⌘ *Author's Choice*

Proteomic Signatures in Plasma during Early Acute Renal Allograft Rejection*[S]

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Acute graft rejection is an important clinical problem in renal transplantation and an adverse predictor for long term graft survival. Plasma biomarkers may offer an important option for post-transplant monitoring and permit timely and effective therapeutic intervention to minimize graft damage. This case-control discovery study ($n = 32$) used isobaric tagging for relative and absolute protein quantification (iTRAQ) technology to quantitate plasma protein relative concentrations in precise cohorts of patients with and without biopsy-confirmed acute rejection (BCAR). Plasma samples were depleted of the 14 most abundant plasma proteins to enhance detection sensitivity. A total of 18 plasma proteins that encompassed processes related to inflammation, complement activation, blood coagulation, and wound repair exhibited significantly different relative concentrations between patient cohorts with and without BCAR (p value <0.05). Twelve proteins with a fold-change ≥ 1.15 were selected for diagnostic purposes: seven were increased (titin, lipopolysaccharide-binding protein, peptidase inhibitor 16, complement factor D, mannose-binding lectin, protein Z-dependent protease and β_2-microglobulin) and five were decreased (kininogen-1, afamin, serine protease inhibitor, phosphatidylcholine-sterol acyltransferase, and sex hormone-binding globulin) in patients with BCAR. The first three principal components of these proteins showed clear separation of cohorts with and without BCAR. Performance improved with the inclusion of sequential proteins, reaching a primary asymptote after the first three (titin, kininogen-1, and lipopolysaccharide-binding protein). Longitudinal monitoring over the first 3 months post-transplant based on ratios of these three proteins showed clear discrimination between the two patient cohorts at time of rejection. The score then declined to baseline following treatment and resolution of the rejection episode and remained comparable between cases and controls throughout the period of quiescent follow-up. Results were validated using ELISA where possible, and initial cross-validation estimated a sensitivity of 80% and specificity of 90% for classification of BCAR based on a four-protein ELISA classifier. This study provides evidence that protein concentrations in plasma may provide a relevant measure for the occurrence of BCAR and offers a potential tool for immunologic monitoring. ***Molecular & Cellular Proteomics 9:1954–1967, 2010.***

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Although advances in immunosuppression have increased the success of renal transplantation continuously during the past decades, immunological injury to the graft remains a critical barrier to long term survival (1–4). Both innate and immune responses are implicated in the process of graft rejection (5–7). Major and minor histocompatibility antigens expressed on graft tissue are quickly identified following implantation through direct or indirect pathways of the innate response, and consequent activation of T-cell and B-cell components of the host adaptive immune response leads to cellular and antibody-mediated injury to numerous structural components of the grafted organ (6). The resulting inflammatory sequence comprising cellular infiltration, antibody production, complement deposition, and activation of the coagulation cascade can be identified by histological changes on allograft biopsy (biopsy-con-

firmed acute rejection (BCAR)[1]), which is the current standard for diagnosis and therapy (8). Although acute rejection may respond satisfactorily to treatment, it is tightly linked to the chronic immunological injury and premature failure of the graft (3).

We have shown that recipient gene expression is profoundly altered following graft implantation, and changes in the transcriptome occur that are characteristic of rejection injury (9). The biological functions of the genes differentially expressed encompass major biological categories of cellular processes related to immune signal transduction, cytoskeletal reorganization, and apoptosis and emphasize the participation of the cytokine-activated JAK-STAT pathway and interferon-γ signaling in lymphocyte activation proliferation, chemotaxis, and adhesion (9). Changes in gene expression provide only a partial understanding of the biological events triggered by transplantation, however, because they do not necessarily indicate the parallel inflammatory changes mediated by proteins. A more comprehensive understanding of these biological events therefore requires a detailed knowledge of the changes in protein expression and function that may be found in the plasma (10, 11).

The rapid evolution of quantitative proteomics technologies over the last two decades has enabled protein expression profiling of many human diseases (10, 12). To date, proteomics analysis in human renal transplantation has focused principally on urine for both technological and biological reasons and has provided valuable insight into the biology of graft injury (12–17). However, the use of urine as a starting matrix is complicated because it is removed from the immune events that occur in the blood compartment, and the varying pH of urine can lead to degradation of proteins of interest (18). The peripheral blood is rapidly emerging as a viable matrix for proteomics measurements as recently shown for peripheral leukocytes in renal transplantation (19).

The plasma proteome corresponds closely with dynamic gene expression, and the field of convergent functional biology is consequently a focus of intense investigation in many disease states (20, 21). Characterization, identification, and quantification of plasma protein content have progressively improved the understanding of the plasma proteome (22–24), although exploration of biomarkers within this matrix has been extremely challenging. This is due both to the extreme dynamic range of protein concentrations, extending from 10^{-6} to 10^{3} μg/ml (22, 25), and to the fact that a small number of abundant plasma proteins constitute 99% of the total protein mass with many proteins of potential interest existing at very low concentrations (22). Quantitative proteomics analysis in a dynamic biological process such as transplantation is particularly complex due to within- and between-individual variability reflecting differences in graft and patient recovery, immunosuppressive treatments, infection, and other patient-specific events following implantation of the new organ. Experimental animal studies indicate that the peripheral blood proteome may offer important diagnostic information in detecting graft rejection, but no studies have yet explored the role of plasma proteins in human renal transplantation (12, 15).

We have used iTRAQ-MALDI-TOF/TOF methodology, in light of its reliability, reproducibility, sensitivity, and large dynamic range (26, 27), to examine the differential patterns of relative concentration in the human plasma proteome during early graft rejection following renal transplantation. The aim of this study was to identify plasma protein diagnostic markers of acute renal allograft rejection. These proteomics analyses have the potential to provide an unbiased profiling and a deeper understanding of the underlying mechanisms of acute rejection. The results will be relevant as a first step toward the development of a minimally invasive, sensitive, and specific modality for diagnosing rejection with promising clinical benefit for the care of renal transplant patients.

EXPERIMENTAL PROCEDURES

Study Population—This prospective longitudinal study was conducted at the University of British Columbia and was approved by the human research ethics board at that institution. All subjects who received a renal transplant from January 2005 to December 2007 were invited to participate, and those who agreed and signed consent forms were enrolled in the study. Patients were followed routinely at the transplant center, and blood and urine samples were obtained prior to and serially post-transplant at 0.5, 1, 2, 3, 4, 8, 12, and 26 weeks; then every 6 months through year 3; and also at the time of suspected rejection. Graft biopsy tissue was obtained pretransplant and at the time of all biopsies performed post-transplant. Blood samples from normal healthy controls served as reference samples. Samples from cases, controls, and comparators were treated identically.

All rejection episodes were diagnosed by conventional clinical and laboratory parameters, confirmed by biopsy, and graded according to the Banff 97 working classification of renal allograft pathology (28). Banff categories 2 and 4 (antibody-mediated or acute/active cellular rejection) were considered significant. Category 3 (borderline change) was only considered significant if associated with graft dysfunction and treated for rejection. All baseline demographic and follow-up data were recorded in the transplant program electronic database, and there was no loss to follow-up during the period of study.

