New developments in transplant proteomics

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Purpose of review
Despite modern immunosuppression, renal allograft rejection remains a major contributor to graft loss. Novel biomarkers may help improve posttransplant outcomes through the early detection and treatment of rejection. Our objective is to provide an overview of proteomics, review recent discovery-based rejection studies, and explore innovative approaches in biomarker development.

Recent findings
Urine MMP7 was identified as a biomarker of subclinical and clinical rejection using two-dimensional liquid chromatography tandem–mass spectrometry (LC-MS/MS) and improved the overall diagnostic discrimination of urine CXCL10 : Cr alone for renal allograft inflammation. A novel peptide signature to classify stable allografts from acute rejection, chronic allograft injury, and polyoma virus (BKV) nephropathy was identified using isobaric tag for relative and absolute quantitation (TRAQ) and label-free MS, with independent validation by selected reaction monitoring mass spectrometry (SRM-MS). Finally, an in-depth exploration of peripheral blood mononuclear cells identified differential proteoform expression in healthy transplants versus rejection.

Summary
There is still much in the human proteome that remains to be explored, and further integration of renal, urinary, and exosomal data may offer deeper insight into the pathophysiology of rejection. Functional proteomics may be more biologically relevant than protein/peptide quantity alone, such as assessment of proteoforms or activity-based protein profiling. Discovery-based studies have identified potential biomarker candidates, but external validation studies are required.

Keywords
activity-based protein profiling, aptamer, mass spectrometry

INTRODUCTION

Transplantation is the therapy of choice for many patients with end organ failure. A major challenge in transplantation is how to optimize immunosuppressive therapy to balance the risk of rejection from underimmunosuppression against the risk of infection and malignancy from overimmunosuppression [1,2]. The ideal regimen would provide the minimum immunosuppression to prevent subclinical and clinical rejection, while limiting infections or other complications that have a negative impact on graft or patient survival. Developing tailored immunosuppression requires sensitive, noninvasive tools for serial monitoring following drug minimization/withdrawal protocols to detect subclinical inflammation prior to injury and to follow the response to antirejection treatment [3,4].

WHY PROTEOMICS?

The human genome consists of approximately 19 000 protein-encoding genes, many of which may code for multiple splice variants [5]. This results in the potential to generate a large number of protein species. Although transcriptomics may provide more complete coverage of transcribed RNA, this information is not an accurate correlate of protein expression because of the many posttranslational regulatory mechanisms that control protein expression [6–8]. Mass spectrometry (MS)-based proteomics currently offers the most comprehensive

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KEY POINTS

- Proteomics offers in-depth pathophysiological information that may enhance our understanding of underlying renal disease processes and identify novel biomarkers for rejection.
- Rigorous technical and clinical validation studies are required to bring a novel biomarker from bench-to-bedside.
- Based on current levels of evidence, there are no proteomics markers that are ready for clinical translation.
- New proteomics technologies may offer novel approaches to gain fresh insight into the pathophysiological changes associated with rejection.

unbiased high-content analytical approach to determine protein composition. Each tightly regulated stage of transcription, translation, and posttranslational modification can lead to different protein functions and/or interactions. Thus, proteomic analysis may provide more direct insights into functionally relevant downstream events than monitoring transcripts does.

The evolution of MS-based analytical techniques has led to an increasing depth of human proteome coverage. Kim *et al.* [9] recently reported a draft map of the human proteome, using high-resolution Fourier transform MS in 30 histologically normal tissue samples (www.humanproteomemap.org). The kidney proteome alone consists of approximately 4000 proteins [10], yet only a fraction has been functionally characterized. Similarly, more than 2400 proteins have been identified in the normal urinary proteome [11–13], yet only a few, such as albuminuria and total proteinuria, are routinely used in clinical practice, suggesting a major untapped resource of biological information that originates directly from the kidney.

PROTEOMICS BIOMARKER DEVELOPMENT: RIGOROUS EVALUATION FROM BENCH-TO-BEDSIDE

Proteomics has largely been used as a discovery tool. However, there are several important considerations when evaluating proteomic data and their interpretation:

(1) What is the confidence of the protein/peptide identification?
(2) Were technical or biological replicates performed?
(3) Were the results quantitatively validated?
(4) Was multiple hypothesis testing accounted for? What assumptions were used? [14]
(5) Was validation performed, such as with an independent validation set or leave-one-out cross-validation [15]?

