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REVIEW

Proteomics for rejection diagnosis in renal transplant patients: Where are we now?

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Abstract

Rejection is one of the key factors that determine the long-term allograft function and survival in renal transplant patients. Reliable and timely diagnosis is important to treat rejection as early as possible. Allograft biopsies are not suitable for continuous monitoring of rejection. Thus, there is an unmet need for non-invasive methods to diagnose acute and chronic rejection. Proteomics in urine and blood samples has been explored for this purpose in 29 studies conducted since 2003. This review describes the different proteomic approaches and summarizes the results from the studies that examined proteomics for the rejection diagnoses. The potential limitations and open questions in establishing proteomic markers for rejection are discussed, including ongoing trials and future challenges to this topic.

Key words: Kidney transplantation; Acute rejection; Chronic rejection; T cell-mediated rejection; Antibody-mediated rejection; Long-term outcome; Graft failure; Biopsy; Non-invasive markers; Proteome; Proteomics; Mass spectrometry; Diagnostic marker; Study design; Diagnostic trial

Core tip: Timely detection and treatment of acute and chronic rejection is important to maintain the allograft function in renal transplant patients. Allograft biopsies are unsuitable for continuous monitoring for rejection. This review summarizes the past experience with proteomic approaches to diagnose rejection non-invasively. Potential limitations and open questions

in establishing proteomic markers for rejection are discussed, including ongoing trials and future challenges to this topic.

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INTRODUCTION

Since 2003, proteomics in blood and urine has been explored for non-invasive rejection diagnosis in renal transplant patients. In this review, we summarize and discuss the approaches and results of previous proteomic studies on the background of the heterogeneous and complex condition "allograft rejection". Ongoing studies on this topic are reported and future challenges in establishing proteomic markers for rejection are discussed.

IMPORTANCE OF REJECTION FOR THE LONG-TERM ALLOGRAFT OUTCOME

Despite all improvements in immunosuppressive protocols and patient surveillance after kidney transplantation, allograft rejection remains a significant adverse factor for the long-term allograft survival. In a previous study, both T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR) were reported as leading causes of graft failure in a substantial proportion of patients[1]. Acute TCMR is most prevalent in the first year after transplantation and has been suggested as a trigger for subsequent development of ABMR[2]. ABMR often evolves over prolonged time and may become chronic, with appearance of donor-specific antibodies first, followed by acute injury of peritubular and glomerular capillaries which in the later course leads to transplant glomerulopathy and tubulointerstitial scarring[3]. Some patients may also present with concomitant findings of TCMR and ABMR (*i.e.*, mixed rejection)[4]. Consequently, early recognition of rejection is important during the entire post-transplant course on a continuous basis to treat the rejection timely and to adjust the maintenance immunosuppression in order to prevent further rejection episodes and chronification of the rejection.

Monitoring for rejection is a challenge and has not been satisfactorily solved. Regular measurement of serum creatinine or cystatine C to detect declining allograft function (which then triggers an allograft biopsy) is insensitive and is a late indicator when tissue injury has already taken place[5]. Some patients may present with increased proteinuria but similar to declining graft function, this can only indicate established injury and is non-specific as to the cause of injury[6]. In the case of ABMR, monitoring for donor specific antibodies may identify patients at risk; however, in our experience full-blown histopathologic features of ABMR can be present without detectable antibodies using currently available assays. Many transplant centres have turned to protocol biopsies to evaluate the course of the allograft. Protocol biopsies may give valuable information, *e.g.,* on silent and early rejection processes, toxicity of medical treatments, BK virus infection and development of chronic scarring processes[5]. However, continuous monitoring for rejection over the entire post-transplant course would require performing biopsies unrealistically often.

