goal of this study was to employ a Targeted Urine Proteome Assay (TUPA) to identify a panel of biomarkers that can best distinguish DGF versus IGF with minimal lag time (e.g., 6–18 hrs following transplant) and with high sensitivity and specificity. TUPA uses a robust mass spectrometry approach, called scheduled Multiple Reaction Monitoring (MRM), that we have developed to quantify up to 167 urine proteins simultaneously on one urine sample [22].

Materials and Methods

The following provides a brief overview of the experimental procedures with a more detailed description in Supporting Information.

Extended Multiple Reaction Monitoring (xMRM)

As described previously [22], TUPA uses a scheduled LC-MRM assay that was developed using a previously described workflow [23]. This was carried out on an AB SCIEX 5500 Q-TRAP to potentially quantify the level of expression of 167 proteins in human urine based on 896 peptide transitions in TUPA that were determined to be free of interference (Supporting Table 1) and that include two transitions each from 224 naturally occurring “light” and matching “heavy” stable isotope labelled internal peptide standards (SIS). Except where indicated in Supporting Table 1, the MRM method used peak windows of 5 min and a cycle time of 2.5 sec. All transitions were weighted equally. The resulting LC-xMRM data were processed and quantitated using AB Sciex MultiQuant™ 2.1 software (research version). The SignalFinder™ 2 algorithm (research version) was used to integrate and score peak groups.

ELISA Assays

C4b-binding Protein Alpha Chain (C4BPA) was quantified with sandwich ELISA kit EC2202-1 from Assaypro. This colorimetric assay has a range of 0.938 – 60 ng/ml. Serum amyloid P component (SAMP) was quantified using sandwich ELISA kit ab137970 from Abcam. This colorimetric assay has a sensitivity of >0.06 ng/ml and a range of 0.156 – 10 ng/ml. Both ELISAs were carried out after diluting urine samples 60-fold with water.

Statistical Analyses

The MRM data are reported as the ratio of the relative level of expression of each protein in the TUPA assay relative to the internal standard in DGF versus IGF samples. The relative

level of expression of each protein was calculated by first determining the light to heavy peak area ratio for each transition pair in each of the three technical replicates for each sample. These data were then used to calculate the median transition peak area ratio for each sample. The protein fold-change in each biological sample was then calculated from the mean of the “light”/“heavy” intensity ratios for each of the transitions that were used to interrogate the level of expression of each peptide. The protein fold-changes for each group of DGF, SGF, and IGF samples were then calculated from the average protein fold-changes for each of the respective biological samples. To control the false discovery rate, the *p* values for the protein fold-changes were adjusted using Benjamini-Hochberg’s approach [24].

Coefficient of Variations (CVs) were calculated at the transition level by first filtering out heavy transition peaks with signal/noise (S/N)<5 and light transition peaks with S/N<3. For those sets of technical replicates with two or three quantified peaks, the CVs were calculated across the two or three technical runs for each transition from each biological sample. The resulting CVs were then averaged over the biological samples within the DGF, SGF, and IGF groups to determine an average technical level CV.

Random Forest (RF) analyses were carried out by generating 10,000 random trees using an R-script based on the approach described by Breiman [25]. To begin each RF analysis, 25 of the 37 (IGF + DGF) samples were randomly selected as the training set with the remaining 12 samples then serving as the test set. This procedure was repeated 10x and the resulting average and standard deviation data for the correctly and incorrectly classified samples in the test sets were compiled and used to calculate the corresponding sensitivity and specificity. To estimate their relative importance, the average Mean Decrease in Accuracy (MDA) of each transition was determined for the 10 RF analyses and the protein level MDA was calculated from the mean of the transition level MDAs that were used to interrogate the level of expression of each protein. The average AUCs for the receiver operating characteristic (ROC) curves were calculated with the performance function in the ROCR package [26] from the mean of the 10 RF analyses that were carried out for each set of biomarkers.

Data Sharing and Dissemination

Data from this study are permanently archived in the Yale Protein Expression Database (YPED) [27, 28]. Data in YPED are stored in MIAPE compliant formats [29] that utilize the current HUPO Proteomics Standards Initiative modules (<http://www.psidev.info/miape>) and their associated XML markup languages (e.g. mzML format for storing mass spectrometry data). In addition, all raw data from the triplicate LC-MRM analyses of 52 samples has been uploaded to PeptideAtlas [30] and can be accessed at <http://www.peptideatlas.org/PASS/PASS00912> with the password: BH6789sd.

Ethical Considerations

All patients who provided urine samples for this study, which was covered by HIC# 60600-1529, signed an informed consent document approved by the participating sites and the Yale Human Investigations Committee.