Study Design—To ensure selection of precise homogeneous phenotypes and to minimize biological variability, patients included in the current proteomics study were less than 75 years of age; were not receiving immunosuppression prior to transplantation; had not received pretransplant immunological desensitization; had received a first kidney transplant from a deceased or non-HLA-identical living donor; had a negative antidonor T-cell cross-match; had not received depleting antibody induction therapy; were able to receive oral med-

[1] The abbreviations used are: BCAR, biopsy-confirmed acute rejection; iTRAQ, isobaric tagging for relative and absolute protein quantification; PGC, protein group code; SVM, support vector machine; AUC, area under the receiver operating characteristic curve; W1, week 1 post-transplant; IPI, International Protein Index; eBayes, empirical Bayes; TTN, titin; LBP, lipopolysaccharide-binding protein; CFD, complement factor D; AFM, afamin; VASN, vasorin; SHBG, sex hormone-binding globulin; LCAT, phosphatidylcholine-sterol acyltransferase; MBL, mannose-binding lectin; MSP, macrophage-stimulating protein; PI16, peptidase inhibitor 16; B2M, β_2-microglobulin.

ication; received a standard post-transplant immunosuppression regime consisting of basiliximab with tacrolimus and mycophenolate administered as described previously (9); had immediate graft function; and had no clinical or laboratory evidence of infections, disease recurrence, and other major co-morbid events. Samples collected from all selected patients at different time points were processed by iTRAQ and ELISA (supplemental Fig. A.3).

A closed cohort case-control design (29) was used to identify a panel of proteomic markers of renal acute rejection comparing plasma protein ratios (relative to a normal pooled control sample) at the time of BCAR from patients with (case) and without (control) BCAR. As most rejection episodes occurred at week 1 post-transplant (W1) and to ensure a rigorous diagnostic discovery analysis, all W1 rejection samples were included in the discovery cohort (cases) and matched ~1:2 with W1 samples from patients who did not have clinical or histological evidence of rejection (controls) during the first 3 months post-transplant. All remaining iTRAQ samples at other time points were used to illustrate the performance of the identified markers across time.

iTRAQ Study Design—A full description of this section is given in the supplemental material. The discovery proteomics analysis was performed using iTRAQ-MALDI-TOF/TOF methodology. The multiplexing capability of iTRAQ technology allows simultaneous processing of four samples per experimental run. To ensure interpretable results across different experimental runs, a reference sample was processed together with three patient samples in all iTRAQ runs. The reference sample consisted of a pool of plasma from 16 healthy individuals and was consistently labeled with iTRAQ reagent 114. Patient samples were randomly labeled with reagents 115, 116, and 117. Each iTRAQ run enabled the identification and quantitation of proteins of three patient samples relative to the reference sample (*i.e.* levels of labels 115, 116, and 117 relative to 114).

iTRAQ Data Acquisition—Briefly, peripheral blood samples were drawn into EDTA tubes and stored on ice before processing. Plasma was separated and stored at −80 °C until selected for analysis. Immunoaffinity chromatography (GenWay Biotech, San Diego, CA) was used to deplete plasma samples of the 14 most abundant plasma proteins (albumin, fibrinogen, transferrin, IgG, IgA, IgM, haptoglobin, α_2-macroglobulin, α_1-acid glycoprotein, α_1-antitrypsin, apolipoprotein A-I, apolipoprotein A-II, complement C3, and apolipoprotein B). The quality of the depletion is described in the supplemental material. Depleted plasma protein samples were digested with trypsin and labeled with iTRAQ reagents according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The resulting iTRAQ-labeled peptides were pooled and acidified to pH 2.5–3.0 with concentrated phosphoric acid (ACP Chemicals Inc., Montreal, Quebec, Canada). Pooled labeled peptides were separated by two-dimensional liquid chromatography, spotted directly onto 384-spot MALDI ABI 4800 plates, and analyzed by a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems); acquisition time ranged from 35 to 40 h. Data were analyzed using ProteinPilot™ software v2.0 with the integrated Paragon™ search algorithm and Pro Group™ algorithm (30) (Applied Biosystems) searching against the International Protein Index (IPI HUMAN v3.39, 69,731 entries) database (31). The precursor tolerance was set to 150 ppm, and the iTRAQ fragment tolerance was set to 0.2 Da. Identification parameters were set for trypsin cleavages and cysteine alkylation by methyl methanethiosulfonate with special factors set at urea denaturation and an identification focus on biological modifications. To maximize protein coverage from multiple experiments, the detected protein threshold was set to Unused ProtScore >0.70 (equivalent to an 80.0% confidence interval). Full details are given in the supplemental material.

For each iTRAQ run, ProteinPilot assembled identified peptide data into a list of identified proteins organized using (local) protein groups (equally ranked proteins with the same "N" parameter in the protein summary) to avoid redundancies (32). For each group, ProteinPilot estimated three protein ratios: levels of labels 115, 116, and 117 relative to 114. Protein ratios were computed as weighted geometric means of most of the individual peptides contributing to the identification of the group. These ratios were corrected for experimental bias using the Auto Bias correction option in the Pro Group algorithm. As the resulting protein groups may contain more than one protein identity, an in-house algorithm, called Protein Group Code Algorithm (PGCA) was used to link (local) protein groups across different iTRAQ experiments.

The Protein Group Code Algorithm creates *global* protein groups using overlapping (or connected) local protein groups from multiple runs (an example is given in the supplemental material). A common identification code, also referred to as the protein group code (PGC), is assigned to all proteins in a global group. This code was used to link groups of proteins across different experimental runs, allowing the comparison of related proteins, including homologous proteins, redundant proteins, and proteins from the same families, if they could not be distinguished based on the observed peptide data. PGCs with differential relative levels between patients with and without BCAR were also manually inspected to ensure that each protein in a group was identified in at least two samples from the current study. Further details on protein identification and quantitation are given in the supplemental material.

Statistical Discovery Analysis—A single sample per patient was used in the discovery analysis to maintain usual assumptions of independence between samples in statistical tests. A one protein at a time evaluation of differential relative levels was performed using a robust moderated *t* test (eBayes (33)) on a set of proteins that were detected in at least two-thirds of the samples with and without BCAR. Using the robust eBayes approach decreases the number of false positives caused by artificially low sample variance estimates when the sample size is small and reduces the adverse effect of observations deviating from the bulk of the data (33). A panel of PGCs with mean relative concentrations differing significantly between BCAR-positive and -negative (p value <0.05) was identified. A score generated by support vector machine (SVM) with linear kernel was used to illustrate main properties of the panel. In SVM, missing relative concentrations for each protein not detected in patient sample(s) and/or pooled control were imputed using *k*-nearest neighbor imputation (34). Classification performance measures (sensitivity, specificity, and area under the receiver operating characteristic curve (AUC)) and the robustness of panel identification were estimated by a leave-one-out cross-validation. At each step of the cross-validation, one sample was left out (test set) for classification, and the remaining samples (training set) were used to identify a panel and/or to build a classifier using SVM. This process was repeated until all samples were used as the test set once. The (overall) performance measure was estimated by the average classification performance from all runs. The AUC was computed using the ROCR package (35).

Knowledge mining using MetaCore analysis (GeneGo, Inc.) and public databases (*e.g.* PubGene) as well as literature mining (*e.g.* PubMed) was performed for biological interpretation of the results. Gene ontologies and networks in MetaCore were prioritized based on their statistical significance.