Due to this diagnostic dilemma, there is clearly a need for sensitive, non-invasive methods to monitor for rejection and to detect rejection at an early stage. Such tests could be performed regularly to identify those patients who need further workup by an allograft biopsy. Several molecules in blood and urine have been evaluated (either as a single marker or as a combination of markers) based on the hypothesis that blood and urine can reflect the molecular processes in the allograft. In theory, testing for markers of rejection in blood and urine could even outperform the diagnosis by biopsy, which is prone to sampling errors and inter-observer variability. However, none of these tests has gained widespread clinical use[5].

RATIONALE FOR A MULTI-MARKER APPROACH TO DIAGNOSE REJECTION

Rejection is a heterogeneous process[7-9] and therefore it is unlikely that a single marker or small number of markers can reflect all facets of rejection reliably. Heterogeneity refers to the entities of T cell- and antibody-mediated rejection but also to the sites of immunological attack and to the morphological severity as specified by the Banff classification[7] and shown in Figure 1. Also, as a reflection of the severity the rejection may be subclinical, *i.e.*, without a concomitant decline in allograft function or clinical with accompanying graft dysfunction[10]. As outlined in Figure 1, rejection is a disease process that extends from the activation of the immune system to the scarring of injured renal structures. This implies that time-dependent features may also be important to consider in terms of early and later stages of rejection. Given these facts, the hypothesis of multi-marker approaches is that a panel of molecules is better suited to detect the diverse aspects of rejection than a single molecular marker. In fact, gene expression analysis of allograft biopsies has demonstrated that different types of rejection present with distinct molecular phenotypes, containing a wide array of chemokines, cytokines and other regulatory molecules[11]. Some of these phenotypic signatures should be detectable in blood and urine and usable for the rejection diagnosis.