Results

Verification of Potential Biomarkers with TUPA

The TUPA scheduled LC-MRM analysis was performed on 16 IGF, 15 SGF and 21 DGF urine samples. These assays provided relative quantitation of 107 urinary proteins (Table 1). The average CV for triplicate analyses of the naturally occurring “light” and matching “heavy” SIS peptide transitions for the 107 proteins were 30.3% and 31.0%, respectively (Supporting Tables 2–4), well within the 20–35% “best practices” criterion established by the NIH Workshop for research use of MRM assays [31]. As detailed in Supporting Table 4, 92.2% of the 240 transitions for the 107 proteins were quantified with a S/N ratio >3.0 and, on average, these transitions were quantified in 71.0% of the samples. Comparison of the relative expression in DGF and IGF samples revealed that 62 of the 107 proteins had a DGF/IGF ratio that was below one (range: 0.069 to 0.961, average FC = 0.455) while 45 proteins had a DGF/IGF ratio that was above one (range: 1.032 to 4.672, average FC = 2.009)(Table 1). Fifty-one of these proteins were significantly up- or down-regulated in DGF as compared to IGF urine with adjusted *p* values of <0.05. TUPA analysis of the 15 SGF samples identified 107 proteins that had SGF/IGF fold changes from 0.346 to 2.394, with 76% of these changes being in the same direction as that seen in the DGF/IGF analysis.

Random Forest Analyses of the 51 Differentially Expressed TUPA Proteins

To determine which of the 51 differentially expressed proteins would best distinguish patients with DGF from those with IGF, we used RF as our classifier and chose an adjusted *p* value <0.05 as our significance threshold (Supporting Figure 1). RF analysis of the 112 interference-free transitions from the 51 proteins correctly classified 73.0% of the IGF and 69.4% of the DGF samples based on the corresponding specificity and sensitivity, respectively (Table 2, Supporting Table 5). As a result of selection bias, these estimated accuracies may be slightly optimistic [32]. To identify the most important classifiers from within the 112 transitions from these 51 proteins, the MDA value for each transition was determined from each of 10 separate RF analyses. The resulting average MDA values ranged from 15.05 for Transition #21 (from C4BPA_HUMAN.EDVYVVGTVLR.2y6) to –2.21 for transition #46 (from GGH_HUMAN.FFNVLTTNTDGK.2y10) (Supporting Table 6), where the more positive MDA corresponds to the greater importance of that transition in accurately classifying the sample. Of note, the R^2 between the MDA values from the two transitions from each peptide was 0.42 (Supporting Figure 2). The relatively low R^2 probably reflects differential levels of interference (albeit all <30%) in the two different transitions from each peptide [22].

Analysis of the MDA ranking revealed a step down in MDA between SAMP (#2 on the rank order list) and IGSF8 and a second step down between APOA2 (#12 on the list) and BIN1 (Figure 1, arrows). To determine if we could achieve better classification of DGF samples, we used this step-down in rank order of MDA values to perform iterative RF analysis and compare the accuracy of DGF classification using all 51 differentially expressed proteins versus the top 12, 6, 4 and 2 proteins, respectively. Of note, the top 12 proteins as judged by MDA all have adjusted *p* values <0.0128 for their relative level of DGF/IGF expression (Table 1). Progressively limiting the number of proteins in the RF analysis from 51 to 12 to

6 to 4 resulted in improved accuracy of classification of DGF and IGF samples with a step-wise increase in AUC from 0.82 to 0.89 (Table 2), whereas further reduction to the top 2 proteins led to a drop-off in classification accuracy (AUC 0.754, see Supporting Tables 7–13 and Supporting Figures 3–6 for details). Based on achieving the highest observed accuracy of 77.5% (Supporting Figure 3), sensitivity of 77.4%, specificity of 82.6% and AUC of 0.89, 4 proteins [C4BPA, IGSF8, SAMP, and Guanylin (GUC2A)] appear to provide the best biomarker panel from within the 107 proteins that were characterized by the TUPA MRM assay.

Additional Characterization and Validation of the Top 4 Proteins as IGF Biomarkers

Since SGF is a condition that is intermediate between DGF and IGF, we compared the trajectory of expression of the top 4 proteins in patients with DGF, SGF and IGF. Confirming the ability of this biomarker panel to accurately reflect the spectrum of kidney outcomes following transplantation, the relative expression of IGSF8 and GUC2A progressively decreased going from IGF to SGF to DGF while the expression of C4BPA and SAMP progressively increased (Figure 2). These findings indicate that increasing levels of C4BPA and SAMP and decreasing levels of IGSF8 and GUC2A are indicative of injury in the transplanted kidney.