Technical Validation—Five proteins were analyzed by ELISA based on commercially available kits following the manufacturers' directions: complement factor D (R&D Systems, Minneapolis, MN and Diapharma, West Chester, OH), phosphatidylcholine-sterol acyltransferase (LCAT) (Alpco Diagnostics, Salem, NH), sex hormone-binding globulin precursor (Alpco Diagnostics, Salem, NH), factor IX (Diapharma), and adiponectin (R&D Systems and Diapharma). All samples were analyzed in triplicates.

A robust *t* test (M-estimator) was used to analyze differences in protein concentrations between patients with and without BCAR. Cor-

TABLE I
Demographic and clinical characteristics of study subjects

Numbers in parentheses are percentages unless otherwise stated. pos, positive; neg, negative; ND, not determined; PRA, panel-reactive antibody; FCXM, flow cytometry cross-match; Tx, transplant; GFR, glomerular filtration rate.

Characteristics	BCAR	No BCAR
Subjects	11	21
Mean age (S.D.)	42 (12)	49 (11)
Male	8 (73%)	13 (62%)
Ethnicity		
Caucasian	9 (82%)	15 (71%)
South Asian	0 (0%)	2 (10%)
East Asian	1 (9%)	1 (5%)
Other	1 (9%)	3 (14%)
Primary disease		
Glomerulonephritis	5 (45%)	4 (19%)
Polycystic kidney disease	0 (0%)	5 (24%)
Diabetic nephropathy	1 (9%)	1 (5%)
Other	5 (45%)	11 (52%)
Donor type		
Living donor	8 (73%)	13 (62%)
Deceased donor	3 (27%)	8 (38%)
Immunology		
PRA (positive)	0	1
Donor-specific antibody	0	0
T-cell FCXM (pos/neg/ND)	0/8/3	0/13/8
B-cell FCXM (pos/neg/ND)	0/8/3	0/9/12
Estimated GFR (ml/min/1.73 m^2)		
Week 1 post-Tx	27 ± 10	43 ± 14
Month 3 post-Tx	48 ± 12	51 ± 9
Month 12 post-Tx	54 ± 13	53 ± 15

relations between ELISA and iTRAQ protein levels for 29 samples processed on both platforms were also examined using the Spearman correlation coefficient. An initial estimation of classification performance based on ELISA measurements was performed by a leave-one-out cross-validation using SVM. Nested classifiers were evaluated with proteins added sequentially based on best performance. All of the statistical analyses were implemented using R version 2.10.1 (36).

RESULTS

Subjects—A total of 27 of 305 subjects (8.8%) developed acute rejection of Banff grade ≥1a during the first 3 months post-transplant. A case selection criteria for a precise phenotype was applied, selecting patients with immediate graft function, grade ≥1a rejection on biopsy (range, 3–10 days; mean, 7 days; histology: grade 1a, 7; grade 1b, 1; grade 2a, 3; C4d-positive, 2), and absence of infection or other confounding co-morbid events that might confound plasma proteomic expression. A total of 11 of the 27 patients in the study (40.7%) fulfilled this criteria and were selected as cases. A further 21 subjects with a similar precise phenotype who had immediate graft function but no clinical or histological evidence of rejection for at least 6 months following transplantation and no confounding clinical co-morbid events were selected as controls. Demographic details are shown in Table I. Graft function was significantly inferior in cases with rejection at the 1st week post-transplant (27 ± 10 *versus* 43 ± 14 ml/min/1.73 m^2, p value = 0.004) but was comparable between cases and controls by month 3 (48 ± 12 *versus* 51 ± 9 ml/min/1.73 m^2, p value = 0.359) and remained clinically stable with good allograft function throughout a 12-month period of observation (54 ± 13 *versus* 53 ± 15 ml/min/1.73 m^2 at month 12, p value = 0.859). The distribution of longitudinally collected samples from the 32 subjects in the selected discovery cohort is illustrated in supplemental Fig. A.3.

Discovery Analysis—Plasma samples from the 11 case subjects at the time of biopsy-confirmed rejection (W1) were compared with samples obtained at the equivalent time from the 21 controls without rejection. These samples were processed in 26 different iTRAQ runs (together with other samples not included in the discovery study), resulting in 855 cumulative PGCs from all runs. Among these PGCs, 144 were detected in at least eight of 11 experimental runs from the rejection samples and in at least 14 of 21 from the controls. A quality summary of the identification of these proteins, included in supplemental Fig. A.5, shows that 65% of these 144 analyzed PGCs were identified based on five or more distinct peptides, and only 8% were identified based on fewer than two peptides (on average over all iTRAQ runs). Overall, these results demonstrate a strong identification of the analyzed proteins.

Analysis of the 144 PGCs with robust eBayes identified a total of 18 PGCs with significant differential relative concentrations (p value <0.05) between the two groups (Table II and Fig. 1). Thirteen of the 18 PGCs were up-regulated (titin (TTN), lipopolysaccharide-binding protein (LBP), vasorin (VASN), brain rescue factor-1/hepatocyte growth factor-like protein (MSTP9/MST1), peptidase inhibitor 16 (PI16), complement factor D (CFD), complement component 2 (C2), mannose-binding lectin 2 (MBL2), protein Z-dependent protease (SERPINA10), complement component C9 (C9), β_2-microglobulin (B2M), complement C1s (C1S), and coagulation factor IX (F9) and five were down-regulated (kininogen-1 (KNG1), afamin (AFM), serine protease inhibitor (SERPINA5), LCAT, and sex hormone-binding globulin (SHBG)) in patients with rejection compared with those without rejection. The volcano plot in Fig. 1*A* illustrates the magnitude of differentiation in relation with the statistical significance for the 18 identified groups. Fig. 1*B* shows the differentiation between the groups with and without BCAR for the peptides used to infer one of the 18 PGCs (other cases and further details on this plot are given in the supplemental material). In accordance with the general guideline for proteomics data publications, all these PGCs were identified based on an average of two or more distinct peptide sequences (Table III and supplemental Fig. A.5). Other quality parameters were examined and are reported in Table III as well as in the supplemental material. On average, Unused ProtScore (a measure of confidence in protein identification) was 17, percent coverage (percentage of total protein sequence covered by the identified peptides) was 27, and the error factor (a measure of confidence in protein quantitation related with the between-peptide variation) was 1.4.

TABLE II

Plasma proteins with differential relative concentrations at p value <0.05

Accession numbers of all proteins in each PGC, corresponding genes (gene symbol) and protein names, *p* values calculated by the robust eBayes test, and fold-changes with directions (plus and minus signs for up- and down-regulated in BCAR-positive relative to -negative, respectively) are given. HMW, high molecular weight; LMW, low molecular weight.