Furthermore, it is important to determine the clinical significance of potential biomarkers. Biomarker development has been broadly categorized into three stages: discovery, performance evaluation, and impact determination [16], with increasing degrees of evidence required at each stage [4,17]. We will review recent proteomic studies of renal allograft rejection within this context and present new proteomics technologies that may be applicable to novel biomarker development.

PROTEOMIC STUDIES OF REJECTION

In-depth two-dimensional liquid chromatography tandem–mass spectrometry proteomics: urine MMP7

We performed a discovery-based analysis using two-dimensional liquid chromatography tandem–mass spectrometry (LC–MS/MS) in selected cases and normal histology/stable graft function controls, and identified urinary MMP7 as a novel rejection marker [18▪]. ELISA validation demonstrated that urinary MMP7 : creatinine ratio (MMP7 : Cr) is elevated in cellular rejection compared with noninflamed renal allografts, such as stable transplants and interstitial fibrosis/tubular atrophy. Interestingly, urine MMP7 : Cr was elevated in cellular rejection, BKV nephropathy, and acute tubular necrosis, but not in antibody-mediated rejection. We evaluated patients with subclinical rejection and demonstrated that elevated urinary MMP7 : Cr outperforms routine clinical monitoring [18▪]. These findings are consistent with an independent cohort that also demonstrated urine MMP7 : Cr elevation in subclinical rejection compared with normal histology/stable graft function controls [19].

To further assess the utility of MMP7 : Cr, we compared it with urinary CXCL10 : Cr that has been demonstrated to be a noninvasive marker of renal allograft rejection [20–24]. Adding urine MMP7 : Cr to CXCL10 : Cr significantly improved the discrimination of inflammatory renal allograft disorders, using a net reclassification and integrated discrimination index analysis [18▪]. This is one of few studies to evaluate the combined diagnostic utility of a novel biomarker with other established markers of rejection; however, these results need to be independently validated.

iTRAQ-based differential protein profiling of plasma

iTRAQ is a differential protein profiling proteomic technique using isobaric tags for the simultaneous relative or absolute quantitation of proteins in multiple samples. Peptides in a sample are labeled with one of four isobaric, isotope-coded tags via their N-terminal or lysine side chains. The tags have a distinct mass reporter ion and do not rely on a mass/charge separation [25,26]. This technique allows for the relative abundance comparison of four samples simultaneously, with high labeling efficiency.

Freue *et al.* [27] performed a discovery-based case–control study in 11 patients with biopsy-proven acute rejection and 21 patients with stable graft function, but not matching normal histology controls. Their top three differentially expressed plasma proteins (minimum fold-change $\geq$1.15) on principal component analysis (PCA) were titin, kininogen-1, and lipopolysaccharide-binding protein. They performed ELISA validation for factor 9, complement factor D, phosphatidylcholine–sterolacyl transferase, sex hormone-binding globulin, and adiponectin; but not their top three PCA candidates. The strength of this study included their prospective, observational cohort with carefully selected cases and controls and the longitudinal nature that allowed them to demonstrate the evolution of their proteomic signature over time.

iTRAQ-based differential protein profiling of urine

Sigdel *et al.* performed urine iTRAQ labeling in independent training and validation sets ($n=108$ and 154 patients) with clinical biopsy-confirmed acute rejection ($n=74$), stable graft function ($n=74$), chronic allograft nephropathy (CAN, $n=58$), BKV nephropathy ($n=38$), nephrotic syndrome ($n=8$), and healthy controls ($n=10$), and identified 958 proteins. The top 10 most significantly elevated proteins in acute rejection versus stable grafts were actin, cytoplasmic 1; histone H4; fibrinogen gamma chain; keratin, type I cytoskeletal 18; fibrinogen beta chain; human leukocyte antigen (HLA)-DRB1; fibrinogen alpha chain; keratin, type I cytoskeletal 14; keratin, type II cytoskeletal 7; and dipeptidyl peptidase 4 ($n=9$ had $P<0.01$, minimum fold increase >1.5) [28].