ELISA Confirmation of Two of the Most Significant Protein Fold-Changes

To independently determine the expression of these biomarkers in IGF, SGF and DGF patients, ELISA assays were carried out for the two proteins from the top four for which there were commercially available standardized kits to enable quantification in human urine. These two proteins, C4b-binding protein alpha chain (C4BPA) and serum amyloid P-component (SAMP), were the two with the greatest MDA effect. As summarized in Table 3, the relative expression values for C4BPA and SAMP that were determined by ELISA were in close agreement with those determined by MRM for the 21 DGF, 15 SGF, and 16 IGF samples and showed the same trend of a progressive rise from SGF/IGF to DGF/IGF. Of note, the ELISA assays of C4BPA expression were at the upper limit of the assay in 10 of the 21 DGF samples, making the ELISA value an underestimate of the true C4BPA level in those urines, and thus the calculated DGF/IGF ratio of 3.824 is likely to be less than the actual ratio. Overall, the four ELISA and MRM expression ratios in Table 3 were within an average of $\pm 21\%$ of each other, in agreement with the expected variance in the MRM data.

As expected, the Boxplots in Supporting Figure 7 indicate that the median concentration of C4BP in the SGF samples (568 ng/ml) was between that for the DGF (3,080 ng/ml) and IGF samples (373 ng/ml). Similarly, the median concentration of SAMP in the SGF samples (376 ng/ml) was between that for the DGF (630 ng/ml) and IGF (178 ng/ml) samples. The scatter plots in Supporting Figure 8 show the optimum breakpoints for classifying DGF versus IGF urine samples based on their ELISA-determined C4BP (Fig. 8A, breakpoint = 1,144 ng/ml), SAMP (Fig. 8B, breakpoint = 426 ng/ml), mean (C4BP + SAMP) (Fig. 8C, breakpoint = 753 ng/ml), and mean (C4BP + C4BP-normalized SAMP) (Fig. 8D, breakpoint = 1,184 ng/ml) concentrations. Supporting Table 16 shows that use of these breakpoints to differentiate DGF from IGF samples provided an overall accuracy of classification of 86.1% with the highest specificity of 93.8% achieved with the use of the SAMP breakpoint and the

highest sensitivity of 95.0% achieved with the use of an average (C4BP + C4BP-normalized SAMP) breakpoint.

Discussion

The association between AKI and, in the setting of transplant, DGF and subsequent chronic kidney disease is well known. In addition to the deleterious effects of the initial injury, maladaptive attempts at repair are believed to promote the long term sequelae of excessive inflammation and subsequent fibrosis. For transplant patients in particular, given all allografts suffer some degree of cold ischemia, adaptive as opposed to maladaptive responses to structural kidney injury likely determine how quickly and to what extent allografts begin functioning. Identification of early biomarkers capable of discriminating DGF from IGF would not only allow rapid targeting of treatments and appropriate alterations in immunosuppressant regimens for those patients at highest risk of DGF but also would identify new pathways of repair and therapeutic targets. To identify early biomarkers capable of discriminating DGF from IGF with sufficient statistical power to draw firm conclusions, we used a non-biased Targeted Urine Proteome Assay (TUPA) that is based on a well proven mass spectrometry approach, scheduled Multiple Reaction Monitoring (MRM) [22].

TUPA has the potential ability to quantify 167 urine proteins that were chosen based on both discovery proteomics in transplant patients and a literature search of proteins differentially expressed in the setting of AKI [22]. After using data quality considerations that were based on NIH guidelines, TUPA LC-MRM analyses of 16 IGF and 21 DGF urine samples successfully quantified 107 of those 167 proteins. Rigorous statistical analysis of these data and an iterative Random Forest approach then was used to identify the top 4 protein (C4BPA, GUC2A, IGFSF8, SAMP) biomarker panel that had an AUC of 0.891 with a specificity of 82.6% and sensitivity of 77.4%.

The ability to anticipate which patients will have DGF and which IGF could result in both immediate improvements in patient care and open the door for the development of new targeted treatments. Such changes in care may consist of better management of patients destined for DGF as well as its prevention. Patients with a urinary panel reflecting high risk for DGF could be started on one of the calcineurin inhibitor sparing regimens, which have been shown to associate with lower rates of DGF [33]. Changes in management in addition to the immunosuppressive regimen could also be considered. For example, patients with DGF are typically suffering from acute tubular necrosis (ATN) and there is data showing the deleterious effects of excessive volume infusion in patients with ATN [34]. If those patients destined to suffer DGF are identified, typically brisk post-operative fluid rates could be titrated down. Finally, functional and bioinformatic analyses of proteins that are differentially expressed in patients with DGF may identify pathways integral to an adaptive response to transplant-associated ischemia-reperfusion injury and thereby provide specific therapeutic targets for the prevention of DGF.