PGC[b]	Accession number	Gene symbol[b]	Protein name	*p* value[b]	Fold-change[b]
111	IPI00759754.1	*TTN*	Isoform 1 of Titin	<0.001	+1.21[a]
	IPI00749039.2	*TTN*	titin isoform N2-A		
	IPI00179357.2	*TTN*	Isoform 7 of Titin		
	IPI00023283.3	*TTN*	Isoform 2 of Titin		
	IPI00759542.1	*TTN*	Isoform 8 of Titin		
	IPI00759637.1	*TTN*	Isoform 4 of Titin		
	IPI00759613.1	*TTN*	Isoform 5 of Titin		
	IPI00375499.2	*TTN*	titin isoform novex-2		
	IPI00375498.2	*TTN*	titin isoform novex-1		
	IPI00455173.4	*TTN*	Isoform 3 of Titin		
	IPI00412307.8	*TTN*	2268-kDa protein		
	IPI00884109.1		Cellular titin isoform PEVK variant 3 (fragment)		
18	IPI00789376.1	*KNG1*	KNG1 protein	0.001	−1.18[a]
	IPI00797833.3	*KNG1*	Kininogen-1		
	IPI00032328.2	*KNG1*	Isoform HMW of kininogen-1 precursor		
	IPI00215894.1	*KNG1*	Isoform LMW of kininogen-1 precursor		
108	IPI00032311.4	*LBP*	Lipopolysaccharide-binding protein precursor	0.006	+1.22[a]
222	IPI00395488.2	*VASN*	Vasorin precursor	0.007	+1.14
23	IPI00019943.1	*AFM*	Afamin precursor	0.007	−1.29[a]
224	IPI00873854.1	*MSTP9*	64-kDa protein	0.009	+1.09
	IPI00292218.4	*MST1*	Hepatocyte growth factor-like protein precursor		
	IPI00384647.1	*MST1*	Hepatocyte growth factor-like protein homolog		
	IPI00718805.1	*MSTP9*	Brain rescue factor-1		
	IPI00847702.2	*MST1*	14-kDa protein		
135	IPI00301143.5	*PI16*	Isoform 1 of peptidase inhibitor 16 precursor	0.013	+1.25[a]
	IPI00845506.1	*PI16*	Isoform 2 of peptidase inhibitor 16 precursor		
97	IPI00007221.1	*SERPINA5*	Plasma serine protease inhibitor precursor	0.019	−1.22[a]
104	IPI00165972.3	*CFD*	Complement factor D preproprotein	0.020	+1.43[a]
38	IPI00303963.1	*C2*	Complement C2 precursor (fragment)	0.019	+1.09
	IPI00643506.3	*C2*	Complement component 2		
116	IPI00004373.1	*MBL2*	Mannose-binding protein C precursor	0.021	+1.37[a]
125	IPI00007199.4	*SERPINA10*	Protein Z-dependent protease inhibitor precursor	0.023	+1.23[a]
26	IPI00022395.1	*C9*	Complement component C9 precursor	0.029	+1.13
230	IPI00022331.1	*LCAT*	Phosphatidylcholine-sterol acyltransferase precursor	0.030	−1.18[a]
103	IPI00868938.1		β_2-Microglobulin	0.032	+1.27[a]
	IPI00796379.1	*B2M*	B2M protein		
	IPI00004656.2	*B2M*	β_2-Microglobulin		
69	IPI00219583.1	*SHBG*	Isoform 2 of sex hormone-binding globulin precursor	0.032	−1.19[a]
	IPI00023019.1	*SHBG*	Isoform 1 of sex hormone-binding globulin precursor		
29	IPI00749179.2	*C1S*	Uncharacterized protein C1S	0.040	+1.08
	IPI00017696.1	*C1S*	Complement C1s subcomponent precursor		
100	IPI00296176.2	*F9*	Coagulation factor IX precursor	0.043	+1.09
	IPI00816532.1	*F9*	Coagulation factor IX (fragment)		

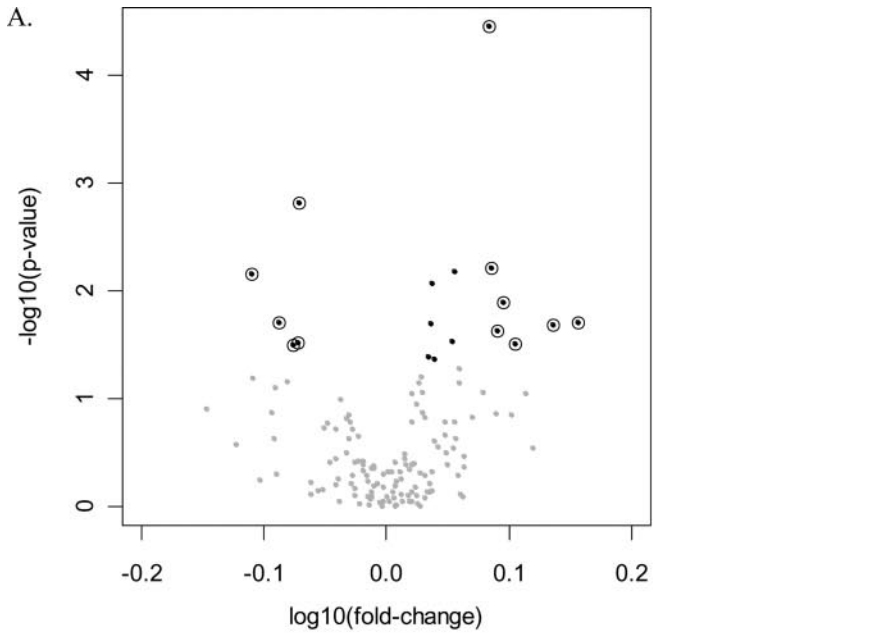
[a] PGCs with fold-change ≥1.15 included in the multivariate classifier.

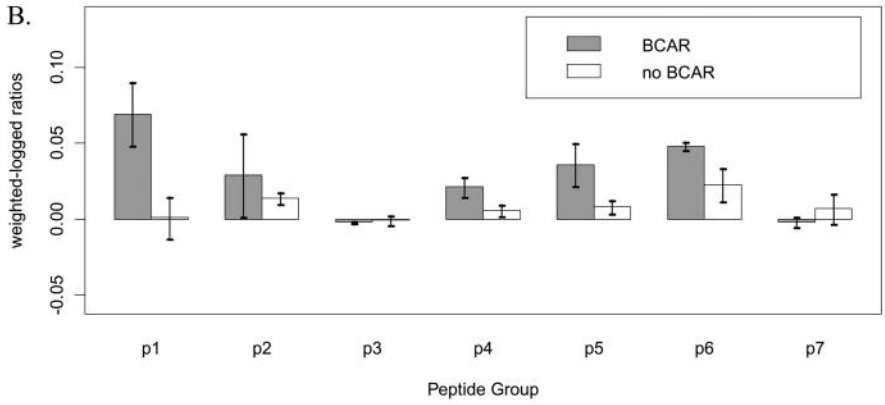
[b] The values of the measures in these columns correspond to the whole protein group code (PGC) and not to a particular protein identifier.

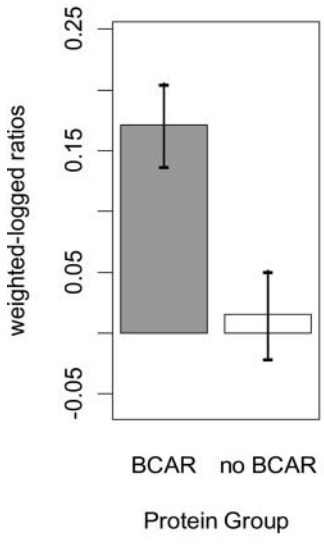
Further details on protein identification, quantitation, and variation are given in the supplemental material.

The biological processes encompassed by the 18 differentially expressed PGCs are shown in Fig. 2. These include humoral responses mediated by circulating antibody, complement activation, activation of the coagulation cascade, and other components of the acute inflammatory response. Analysis of gene-gene and protein-protein networks based on exploration of public knowledge databases (MetaCore analysis (GeneGo, Inc.)) revealed that activation of the complement cascade, the kallikrein-kinin system, involvement of phagosomes in antigen presentation, and the coagulation cascade were prominently represented among the differentially expressed PGCs.