ELISA validation with fibrinogen beta, fibrinogen gamma, SUMO2, and HLA-DRB1 demonstrated that fibrinogen beta and gamma were only borderline elevated in acute rejection versus stable allografts ($P=0.04$ for both). HLA-DRB1 and SUMO2 were significantly higher in rejection versus stable allografts [28]. The strengths of this study are the independent training and validation sets and inclusion of other renal allograft injury phenotypes. It is notable that Sigdel *et al.* [29] used an LC–MS/MS approach with spectral counting to estimate the relative abundance of urine proteins in rejection versus stable controls. This alternative approach identified a different and nonoverlapping set of rejection candidates, with the exception of HLA-DR.

Urine proteomics: iTRAQ, label-free liquid chromatography–mass spectrometry, and selected reaction monitoring mass spectrometry

In a key article this year, Sigdel *et al.* evaluated 396 urines with matched histology in patients with normal histology/stable graft function ($n=117$), biopsy-proven clinical rejection ($n=112$), CAN ($n=116$), and BKV nephropathy ($n=51$). Differential protein profiling with iTRAQ labeling in 108 urines identified 958 proteins that segregated the four clinical phenotypes on PCA. In a complementary approach, label-free LC–MS was performed in 137 unique urines and identified 1574 proteins that also segregated the four clinical phenotypes on PCA [30▪▪].

They also compared their urine proteomics and renal allograft transcriptomic data, and this demonstrated only partial agreement between dysregulation of tissue genes and urine proteins: 50% acute rejection, 67% CAN, and 42% BKV nephropathy [30▪▪]. This observed disparity emphasizes the importance of downstream proteomics analysis for functional relevance.

They developed minimal peptide signatures and validated them with selected reaction monitoring MS (SRM-MS) using independent training and validation sets ($n=80$ and 71, respectively) to characterize diagnostic performance: acute rejection (AUC 0.94), CAN (AUC 0.995), and BKV nephropathy (AUC 0.83). The 11 acute rejection peptide fragments were from cartilage oligomatrix protein; proepidermal growth factor; brevican core protein; zinc-alpha-2-glycoprotein; fibrinogen alpha chain; histone H2B type 1-B; keratin, type II cytoskeletal 75; keratin, type I cytoskeletal 15; dystroglycan; immunoglobulin heavy variable chain; and haptoglobin [30▪▪].

These results represent a detailed analysis of the urinary proteome in renal allograft injury. The strengths include the carefully characterized clinical cohort, complementary proteomics approaches, integration with renal transcriptome data, and quantitative validation with SRM-MS. Nevertheless, the lack of overlap in candidates from this study compared with their earlier work [28,29] and others

[18▪,27] is notable. Therefore, these findings remain to be independently validated in a prospective, observational cohort. Indeed, the only promising proteomic signature [31–33] that has ever been independently evaluated in a large, prospective multicenter cohort of incident renal transplant patients ($n = 280$, CTOT-01 study) was ultimately found to be nondiagnostic for rejection [34], emphasizing the critical need for independent validation studies.

Intriguingly, Sigdel *et al.* [35] performed a pilot study of urinary exosome analysis in kidney transplant patients ($n = 30$) and identified differential exosomal protein expression in patients with acute rejection using an iTRAQ approach. They identified three proteins unique to the urinary exosomal fraction and acute rejection: calcium-activated chloride channel regulator 1, vitamin K-dependent protein S, and KIAAO 753 – an uncharacterized protein associated with protein degradation [35]. It remains to be seen whether integration of these urinary exosome data with their unfractionated whole urine data (reported above) will improve the overall diagnostic discrimination for rejection, BKV, and CAN.

Proteoform characterization

Savaryn *et al.* [36▪] recently performed a top-down proteomics analysis of peripheral blood mononuclear cells comparing patients with stable graft function/normal histology ($n = 20$) versus patients with clinical indication biopsy-proven cellular rejection ($n = 20$). The focus of their discovery-based analysis was to characterize differential proteoform expression, thereby accounting for alternative splice variants, different translational start sites, proteolytic processing, and posttranslational modifications. This unique in-depth analysis identified 27 differentially abundant proteoforms below a false discovery rate of 1%, of which 26 were histone H2A proteoforms elevated in healthy transplants and the remaining was a 60S ribosomal protein L36 proteoform elevated in rejection [36▪]. The strength of this study was their detailed proteoform analysis, which may identify the most functionally relevant protein species by accounting for both splice variants and posttranslational modifications.