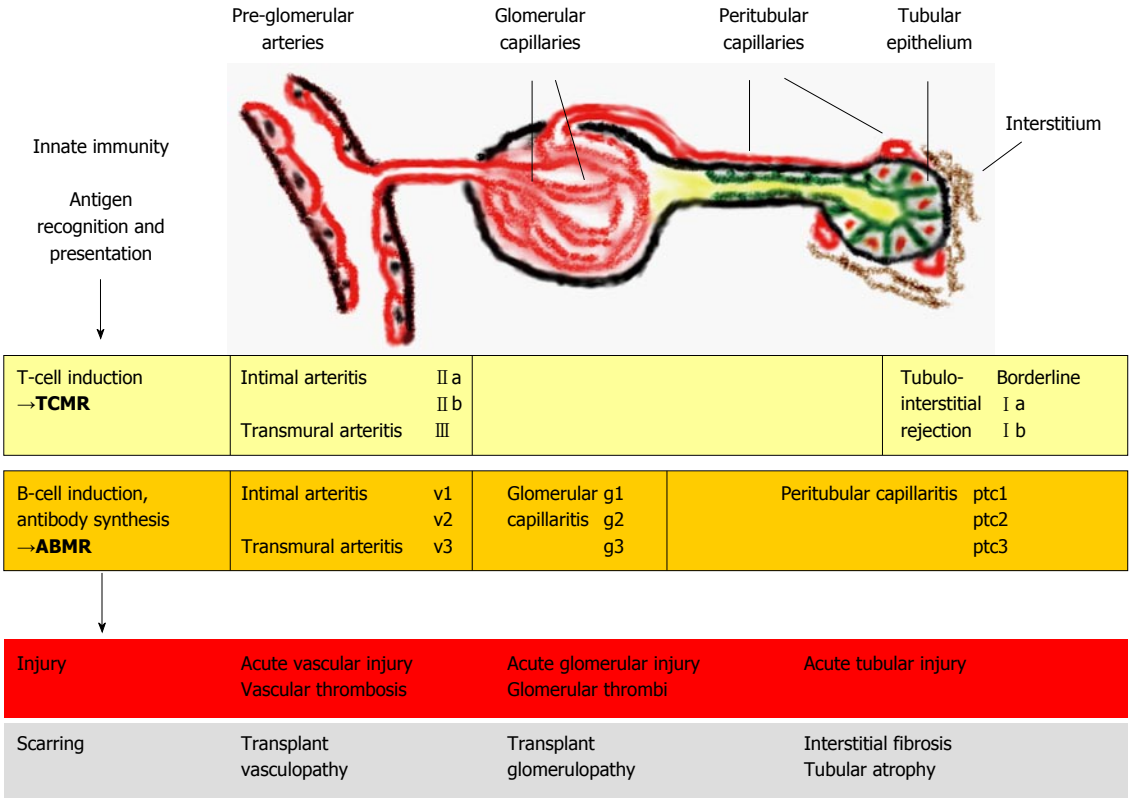

Figure 1 Kidney allograft rejection types, histological sites of injury and underlying mechanisms. TCMR includes recognition and presentation of donor antigens by antigen-presenting cells to T cells, which become activated and undergo proliferation. Activated T-cells invade vascular, tubular and interstitial structures. Vascular rejection often presents with some degree of tubulointerstitial inflammation; however pure cases of vascular rejection ("v-only") can be observed[8]. In ABMR, activated T cells induce B cells to undergo plasma cell proliferation resulting in the production of donor-specific antibodies. Antibody-mediated injury to pre-glomerular arteries, glomerular and peritubular capillaries is mediated by local activation of complement factors however, non-complement-fixing antibodies may also play a role in some cases[9]. Isolated findings of glomerular and peritubular capillaritis or pre-glomerular arteritis may be present or a combination of these features[7]. TCMR and ABMR can occur simultaneously (*i.e.*, mixed rejection)[4]. The rejection processes can lead to different histological forms of injury and if not successfully treated, to scarring. The Banff classification[7] associates the elementary lesions of glomerular (g) and peritubular capillaries (ptc) and pre-glomerular vessels (v) to ABMR. TCMR includes tubulointerstitial infiltration (Borderline, Ⅰ) and arteritis of pre-glomerular vessels (Ⅱ-Ⅲ). Banff grades (a-b, Ⅱ-Ⅲ, v1-3, g1-3, ptc1-3) denote different severities of the lesions. TCMR: T cell-mediated rejection; ABMR: Antibody-mediated rejection.

It is important to note that the rejection process induces host responses like repair and healing mechanisms including scarring processes which contribute to molecular signatures[12] (Figure 1). On theoretical grounds, marker sets for the diagnosis of rejection should be distinct from those signatures as they rather reflect the sequel of rejection instead of depicting specifics of the rejection process itself. As an example, urinary β2-microglobulin or fragments of it have been reported as potential indicators of rejection[13,14]. Further analysis however showed that increased urinary β2-microglobuline-derived peptides are similarly present in pure cases of acute tubular injury[15] and in cases with tubular atrophy and interstitial fibrosis[16,17], without any evidence of rejection.

To date, several approaches have been employed to establish multi-marker models for the non-invasive diagnosis of rejection. Gene expression, RNA analysis and proteomics are the commonest whereas fewer studies concentrated on microRNA analysis[18], metabolomics[19] and lipidomics. This review focuses on proteomics in blood and urine of kidney transplant patients to diagnose rejection.

PROTEOME ANALYSIS

The proteome is the whole set of proteins present in an organism or in one of its functional or structural units at a given state. Compared to the transcriptome or the metabolome, the proteome is the most functional compartment and is subject to continuous and dynamic changes either in response to external stimuli or alterations in homeostasis[20]. In recent years, clinical research mainly focused on the detection of single proteins by immunological techniques. This hypothesis-driven approach requires precedent knowledge on the functional characteristics of a specific protein target. Proteome analysis in contrast is hypothesis-free since it explores a biological sample in its proteomic entirety. Therefore, by comparison of the proteomic content at two or more distinct conditions (*e.g.*, diseased and non-diseased) all differently expressed proteins may be captured as potential differentiating markers. Technically, proteomic technologies rely on the physicochemical properties of the proteins instead of immunological properties, which are required for antibody-mediated analyte detection.