A more conservative subset of 12 PGCs with a fold-change ≥1.15 was further examined: seven of these were up-regu-







Protein Group	Protein Name	number of BCAR samples (total 11)	number of noBCAR samples (total 21)
104	Complement Factor D	10	18
Peptide Group	**Sequence**	**number of BCAR**	**number of noBCAR**
p1	ATLGPAVRPLPWQR	10	18
	ATLGPAVR		
	PLPWQR		
p2	DSLQHVLLPVLDR	2	12
	RPDSLQHVLLPVLDR		
p3	DVAPGTLCDVAGWGIVNHAGR	4	10
p4	GDSGGPLVCGGVLEGVVTSGSR	6	10
p5	LMCAESNR	4	5
	LMCAESNRR		
p6	LYDVLR	8	17
p7	VQVLLGAHSLSQPEPSK	4	6
	SLSQPEPSK		
	VQVLLGAHSLSQPEPSKR		

FIG. 1. **Differential concentration of PGCs between subjects with and without BCAR detected by iTRAQ.** *A*, points in *gray* indicate the 144 PGCs identified in at least two-thirds of the samples from patients with and without BCAR, whereas those in *black* indicate the 18 PGCs that differed significantly (p value <0.05) between subjects with or without BCAR. *Circles* indicate the 12 PGCs with fold-changes ≥ 1.15. The *x axis* shows the logarithm (base 10) of the ratio between median relative levels in patients with and without BCAR. The *y axis* shows the $-\log_{10} p$ values. *B*, *gray* and *white bars* represent the averages of the weighted-logged peptide ratios (base 10) in samples with and without BCAR, respectively. Groups of trypsin-cleaved and miscleaved trypsin peptides and the number of samples in which each group was detected are summarized in the adjacent table. Similarly, the averages of the logged PGC ratios (base 10) for the samples with and without BCAR are represented with *gray* and *white bars*, respectively, in a separate plot. *Vertical lines* in all plots represent S.E.

TABLE III

Identification parameters and protein information

The values reported in the "Unused" column correspond to the median of the Unused ProtScores calculated by ProteinPilot for the top protein in each group for each iTRAQ run. Unused values equal to 1.3 and 2.0 are equivalent to a 95 and 99% confidence, respectively. Similarly, "Coverage" and "Error factor" represent the median of percent coverage and error factor measures calculated by ProteinPilot for each group in each iTRAQ run. "Peptide count" shows the average of unique peptide counts, excluding miscleavages, used for protein identification and quantitation by ProteinPilot in each iTRAQ run. Definitions of these measures are explained further in the supplemental material. The number of samples in the rejection (AR) and non-rejection (NR) groups in which each protein group was not detected is reported in "Missing AR/NR," respectively. "Length" and "pI/molecular mass" contain the number of amino acids in each sequence and the isoelectric point/molecular mass (kDa) for each protein, respectively.

PGC[a]	Accession number	Unused[a]	Coverage[a]	Error factor[a]	Peptide count[a]	Missing AR/NR[a]	Length	pI/molecular mass
111	IPI00759754.1	4.97	7.78	1.55	6.9	0/0	34,350	6.01/3,816.19
	IPI00749039.2						33,423	6.16/3,713.67
	IPI00179357.2						33,615	6.13/3,734.82
	IPI00023283.3						34,258	6.01/3,805.87
	IPI00759542.1						34,474	6.02/3,829.87
	IPI00759637.1						33,445	6.15/3,716.2
	IPI00759613.1						32,900	6.09/3,653.26
	IPI00375499.2						27,118	6.31/3,014.03
	IPI00375498.2						27,051	6.36/3,006.83
	IPI00455173.4						26,926	6.35/2,993.01
	IPI00412307.8						20,476	6.22/2,268.36
	IPI00884109.1						391	4.92/43.48
18	IPI00789376.1	49.91	58.72	1.62	9.4	0/0	291	6.27/33.08
	IPI00797833.3						427	6.29/47.9
	IPI00032328.2						644	6.34/71.96
	IPI00215894.1						427	6.29/47.88
108	IPI00032311.4	12.12	25.68	1.4	5.5	1/0	481	6.23/53.38
222	IPI00395488.2	7.44	16.86	1.54	5.6	1/0	673	7.16/71.71
23	IPI00019943.1	40.04	41.57	1.15	19.5	0/0	599	5.64/69.07
224	IPI00873854.1	5.93	18.03	1.31	6.8	2/0	565	7.21/64.16
	IPI00292218.4						711	7.98/80.32
	IPI00384647.1						567	7.21/64.12
	IPI00718805.1						689	8.11/76.75
	IPI00847702.2						127	5.64/13.66
135	IPI00301143.5	4.12	8.59	1.66	2.7	3/0	463	5.24/49.47
	IPI00845506.1						270	5.45/29.67
97	IPI00007221.1	13.49	30.05	1.33	7.4	0/0	406	9.3/45.7
104	IPI00165972.3	7.32	28.46	1.55	3.8	1/3	260	6.82/27.78
38	IPI00303963.1	31.55	35.77	1.17	15.0	0/0	752	7.23/83.27
	IPI00643506.3						525	7.88/58.79
116	IPI00004373.1	12.01	35.48	1.34	5.1	1/0	248	5.39/26.14
125	IPI00007199.4	7.68	17.98	1.48	4.5	0/0	484	7.21/55.11
26	IPI00022395.1	33.73	41.32	1.16	15.3	0/0	559	5.43/63.17
230	IPI00022331.1	5.33	10	1.45	3.7	0/0	440	5.71/49.58
103	IPI00868938.1	4	19.64	2.1	2.5	0/0	101	5.88/11.75
	IPI00796379.1						119	6.06/13.7
	IPI00004656.2						124	6.51/14.42
69	IPI00219583.1	12	32.09	1.35	6.5	0/0	288	5.93/31.83
	IPI00023019.1						402	6.22/43.78
29	IPI00749179.2	37.75	43.43	1.15	15.0	0/0	682	5.08/75.91
	IPI00017696.1						688	4.86/76.68
100	IPI00296176.2	9.41	18.44	1.34	5.3	0/0	461	5.34/51.78
	IPI00816532.1						66	5.05/7.23

[a] The values of the measures in these columns characterize each protein group and do not correspond to a particular protein identifier.

lated in patients with rejection (TTN, LBP, PI16, CFD, MBL2, SERPINA10, and B2M), and five were down-regulated (KNG1, AFM, SERPINA5, LCAT, and SHBG). A principal component analysis can be used to transform the 12 PGCs data into a lower dimensional data set (*i.e.* the principal components) without much loss of information. Fig. 3*A* illustrates the first three principal components of the relative levels of these 12 PGCs, demonstrating the ability of the panel to separate the groups with and without BCAR. Fig. 3*B* illustrates the marginal performance, as estimated by a leave-one-out cross-