The observation that the top 4 proteins change synchronously (C4BPA and SAMP increasing and GUC2A and IGFS8 decreasing) when going from minimal injury (IGF) to

moderate injury (SGF) to severe injury (DGF) provides not only validation of their potential use as biomarkers of injury severity, but also suggests that they can provide important biological insights into the mechanism of that injury. Based on the relative AUCs from the RF analysis of the top 2 versus the top 4 proteins, C4BPA and SAMP contribute ~85% of the ability of the top 4 proteins to differentiate DGF versus IGF samples (Table 2). Co-upregulation of C4BPA and SAMP following kidney injury is in line with their being present in plasma as a 1:1 stoichiometric complex [35]. Due to the equimolar concentrations of these two proteins and the high affinity of their interaction, virtually all SAMP in plasma is thought to be bound to C4BPA [35]. C4BPA binds multiple C4b molecules and thus inhibits assembly of the classical pathway C3 convertase (C4bC2a), dissociates C2a from the convertase, and functions as a cofactor to Factor I in the degradation of C4b [36, 37]. Increasing C4BPA likely would help prevent C1q from activating C4 and inducing membrane attack complex formation and driving further cell death. Indeed, C4BPA enables the “silent” removal of apoptotic and necrotic cells by macrophages [37]. During the natural turnover of cells or in the case of external damage, such as AKI from kidney transplant, apoptotic and necrotic cells must be removed from the system. These cells are recognized by C1q, mannan binding lectin and ficolins, which initiate their opsonization. C4BPA then binds to these cells and together with Factor I, cleaves C3b to iC3b [37]. This allows macrophages to identify the marked cells and to “silently” remove them without initiating inflammation. In fact, macrophages with phagocytosed C4BPA-labeled apoptotic/necrotic cells release anti-inflammatory cytokines [37]. Similarly, SAMP enhances phagocytosis of cell debris and regulates many aspects of neutrophil biology to exert an anti-inflammatory and anti-fibrotic effect [38]. Taken together, the progressive up-regulation of SAMP and C4BPA in SGF and DGF may reflect a biological response to sterile cell death and the need to clear the dead cells without activating terminal complement/MAC and killing more cells.

The two proteins in the top 4 that are down-regulated in DGF are GUC2A and IGSF82 (Figure 2). GUC2A is guanylin, which is an intestinal natriuretic peptide hormone [39]. The major endogenous form of guanylin in human intestine and plasma is proguanylin [40], and the TUPA assay indirectly interrogates the level of expression of guanylin via detection of a tryptic peptide that corresponds to residues 41–49 in proguanylin [22]. Guanylin is highly expressed in the collecting duct of the kidney and stimulates natriuresis, kaliuresis, and diuresis [39]. The progressive decrease in GUC2A in urine on going from IGF to SGF to DGF suggests either reduced expression by the injured collecting duct or reduced filtration and metabolism of plasma proguanylin [40, 41].

Immunoglobulin Super Family Member 8 (IGSF8), which is also called EWI-2 due to the presence of a conserved Glu-Ile-Trp motif, is a membrane adhesion protein that links tetraspanin CD9 and CD81 with the actin cytoskeleton [42, 43]. IGSF8 has been shown to be an activation marker of mature monocytes and dendritic cells that enhanced the migration of activated mature dendritic cells but attenuated their antigen-specific stimulatory capacities [44]. Important functions of recently activated dendritic cells are thus critically modulated by IGSF8 with these functions being linked to IGSF8 complex formation with heat shock protein A8 (HSPA8) [44]. Indeed, it has been proposed that IGSF8 and HSPA8 are members of a broader regulatory circle that fine-tunes the transition between innate and adaptive immune responses and that may provide opportunities for therapeutic treatment of

autoimmune diseases [44]. While additional research is needed to understand the decreased relative expression of IGSF8 in DGF patients, it is tempting to speculate that it may be involved in the immunological response to the newly grafted kidney.