Biomarker research by proteomics is based on the hypothesis that at least one of the following conditions is true: (1) Proteins are differentially expressed from their genes during a disease process; (2) Proteins are subject to differential post-translational modifications due to disease-specific changes in the activity of enzymes; and (3) Proteins are detectable in different amounts due to altered production, degradation or release from cells by the disease process.

Sample matrix

In biomarker research, easily accessible sample matrices like blood or urine are preferred because procurement of tissue relies on invasive methods. Blood has a high dynamic range of protein concentrations, necessitating depletion of the most abundant proteins to improve detection of low abundant protein markers. It is also characterized by lower stability due to high proteolytic activity. Urine on the other hand, has a higher stability and lower complexity than blood. However, urine is in contact with the genital-urinary tract and thus, prone to bacterial contamination. Moreover, the proteomic compounds in urine originate from different sources, namely from the systemic circulation *via* glomerular filtration, from the kidney, and from the urinary tract. The exact contribution by these sources is unknown and may change in disease conditions.

Proteomic workflow

The proteomic workflow includes the preparation of the sample to clear the proteomic content from other compounds, followed by complexity-reducing separation and physicochemical detection methods.

Sample preparation: Before proteomic analysis, a sample usually needs processing to remove insoluble materials like cell debris and interfering salt and lipids. It is however important to note that such preparation steps introduce bias and add variability, and therefore should be restricted to the absolute requirements[21]. Because proteins can be degraded by proteases, heat, bacteria and pH changes, the integrity of the samples should be maintained by applying standardized collection protocols and immediate freezing.

Protein separation: Historically, 2-D gel electrophoresis used to be the principal proteomic separation method[22]. This is now largely replaced by the non-gel based separation methods liquid chromatography (LC) and capillary electrophoresis (CE), which have a higher resolving capacity. Using LC and CE, small proteins and peptides can be directly subjected to mass spectrometry analysis whereas larger proteins have to be cleaved by trypsin before separation and mass detection[23].

Protein ionization: There are many different mass spectrometry methods but they all have in common that proteins and peptides are transferred into ions, which are then subjected to an electric or magnetic field. The subsequent characterization of each ion is based on its mass over charge ratio (m/z). Electron spray ionization, matrix-assisted laser desorption/ionization and surface enhanced laser desorption-ionization are the main ionization techniques used in clinical proteomic studies.

Protein mass detection: The desolvatized ions in the electric or magnetic field are then collected by the mass detector. Many different concepts exist, mostly in respect to how an ionic signal is amplified. "Time of flight", Orbitrap and Triple Quadrupoles are the most commonly used detectors in biomarker research.

Protein quantification

Normally, only relative quantification is possible with mass spectrometry (MS) techniques, based on an approximate proportionality between signal intensity and the relative protein/peptide abundance in a sample. Advanced methods have been developed like "isobaric tags for relative and absolute quantification"[24]. And "multiple reaction monitoring"[25] to compare the protein/peptide abundance between different samples.

Protein sequence identification

In its simple one-dimensional form, mass spectrometry gives mass over charge ratios of peptides and proteins but no information on the amino acid sequence. This may be sufficient to identify and detect proteomic markers for disease conditions simply by their physicochemical characteristics. Nevertheless, identification of the proteins and peptides may be desirable, *e.g.,* to understand pathophysiologic pathways or to transfer the discovered markers to another platform (*e.g.,* ELISA). With tandem mass spectrometry (MS/MS), a MS-detected peptide can be isolated in the first MS dimension and then forced into multiple rounds of collisions in the second MS dimension to generate an ordered fragment ion spectrum[26].