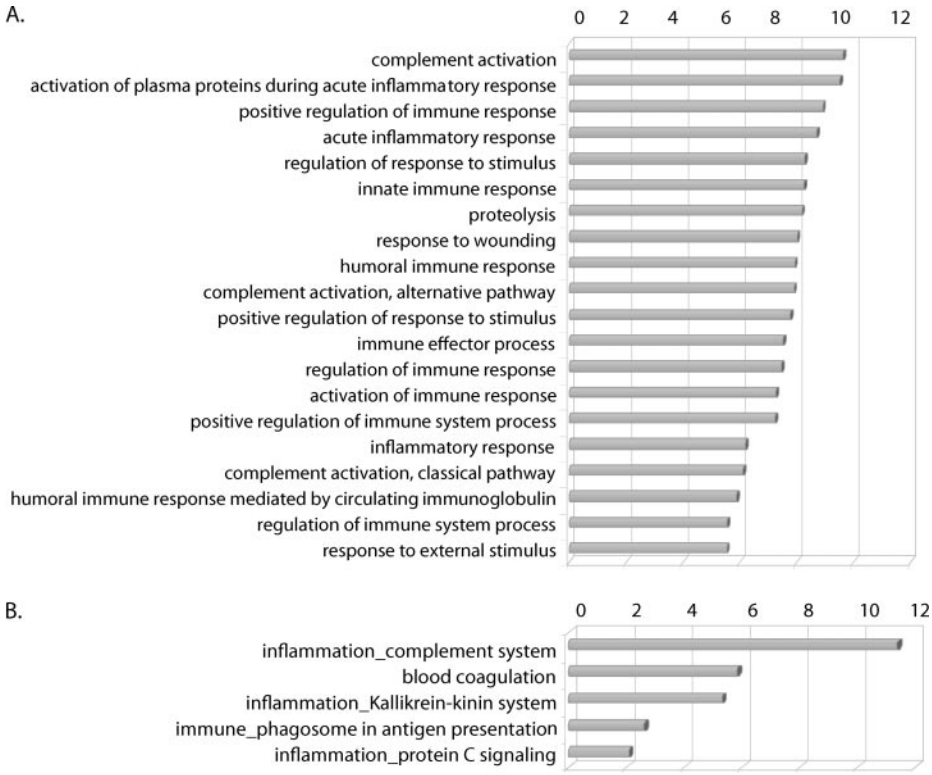


FIG. 2. **Gene ontologies.** The most significantly enriched biological processes (*A*) and biological categories (*B*) based on the 18 protein group codes differentially expressed in BCAR are shown. The *x axis* shows $-\log_{10}$ (*p* values).

validation, achieved when PGCs were sequentially added to an SVM classifier based on a multivariate panel (in order of increasing *p* value). Performance improved (non-monotonically) with the addition of sequential PGCs, reaching a primary asymptote with the first three PGCs (TTN, KNG1, and LBP). Longitudinal monitoring of the SVM score based on ratios of these top three PGCs over the first 3 months post-transplant showed a clear discrimination between the two precise phenotype cohorts at the time of rejection that then disappeared following treatment and resolution of the rejection episode. The score then remained comparable between cases and controls throughout the period of quiescent follow-up (Fig. 3*C*). A leave-one-out cross-validation was used to explore the robustness of the panel identified with a *p* value <0.05 in eBayes test and a fold-change ≥ 1.15. The conservative set of 12 PGCs identified in the complete data analysis appeared in more than 84% of the 32 leave-one-out cross-validation panels for which one sample was left out of the analysis. Furthermore, six PGCs (TTN, KNG1, LBP, AFM, CFD, and SERPINA10), including the three illustrated in Fig. 3*C*, were identified in all cross-validation panels. Performance measures from this cross-validation are presented together with the performance results from the ELISA validation.

Technical Validation—A total of five PGCs were validated by ELISA, including CFD, LCAT, SHBG, F9, and one control (adiponectin) on 29 of the 32 samples in the discovery cohort (see supplemental Fig. A.3). Comparisons between iTRAQ and ELISA results are summarized in Fig. 4*A*. Fig. 4*B* illustrates the differentiation between samples with and without BCAR based on ELISA measurements for four of the five validated. Adiponectin was excluded from Fig. 4*B* as it was run only as a control in the validation. Results demonstrated four important points. First, levels of all five validated proteins were in the same direction (up- and down-regulated in rejection relative to quiescence) in both iTRAQ and ELISA. Second, three of the four proteins (F9, CDF, and SHBG) with significant differential relative levels between subjects with and without rejection in the iTRAQ analysis demonstrated consistent differentiation in ELISA (*i.e.* similar *p* values in both analyses). LCAT showed a weaker correlation than that seen for other proteins. Third, adiponectin (control) was not significant in either of the analyses with similar *p* values and fold-changes. Finally, evidence of a strong correlation between the measurements of both platforms was found in almost all cases. Although the correlation between F9 protein levels determined by iTRAQ and ELISA was low (33%), both platforms discriminated samples with and without rejection. Taken together, the overall results corroborate the results found by iTRAQ and show that measurements from both platforms can be well correlated.

Performance—Leave-one-out cross-validation estimates of sensitivity, specificity, and accuracy from four nested classifiers built using SVM on ELISA data demonstrate the potential gain in classification performance by a panel of proteins (Fig. 4*C*). The sensitivity improved from 30% for a classifier based only on F9 protein to almost 80% for a classifier based on a four-protein panel (F9, SHBG, CFD, and LCAT). The specificity was in all cases above 80%, and the AUC improved from