We believe that the current work represents an important step towards the development of a clinically useful assay to identify DGF patients based on analyses of urine samples that have been collected within 18 hours following kidney transplant from those patients who do not have postoperative anuria. Since ELISA-based assays for 2 of the top 4 proteins provided 86.1% accuracy in classifying DGF versus IGF urine samples, it is likely that development of commercial ELISA assays for the remaining two proteins (IGSF8 and GUC2A) could increase the prediction accuracy to the >90% level that is needed for a clinically useful assay [45]. Indeed, a two-step approach that uses the mean (C4BP + normalized SAMP) concentration to identify DGF patients and then the SAMP concentration to eliminate false-positives potentially would provide 95.0% sensitivity and 93.8% specificity. Another area of interest will be determining if the addition of clinical data such as that in Supporting Table 14 (e.g., cold ischemia time for the donated kidney) into the Random Forest algorithm may further improve the accuracy of classification. In addition, these results should spur research into further optimizing the proposed biomarker panel and into the functions and impacts of the top 4 proteins on graft response in kidney transplant patients. In particular, the large contribution by C4BPA to the ability of the top 4 proteins to classify DGF from IGF patients and its progressive increase on going from IGF to SGF to DGF probably reflects the importance of controlling the complement pathway following kidney transplant and suggests a new area of research that has not yet been well studied for developing treatments or preventive strategies for DGF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AKI	acute kidney injury
AUC	Area Under the Curve
CV	coefficient of variation
DGF	delayed graft function
FC	fold Change
IGF	immediate graft function

IRI	ischemia-reperfusion injury
MDA	Mean Decrease in Accuracy
MRM	Multiple Reaction Monitoring
RF	Random Forest
SGF	slow graft function
SIS	stable isotope-labeled internal peptide standard
TUPA	Targeted Urine Proteome Assay
YPED	Yale Protein Expression Database

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Statement of clinical relevance

Delayed Graft Function (DGF) can occur following deceased donor kidney transplantation and results in worse outcomes. Current approaches for diagnosing DGF may take up to a week and are thus inadequate for early targeted therapy. Identification of early DGF biomarkers would improve treatment for patients at highest risk of DGF and could offer insights into novel therapeutic strategies. To identify early biomarkers for diagnosing DGF, we used the Targeted Urine Proteome Multiple Reaction Monitoring Assay (TUPA) to identify a 4 protein biomarker panel that includes C4b-binding protein alpha, guanylin, immunoglobulin superfamily member 8, and serum amyloid P-component. This panel provided strong discrimination between DGF and immediate graft function with an AUC of 0.89. This important step provides the basis for the development of a clinically useful assay for early prediction of DGF and for the investigation of the function of these proteins following kidney transplantation.

Figure 1.
Ranked protein MDA Values for Random Forest Analysis of the top 51 Proteins.

Figure 2.

Trajectory of Fold-Changes for the Top 4 Proteins. The fold-changes for the top 4 proteins, C4b-binding protein alpha chain (C4BPA), Serum amyloid P-component (SAMP), Immunoglobulin superfamily member 8 (IGSF8), and Guanylin (GUC2A) were determined by TUPA analyses described in the text and summarized in Table 1.

Table 1

Relative Expression of 107 Urine Proteins in Kidney Transplant Patients as Determined by TUPA MRM Analyses¹

Recommended Name	UniProtKB Entry Name	#Tr	#Peptides	DGE/IGF ²			SGE/IGF ³		
				Fold Change	p	Adj. p	Adj. p	Rank#	Fold Change
Apolipoprotein A-II	APOA2_HUMAN	4	2	3.179	0.0001	0.0028		1	1.257
C4b-binding protein alpha chain	C4BPA_HUMAN	2	1	4.672	0.0000	0.0028		2	1.456
Immunoglobulin superfamily member 8	IGSF8_HUMAN	2	1	0.166	0.0001	0.0028		3	0.379
Prosaposin	SAP_HUMAN	2	1	0.090	0.0001	0.0028		4	0.379
Tyrosine-protein kinase receptor UFO	UFO_HUMAN	2	1	0.317	0.0001	0.0028		5	0.595
Serum paraoxonase/arylesterase 1	PON1_HUMAN	2	1	3.732	0.0002	0.0030		6	1.076
Glutathione peroxidase 3	GPX3_HUMAN	2	1	0.241	0.0003	0.0032		7	0.603
Guanylin	GUC2A_HUMAN	2	1	0.069	0.0003	0.0032		8	0.384
Heparin cofactor 2	HEP2_HUMAN	2	1	2.408	0.0003	0.0032		9	1.135
SH3 domain-binding glutamic acid-rich-like protein 3	SH3L3_HUMAN	2	1	0.208	0.0002	0.0032		10	0.572
Vascular cell adhesion protein 1	VCAM1_HUMAN	2	1	0.288	0.0003	0.0032		11	0.570
Myc box-dependent-interacting protein 1	BIN1_HUMAN	2	1	0.251	0.0004	0.0040		12	0.515
Protein AMBP	AMBP_HUMAN	2	1	0.158	0.0005	0.0042		13	0.689
Glutaminyl-peptide cyclotransferase	QPCT_HUMAN	2	1	0.338	0.0006	0.0042		14	0.623
Serum amyloid P-component	SAMP_HUMAN	2	1	2.456	0.0006	0.0044		15	1.236
V-set and immunoglobulin domain- containing protein 4	VSIG4_HUMAN	2	1	0.117	0.0007	0.0044		16	0.520
Cadherin-13	CAD13_HUMAN	4	2	0.224	0.0007	0.0044		17	0.509
Apolipoprotein A-I	APOA1_HUMAN	4	2	3.903	0.0008	0.0045		18	1.047
Osteopontin	OSTP_HUMAN	3	2	0.227	0.0008	0.0045		19	0.455
Cystatin-M	CYTM_HUMAN	2	1	0.177	0.0009	0.0046		20	0.361
Cathepsin D	CATD_HUMAN	2	1	0.360	0.0010	0.0050		21	0.800
Mallase-glucoamylase, intestinal	MGA_HUMAN	2	1	0.471	0.0010	0.0050		22	0.556