Construction of multi-marker diagnostic models

Although average levels of single proteins or peptides may be significantly different between case and control groups large overlap of values is often observed when individual samples are compared with each other[27]. To construct classifiers with as little overlap as possible between case and control groups, biomarkers are often combined into multi-marker sets[28]. This strategy can compensate for analytical variances and biological variability like heterogeneity of the disease process, time-dependent changes, or confounding conditions. The integration of proteins/peptides into a multi-marker set can range from a few individual molecules up to whole "fingerprints" (chromatograms, spectra), depending on the requirements for sensitivity and

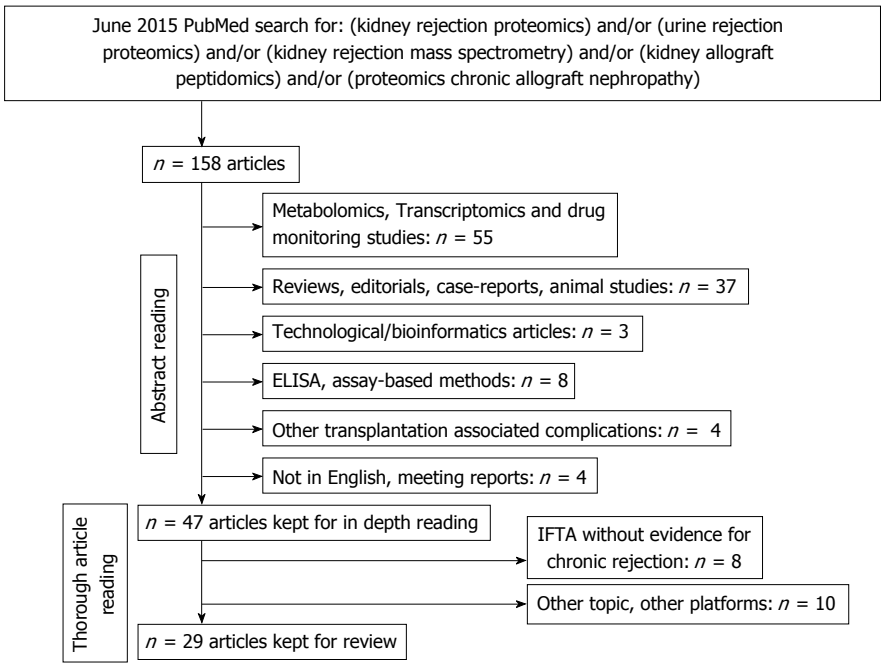

Figure 2 Search strategy for proteomic studies in the field of renal allograft rejection. IFTA: Interstitial fibrosis and tubular atrophy.

specificity and on the complexity of the disease of interest.

Methods to integrate multiple discriminative proteins into a biomarker model can be divided into "linear" and "high dimensional" algorithms, the latter tending to have better results due to a weighted combination of the markers according to the degree of their correlation. Here, the most frequently used algorithms are "support vector machine", adaptive boosting, random forest and neural networks.

PROTEOMIC STUDIES ON RENAL ALLOGRAFT REJECTION

The literature search was done in PubMed using the keywords "kidney, rejection, proteomics, urine mass spectrometry, allograft, peptidomics, chronic allograft nephropathy" in different combinations (Figure 2). Of the 158 publications, 111 were excluded after reviewing title and abstract of each publication. The remaining 47 articles were kept for in depth study. Ten articles were excluded because they concentrated only on technical aspects (n = 4), did not use shotgun proteomic methods (n = 5), or did not examine rejection patients (n = 1).

Examination of patients with chronic rejection/chronic allograft nephropathy was reported in eight studies[16,17,29-34]. However, evaluation of the histomorphological reporting revealed that patients in these studies had merely interstitial fibrosis and tubular atrophy (IFTA; Banff category 5) according to the latest update of the Banff classification[7], without any evidence of acute or chronic rejection. This mistaking is explained by the historical definition of "chronic allograft nephropathy", which does not differentiate between patients with non-specific chronic lesions (IFTA) and patients with signs of chronic rejection. Hence, these studies were considered as non-relevant for the topic "rejection" and excluded from the reporting in Table 1.