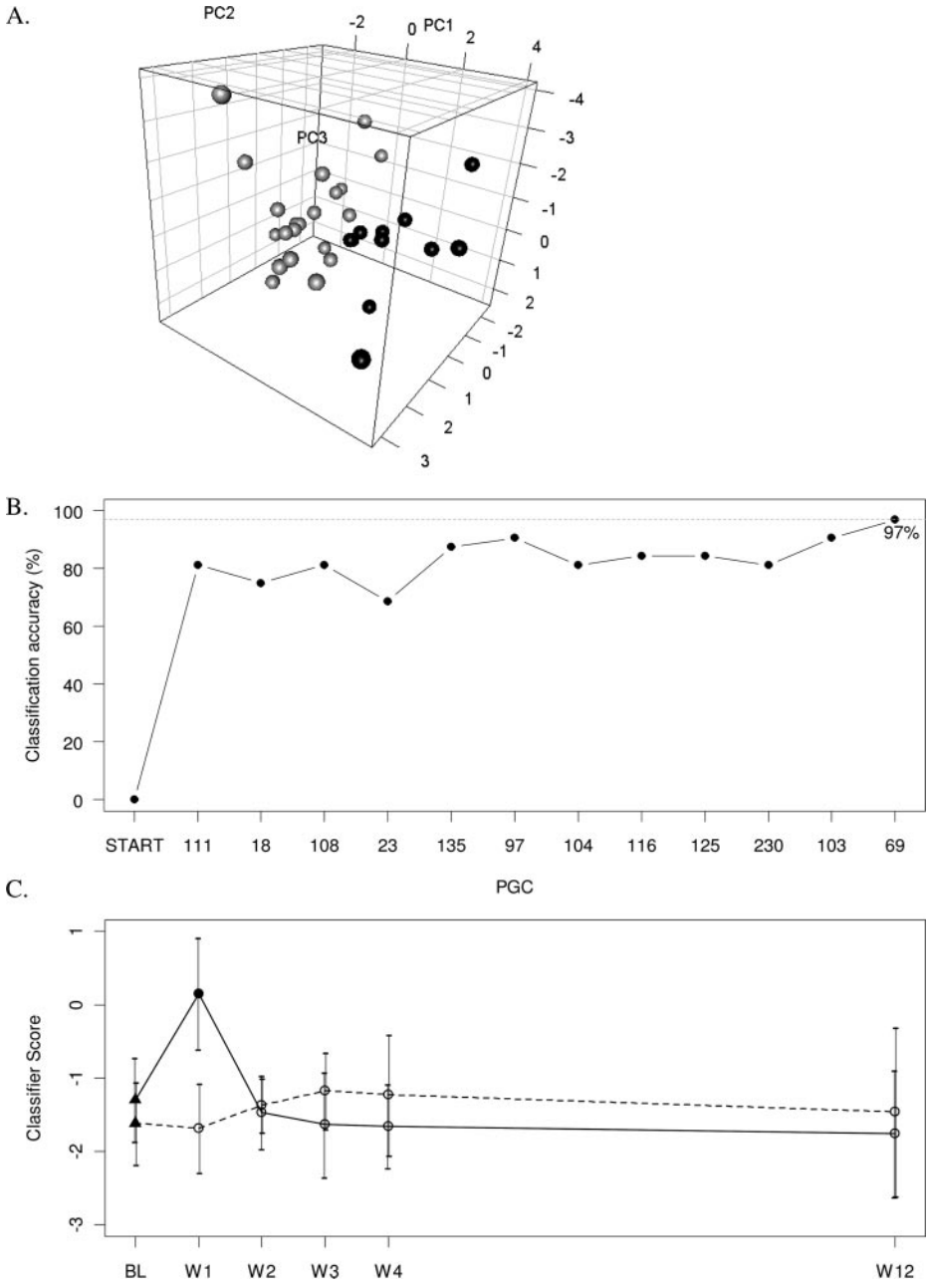


FIG. 3. **Performance of multivariate panel.** *A*, three-dimensional plot of the first three principal components based on 12 PGCs in the panel. *Black* and *gray spheres* represent samples with and without BCAR, respectively. *B*, incremental classification accuracy demonstrating stepwise inclusion of 12 PGCs in the panel. At each step, a classifier score is built using all PGCs to the *left* on the *x axis*, and its corresponding classification accuracy is indicated on the *y axis*. *C*, longitudinal change in average classifier score using the top three PGCs for subjects with (*solid line*) and without (*dashed line*) BCAR and for pre- (▲) and post-transplant samples with (●) and without (○) BCAR. "*BL*" represents the time before transplant (baseline), and other time points correspond to weeks (*W*) after transplant. *Vertical lines* represent S.E.

0.72 to 0.89. In all cases, the classifier was trained only on the training set (*i.e.* excluding the test sample left out), and all samples were processed by an independent platform (ELISA). Thus, results also demonstrate that the ability of the panel identified by quantitative proteomics to discriminate between groups with and without rejection was preserved and validated by ELISA. As test samples in the cross-validation were used to select the analyzed proteins (even when the selection was based on iTRAQ relative levels), the estimated performance measures may be overestimated to characterize the panel as a clinical test. However, similar results were obtained from a full leave-one-out cross-validation based on iTRAQ data (*i.e.* both discovering and training the model based only on the training set and using the resulting classifier to test the sample left out), achieving an AUC of 0.86 (p value = 0.001).

DISCUSSION

Acute graft rejection remains an important complication of renal transplantation, and accurate diagnosis and appropriate treatment are essential to maintain graft function, preserve organ viability, and optimize patient outcomes. Although graft biopsy is the standard for detection and confirmation of acute rejection, it is impractical for routine monitoring because of invasiveness, risk of complications, histological interpretation,

A.

	ELISA		iTRAQ		
Protein	**Robust t-test pval**	**Fold Change**	**Robust eBayes pval**	**Fold Change**	**Correlation ELISA-iTRAQ**
CFD	0.0280	+1.24	0.02	+1.43	0.81
LCAT	0.1087	-1.22	0.03	-1.18	0.24
SHBG	0.0288	-1.31	0.03	-1.19	0.71
F9	0.0004	+1.35	0.04	+1.09	0.33
Adiponectin	0.1048	-1.39	0.08	-1.23	0.86

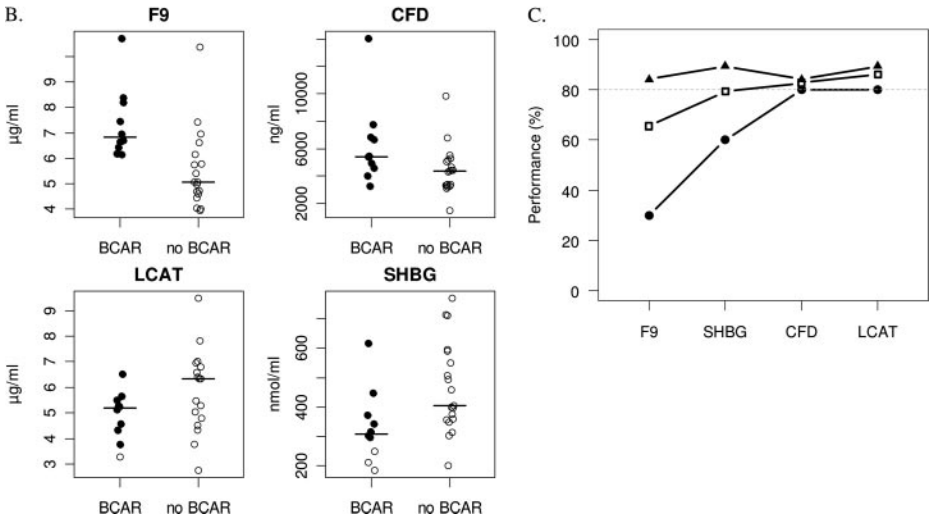


FIG. 4. **Technical validation.** *A*, comparison of ELISA and iTRAQ analyses using 10 samples with BCAR and 19 without BCAR processed in both platforms. *B*, ELISA protein concentrations for four validated proteins from groups with BCAR (*AR*; *filled circles*) and without BCAR (*NR*; *open circles*). *Horizontal lines* represent the median within each group. *C*, classifier performance based on four proteins measured by ELISA: sensitivity (●), specificity (▲), and accuracy (□). *pval*, *p* value.

and cost (37). Alternative diagnostic measures that permit minimally invasive yet highly specific monitoring of allograft rejection are therefore urgently required. Few biomarkers exist for diagnosis of acute graft rejection, and their performance is highly variable (38). Sensitivity and specificity of pharmacokinetic monitoring of immunosuppressive drugs in peripheral blood, the most common assay used routinely in almost all centers for both diagnosis and treatment (39), rarely exceed 70%. Because the index of suspicion for rejection is clinically high in renal transplantation, assay specificity is particularly important to avoid unnecessary biopsy and treatment, and a value of greater than 80% is normally considered appropriate for clinical application.

In this discovery program, we have explored the use of plasma proteomics analysis by iTRAQ-MALDI-TOF/TOF. This methodology has proven reliability and reproducibility with acceptable bias and variability for plasma protein biomarker discovery. Depletion of the most abundant proteins resulted in the identification of ~1000 medium-to-low abundance proteins from 26 iTRAQ runs used to process samples in this study. Of these, 144 PGCs were detected in at least two-thirds of samples from patients with and without BCAR, and 18 PGCs were identified with significant differential relative concentrations between patients with and without acute graft rejection. An initial validation was performed using ELISA. For both iTRAQ and ELISA data, SVM was used to build classifiers based on panels of proteins. As SVM seeks for the hyperplane that represents the largest separation between the two classes *together* as a panel, the identified markers can achieve a satisfactory classification even if single markers may not clearly differentiate the classes (with some fold changes being relatively small but, in general, in the range of other iTRAQ studies (11, 40–42)). For example, if two proteins are compared (*x* and *y* axes), a diagonal line can show a separation of the case and control groups that is not observed using vertical or horizontal lines due to overlap of groups observed on each individual protein.