Recommended Name	UniProtKB Entry Name	#Tr	#Peptides	DGE/IGF ²			SGE/IGF ³	
				Fold Change	p	Adj. p	Adj p Rank#	Fold Change
Lactotransferrin	TRFL_HUMAN	2	1	2.432	0.0011	0.0050	23	1.185
Histone H4	H4_HUMAN	2	1	4.219	0.0015	0.0065	24	1.520
Zinc-alpha-2-glycoprotein	ZA2G_HUMAN	2	1	0.223	0.0015	0.0065	25	0.414
Insulin-like growth factor-binding protein 7	IBP7_HUMAN	2	1	0.455	0.0018	0.0071	26	0.555
Inter-alpha-trypsin inhibitor heavy chain H1	ITIH1_HUMAN	2	1	2.295	0.0018	0.0071	27	1.026
Fatty acid-binding protein, liver	FABPL_HUMAN	2	1	0.241	0.0023	0.0088	28	0.755
Coactosin-like protein	COTL1_HUMAN	2	1	0.281	0.0025	0.0093	29	0.629
Pro-epidermal growth factor	EGF_HUMAN	2	1	0.563	0.0027	0.0093	30	0.403
Tripeptidyl-peptidase 1	TPP1_HUMAN	2	1	0.437	0.0026	0.0093	31	0.564
Complement C3	CO3_HUMAN	2	1	2.426	0.0028	0.0095	32	1.215
Retinol-binding protein 4	RET4_HUMAN	2	1	0.288	0.0033	0.0108	33	0.887
Mimecan	MIME_HUMAN	2	1	0.149	0.0038	0.0115	34	0.485
Thy-1 membrane glycoprotein	THY1_HUMAN	2	1	0.274	0.0037	0.0115	35	0.716
Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2_HUMAN	2	1	2.257	0.0041	0.0122	36	1.018
Alpha-2-macroglobulin	A2MG_HUMAN	2	1	2.330	0.0045	0.0128	37	1.292
Polymeric immunoglobulin receptor	PIGR_HUMAN	4	2	0.331	0.0045	0.0128	38	0.780
Leucine-rich alpha-2-glycoprotein	A2GL_HUMAN	2	1	0.539	0.0050	0.0137	39	0.771
EGF-containing fibulin-like extracellular matrix protein 1	FBLN3_HUMAN	2	1	0.246	0.0057	0.0147	40	0.753
Phosphoinositide-3-kinase-interacting protein 1	P3IP1_HUMAN	3	2	0.314	0.0058	0.0147	41	0.616
Vasorin	VASN_HUMAN	2	1	0.462	0.0057	0.0147	42	0.717
6-phosphogluconate dehydrogenase	6PGD_HUMAN	2	1	2.165	0.0068	0.0169	43	1.291
Fibrinogen beta chain	FIBB_HUMAN	2	1	3.076	0.0071	0.0170	44	0.903
Inter-alpha-trypsin inhibitor heavy chain H3	ITIH3_HUMAN	2	1	1.765	0.0071	0.0170	45	1.280
Complement factor B	CFAB_HUMAN	2	1	2.712	0.0091	0.0211	46	1.179
Fructose-bisphosphate aldolase B	ALDOB_HUMAN	2	1	0.473	0.0115	0.0262	47	0.408
Phosphoglycerate kinase 1	PGK1_HUMAN	2	1	1.605	0.0127	0.0283	48	0.944
Prothrombin	THRB_HUMAN	2	1	0.335	0.0130	0.0283	49	0.430
Gamma-glutamyl hydrolase	GGH_HUMAN	2	1	0.517	0.0144	0.0305	50	0.741