WHAT'S NEW AND COMING IN PROTEOMICS?

Activity-based protein profiling: enzyme activity is more important than quantity

Many proteins including enzymes are maintained in a latent state until their activity is required. Thus, the quantity of a protein may not necessarily reflect its functional activity. However, most proteomic techniques provide information on absolute or relative changes in quantity but not functional status. Activity-based protein profiling (ABPP) was developed to address this problem using the underlying premise that only the catalytic sites of active enzymes will be substrate accessible [37,38]. Activity-based probes are designed with a reactive group that specifically targets residues that belong to the catalytic sites of an enzyme family and a tag that may be used as a reporter or for affinity capture. These features provide a measure of enzyme functional status as well as a basis for isolating and identifying the active enzymes.

The serine hydrolases constitute approximately 1% of the eukaryotic genome and are a large enzyme family of more than 200 members with a range of activities including protease, esterase, lipase, peptidase, and amidase [39]. We optimized a technique for performing serine hydrolase ABPP in urine and identified 13 active serine hydrolases in normal urine [13]. These included luminal regulators of electrolyte homeostasis and components of the innate immune system [13]. This technique offers unique opportunities to correlate differential urinary serine hydrolase activities with renal allograft disorders. Ultimately, if differential enzyme activities exist, this may offer the potential for developing point-of-care assays for relevant enzymes.

Aptamer-based proteomics

Recently, an aptamer-based platform, SOMAscan, has been introduced for targeted proteomic analysis of human samples [40]. Aptamers are short single-stranded oligonucleotides that are capable of binding proteins with high affinity and specificity and can be chemically modified to slow their dissociation rate from proteins [40]. The aptamer-multiplex assay offers a basis for the simultaneous analysis of 1310 proteins in a single 65 μl sample. The assay is highly sensitive, reproducible, with a broad dynamic range over 7 logs quantification [40] and offers significantly increased protein coverage compared with conventional antibody microarrays [41].

Aptamer analysis was used to compare serum protein profiles in patients with microvascular inflammation and clinical antibody-mediated rejection versus patients with normal histology and stable graft function at 24 months. Forty-five proteins were differentially expressed between groups, with an overall increase in proteins related to inflammation and endothelial activation [42]. Several potential biomarker candidates were identified but remain to be verified in larger studies.

CONCLUSION

Proteomics has many powerful tools for interrogating renal disease processes. A major challenge in biomarker-discovery proteomics is the identification of non-overlapping candidates using different approaches. This may be explained in part by a high false discovery rate associated with high dimensional data combined with sparse datasets/low numbers [43]. It is further complicated by the stochastic nature of MS in biologically complex samples. Therefore, this immense promise needs to be tempered by rigorous technical application and quantitative validation, including establishing robust thresholds for acceptable levels of significance to limit false discovery. Furthermore, these analyses need to be constrained by a sensible approach to evaluate clinical utility within a patient-centered context. The majority of transplant proteomic studies report the discovery phase of biomarker development, but very few demonstrate their clinical utility with fully independent validation studies. Ultimately, prospective interventional trials will be needed to demonstrate that using novel biomarkers improves long-term graft outcomes.

Finally, the frequent emphasis on biomarker discovery should not detract from the overall utility of proteomics in reshaping our understanding of transplant pathophysiology. Indeed, proteomics has offered important contributions to characterizing disease processes in C3 glomerulopathy [44], amyloidosis, fibrillary glomerulonephritis [45], and membranous glomerulopathy [46]. These data can be used to shape novel therapeutic approaches, such as terminal complement inhibitors for C3 glomerulopathy, or refine the diagnosis of amyloidosis [47]. Overall, the human proteome has the potential to be a vast resource for understanding underlying pathophysiological processes and improving precision diagnostics.

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Conflicts of interest

There are no conflicts of interest.

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