Identified PGCs associated with BCAR represent a range of biologic processes involved in inflammation, complement activation, blood coagulation, and wound repair, consistent with the current understanding and pathogenesis of acute rejection injury. In addition to these established pathways, this analysis has revealed several novel proteins with unknown roles in acute rejection that may provide insight into new mechanisms of rejection and may provide new targets for therapeutic intervention.

Six of the 18 PGCs identified in the current analysis are involved in the classical, alternative, and mannose-binding activation arms of the complement cascade. Relative concentrations of complement factors C1s, C2, C9, factor D, and mannose-binding protein C (MBL2) were increased in subjects with acute rejection. The role of complement has been primarily related to antibody-mediated immunity, acting to bridge innate and acquired immunity by enhancing antibody responses, mediating inflammation, and stimulating chemotaxis (43). However, recent data have demonstrated that complement can also interact to regulate T-cell responses in the setting of acute rejection (44), and complement deposition has been found in up to 30% of transplant renal biopsies (45), leading to an increased appreciation for the role of this pathway in acute rejection. Increased levels of MBL have also been shown to correlate with poorer survival (46).

LBP is a member of the lipid transfer/lipopolysaccharide-binding family of proteins and was shown to be increased in our acute rejection cohort. LBP acts to transfer lipopolysaccharide to CD14 on monocytes and neutrophils and induce the inflammatory response. Recently, it has gained value in the monitoring of bacterial infection in renal allograft recipients. Levels of LBP have been shown to be elevated in the serum of kidney transplant recipients on the 1st day post-transplant but then return to normal levels, and no rejection-related changes in circulating LBP levels have been reported (47). Our data may indicate a role of LBP in the inflammatory response following rejection.

B2M is a protein associated with major histocompatibility complex class I antigens and has value as a marker for immunologic monitoring with increased levels associated with allograft rejection resulting from increased immune activation (48). Several studies in the 1980s monitored circulating levels of B2M as a biomarker of cardiac or renal allograft rejection (49–51). More recent work has demonstrated some value for B2M as a urinary biomarker of acute allograft rejection in renal transplantation, although it is limited by issues of degradation and lack of specificity (18). B2M is a well known marker of tubular injury in the kidney, and its presence in the plasma of allograft recipients may be indicative of this insult.

Macrophage-stimulating protein (MST1/MSTP9) was significantly increased in our patients with acute rejection. This result is consistent with published work demonstrating elevated plasma levels of MSTP9 in autoimmunity and MSP in critically ill patients with acute renal failure and in recipients of renal allografts during the 1st week after transplantation (52). MSP is a high molecular weight glycoprotein that has been shown previously to be important in wound healing (53–55). Recent reports of MSP levels in renal injury have demonstrated that MSP is up-regulated during tubular cell regeneration and may in this way act to aid recovery from acute kidney injury (56). The vascular response to injury as mediated by TGF-β is also of significance in the development of transplant vasculopathy and rejection (57). Our analysis identified VASN as being up-regulated in acute rejection. Vasorin is a TGF-β-binding protein and has been shown to modulate its activity *in vitro*. The role of TGF-β in rejection is complex, although recent work has shown it to play an important role in chronic rejection (58). The role of vasorin as a binding partner attenuating TGF-β signaling remains to be seen.

This study identified two proteins involved in the coagulation cascade: protein Z-dependent protease inhibitor (SERPINA10) and factor IX. SERPINA10 is a potent inhibitor of coagulation, acting to inhibit factor Xa (59) and degrading factors X and XI (60). Factor IX acts as an activator of factor X, forming the tenase complex with factor VIII. There have been analyses of the effect of renal transplantation on the coagulation system (61), but the effects of changing coagulation parameters on graft outcomes have yet to be determined. Our results suggest that coagulation factors may play an important role in the pathophysiology of acute rejection and may warrant further investigation.

In addition to the broad pathways described above, our analysis identified several proteins not previously associated with acute renal allograft rejection. PI16 has been described as an antihypertrophic protein secreted by cardiomyocytes (62). There have been limited reports of the cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins (CAP) superfamily, of which PI16 is a member, being involved in immunity, but further work remains to be done to establish its role in the pathogenesis of renal allograft rejection. Similarly, AFM, a vitamin E-binding member of the albumin family of proteins (63), has no known role in kidney disease. The role of TTN is also unclear, although the development of anti-titin antibodies has been demonstrated in the autoimmune condition scleroderma (64). Our analysis showed a decreased level in SHBG in acute rejection. Previous work has indicated that SHBG levels fall in male kidney transplant recipients (65), but again, its relevance in acute rejection remains to be seen.

The pilot data presented here are consistent with cellular and molecular signaling pathways known to participate in the immune and inflammatory processes associated with graft rejection. Many of the proteins with differential relative concentrations between samples with and without rejection correlate well with the established understanding of acute renal allograft rejection, although there are several other proteins that have not yet been linked to this condition, providing novel avenues of investigation and potential new targets for drug discovery. The biological functions of the proteins identified complement the functional roles of the genes we have shown previously to be differentially expressed during rejection that encompass major biological processes related to immune signal transduction, cytoskeletal reorganization, apoptosis, lymphocyte activation, proliferation, chemotaxis, and adhesion (9). Because this is a discovery phase, the identified plasma protein markers now will be examined further in combinatorial analyses with genomics and metabolomics data,

and a prospective international clinical validation of these and other discriminating markers is underway.

The methodology used in the current study was designed to ensure rigorous phenotypic comparison and diagnostic simplicity, but this design naturally entails certain potential limitations. The rigorous selection to ensure precise phenotypic homogeneity limited the sample numbers interrogated in the present discovery study, which therefore does not show the performance of the identified proteins under conditions of clinical complexity, such as delayed graft function or coincident infection. It is possible that both the inflammatory injury of rejection and the accompanying decrease in glomerular filtration may contribute to differential alterations in the plasma proteome, and the exact influence of these remains to be determined. Inclusion of these patients in a larger analysis set will now enable us to compare the transcript signals observed in these settings.

In conclusion, quantitative plasma proteomics using iTRAQ methodology has the potential to provide an unbiased profiling and a deeper understanding of acute renal rejection. A panel of 18 plasma proteins that clearly distinguished patients with rejection determined by biopsy from case-matched controls was identified. An initial validation was performed by ELISA for four of these proteins that corroborated most results. However, given that a validation using ELISA is limited by antibody availability, multiple reaction monitoring assays are also being developed to extend mass spectrometry for validation and clinical utility. A larger validation in a new cohort of patients under conditions of clinical complexity is underway to characterize the identified panel using multiple reaction monitoring protein measurements. If confirmed in broader studies, we conclude that the profiling of the plasma proteome or measurement of selected proteins by more specific methods may offer a promising approach to monitor the immunological course in patients following kidney transplantation.

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[S] This article contains 3 supplementary files: a pdf file with an extended explanation of methods and results, and 2 xls files with peptide data for the identified panel of proteins.

[c] Supported by an IBM Institutes of Innovation fellowship award.

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