Recommended Name	UniProtKB Entry Name	#Tr	#Peptides	DGE/IGF ²			SGE/IGF ³	
				Fold Change	p	Adj. p	Adj. p Rank#	Fold Change
Hemoglobin subunit delta	HBD_HUMAN	2	1	4.239	0.0145	0.0305	51	1.072
Collagen alpha-3(VI) chain	CO6A3_HUMAN	2	1	0.360	0.0248	0.0511	52	0.808
Cystatin-C	CYTC_HUMAN	2	1	0.369	0.0285	0.0568	53	1.625
Bone marrow proteoglycan	PRG2_HUMAN	2	1	0.521	0.0287	0.0568	54	0.505
CD59 glycoprotein	CD59_HUMAN	4	2	0.374	0.0323	0.0629	55	0.557
Carbonic anhydrase 3	CAH3_HUMAN	2	1	0.415	0.0330	0.0630	56	0.784
Ribonuclease pancreatic	RNAS1_HUMAN	2	1	0.384	0.0342	0.0643	57	0.843
Fibrinogen gamma chain	FIBG_HUMAN	2	1	2.503	0.0371	0.0684	58	0.911
Nidogen-1	NID1_HUMAN	2	1	0.404	0.0460	0.0834	59	0.535
Vitronectin	VTNC_HUMAN	2	1	1.657	0.0534	0.0952	60	1.290
Lysozyme C	LYSC_HUMAN	2	1	0.484	0.0618	0.1085	61	1.286
Mannan-binding lectin serine protease 2	MASP2_HUMAN	2	1	0.463	0.0686	0.1184	62	0.346
Carbonic anhydrase 2	CAH2_HUMAN	2	1	2.154	0.0710	0.1205	63	0.885
Alpha-1-antitrypsin	AIAT_HUMAN	4	2	0.589	0.0781	0.1306	64	1.094
Trypsin-2	TRY2_HUMAN	2	1	0.482	0.0884	0.1456	65	1.011
Ig alpha-1 chain C region	IGHA1_HUMAN	2	1	1.452	0.0957	0.1552	66	0.857
Apolipoprotein D	APOD_HUMAN	2	1	0.662	0.1158	0.1800	67	0.746
Complement factor I	CFAI_HUMAN	2	1	1.422	0.1155	0.1800	68	1.360
Complement component C9	CO9_HUMAN	4	2	1.801	0.1177	0.1800	69	1.010
Carboxypeptidase N subunit 2	CPN2_HUMAN	2	1	1.309	0.1163	0.1800	70	0.891
WAP four-disulfide core domain protein 2	WFDC2_HUMAN	2	1	0.576	0.1302	0.1962	71	0.701
Band 3 anion transport protein	B3AT_HUMAN	2	1	1.618	0.1320	0.1962	72	0.539
Cadherin-1	CADH1_HUMAN	2	1	1.194	0.1824	0.2638	73	1.232
Complement component C6	CO6_HUMAN	2	1	1.415	0.1803	0.2638	74	1.010
Flavin reductase (NADPH)	BLVRB_HUMAN	2	1	1.947	0.1960	0.2723	75	0.833
Kininogen-1	KNG1_HUMAN	2	1	0.783	0.1917	0.2723	76	1.016
Thyroxine-binding globulin	THBG_HUMAN	2	1	0.824	0.1947	0.2723	77	1.216
Phosphatidylethanolamine-binding protein 4	PEBP4_HUMAN	2	1	0.489	0.1996	0.2738	78	1.362
Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	2	1	1.411	0.2254	0.3031	79	0.858
Superoxide dismutase [Cu-Zn]	SODC_HUMAN	2	1	0.602	0.2267	0.3031	80	0.725

Recommended Name	UniProtKB Entry Name	#Tr	#Peptides	DGE/IGF ²			SGE/IGF ³	
				Fold Change	p	Adj. p	Adj p Rank#	Fold Change
14-3-3 protein epsilon	I433E_HUMAN	2	1	1.792	0.2663	0.3518	81	2.394
Transferrin	TTHY_HUMAN	2	1	0.724	0.2838	0.3703	82	0.930
Peptidoglycan recognition protein 1	PGRP1_HUMAN	2	1	1.032	0.3012	0.3882	83	1.466
Complement C1r subcomponent-like protein	C1RL_HUMAN	2	1	0.768	0.3117	0.3971	84	0.869
N-acetylmuramoyl-L-alanine amidase	PGRP2_HUMAN	4	2	0.785	0.3365	0.4187	85	0.944
Thioredoxin	THIO_HUMAN	2	1	0.775	0.3361	0.4187	86	1.353
Triosephosphate isomerase	TPIS_HUMAN	2	1	1.329	0.3610	0.4439	87	0.695
Collagen alpha-1(XV) chain	COFAL_HUMAN	2	1	0.934	0.3654	0.4443	88	0.713
Folate receptor alpha	FOLR1_HUMAN	2	1	0.823	0.4045	0.4809	89	0.875
Lipopolysaccharide-binding protein	LBP_HUMAN	2	1	1.385	0.4040	0.4809	90	1.770
Protein S100-A6	S10A6_HUMAN	5	3	1.404	0.4212	0.4953	91	0.974
Ig heavy chain V-III region GAL	HV320_HUMAN	2	1	1.156	0.4532	0.5214	92	0.858
Plasma protease C1 inhibitor	IC1_HUMAN	2	1	1.200	0.4523	0.5214	93	0.761
Fibronectin	FINC_HUMAN	2	1	1.178	0.4973	0.5660	94	0.972
Phosphatidylethanolamine-binding protein 1	PEBP1_HUMAN	2	1	0.837	0.5088	0.5731	95	1.340
Ankyrin beta A4 protein	A4_HUMAN	2	1	1.133	0.5201	0.5797	96	1.118
L-lactate dehydrogenase A chain	LDHA_HUMAN	2	1	1.074	0.5804	0.6402	97	1.041
Ig mu chain C region	IGHM_HUMAN	2	1	0.886	0.6075	0.6633	98	0.667
Haptoglobin	HPT_HUMAN	4	2	1.245	0.6363	0.6877	99	1.158
Alpha-1B-glycoprotein	A1BG_HUMAN	2	1	1.093	0.7386	0.7765	100	1.195
Serum albumin	ALBU_HUMAN	2	1	0.919	0.7402	0.7765	101	1.055
Clusterin	CLUS_HUMAN	2	1	1.413	0.7343	0.7765	102	1.308
CD44 antigen	CD44_HUMAN	2	1	0.686	0.8034	0.8346	103	0.676
Angiotensinogen	ANGT_HUMAN	2	1	1.045	0.8132	0.8366	104	1.414
Antithrombin-III	ANT3_HUMAN	2	1	0.961	0.8912	0.9082	105	1.077
Coronin-1A	COR1A_HUMAN	2	1	1.181	0.9159	0.9245	106	0.855
Dermokine	DMKN_HUMAN	2	1	0.504	0.9738	0.9738	107	0.609
Total				237	120			

/ Abbreviation: #Tr = number of transitions for the indicated protein in the TUPA assay. The top 12 proteins, as determined by relative MDA values, are shaded.

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² Calculated by taking the reciprocal of the observed IGF/DGF ratio.
³ Calculated by dividing the observed SGF/DGF by the IGF/DGF ratio.

Table 2

Ability of the TUPA Assay to Classify DGF versus IGF Samples Based on Random Forest Analysis of DGF/IGF Protein Expression^a

#Proteins	AUC		Specificity		Sensitivity	
	Avg.	S.D.	Avg.	S.D.	Avg.	S.D.
Top 51	0.821	0.078	73.0%	15.2%	69.4%	20.6%
Top 12	0.834	0.109	73.6%	19.0%	71.8%	18.5%
Top 6	0.892	0.052	79.2%	23.0%	78.2%	15.9%
Top 4	0.891	0.066	82.6%	19.7%	77.4%	20.2%
Top 2	0.754	0.114	59.8%	19.3%	71.4%	21.3%

^aData are based on the randomly chosen “test set” of 12 samples in each of the 10 Random Forest classifications with each sample being classified 10x for a total of 120 classifications.

Table 3

Average ELISA and MRM Fold-Changes for C4b-binding Protein Alpha Chain and Serum Amyloid P-Component

Recommended Name	UniProt KB Entry	SGF/IGF		DGF/IGF	
		ELISA	MRM	ELISA	MRM
C4b-binding Protein Alpha Chain	C4BPA	1.783 ^a	1.456	3.824 ^a	4.672
Serum Amyloid P-Component	SAMP	1.751 ^b	1.236	2.501 ^b	2.456

^a Ranges and mean for C4BPA ELISA: DGF (n = 20), 340.86 – >3780.0 ng/ml with 10 samples >3780.0 ng/ml, mean = 2578.0 ng/ml; SGF (n = 15), 116.72 – >3780.0 ng/ml with one sample >3780.0 ng/ml, mean = 1202.09 ng/ml; IGF (n = 16), 96.71 – 3744.26 ng/ml, mean = 674.14 ng/ml.

^b Ranges and mean for SAMP ELISA: DGF (n = 20) 80.55 – >630.00 ng/ml with 13 samples >630.00, mean = 514.57 ng/ml; SGF (n = 15) 11.69 – >630 ng/ml with 5 samples >630 ng/ml, mean = 360.34 ng/ml; IGF (n = 16) 5.53 – >630.0 ng/ml with one sample >630.0 ng/ml, mean = 205.75 ng/ml.