The remaining 29 studies[13-15,35-60] are listed in Table 1. Five studies reported a prospective study design[37,41,45,46,57], with assumable random or consecutive sample selection. In the remaining studies, samples seemed to be drawn from a biobank/sample archive not specifically established for the proteome study, without giving details to selection process and randomness of the samples. Most studies were cross-sectional. Nine studies described longitudinal aspects with regard to sample collection[39], profiling of sequential samples or comparison of proteome patterns before and after rejection[13,35,37,41,45,53,60] and to the assessment of graft survival[59].

One third of the study performed proteomic analysis on an independent validation set of samples to confirm the discovered markers. Validation on independent samples was also performed by ELISA assays for the discovered markers[50,51,53,60].

Urine was clearly the diagnostic matrix of choice, with 23 studies compared to the six studies that examined blood samples. In the study of Ling *et al*[40] mRNA expression in biopsies was examined in parallel to the urinary proteome. O'Riordan *et al*[45] stained biopsies to confirm the identified urinary proteomic marker β-defensin-1.

In approximately half of the studies, patients with TCMR were examined, as evident from the reported Banff grades. Patients with ABMR were included in six studies[35,47,48,51,58]; in one study[46] a few patients were reported to have mixed rejection (TCMR + ABMR). In

Table 1 Proteomic studies on renal allograft rejection

Ref.	B/U	Training set	*n*	Validation set	*n*	Proteomic method	Performance	Identified molecules	Remarks
Akkina *et al*[35]	U	C (bx) BL Ⅱa aABMR	13 1 1 1	None		iTRAQ-MALDI-MS/MS	NR	None	Study included healthy individuals. Study concentrates on longitudinal stability of peptides in rejecting and non-rejecting patients
Clarke *et al*[36]	U	C (st) AR	15 15	None		SELDI-TOF-MS	Accuracy 91% Sensitivity 83% Specificity 100% (2-marker classifier)	None	
Freue *et al*[37]	B	C (bx) Ⅰa Ⅰb Ⅱa	21 7 1 3	None		iTRAQ-MALDI-MS/MS	AUC 0.86 Sensitivity 80% specificity 90% (4-marker classifier)	Up-regulated: TTN, LBP, PI16, CFD, MBL2, SERPINA10, B2M Down-regulated: KNG1, AFM, SERPINA5, LCAT, SHBG	ELISA was performed on 4 of the identified markers (coagulation factor IX, SHBG, CFD, LCAT) in blood
Günther *et al*[38]	B	C (st) AR	13 13	C (st) AR	7 7	iTRAQ-MALDI-MS/MS	AUC 0.76 Sensitivity 57% specificity 86%	21 peptides	Different statistical approaches to integrate proteomics and transcriptomic results are presented
Jahnukainen *et al*[39]	U	C (st) Ⅰa-Ⅱb BKV	29 28 21	None		SELDI-TOF-MS	Sensitivity 81% Specificity 84% (100-marker classifier)	None	21 of the 28 rejection samples showed also signs of chronic rejection Article concentrates on differentiation of AR and BKV-NP
Ling *et al*[40]	U	C (bx) AR BKV	10 10 10	C (bx) AR BKV	10 10 4	LC-MALDI-TOF-MS LC-MS/MS	AUC 0.96 (40-marker classifier)	COL1A2, COL3A1, UMOD, MMP-7, SERPING1, TIMP1	Study included healthy individuals and patients with native kidney disease (nephrotic syndrome). Results of proteomic analysis are related to mRNA expression profiling of corresponding biopsies
Loftheim *et al*[41]	U	C (st) BL Ⅰa Ⅱa	6 1 4 1	None		2D LC-MS/MS	NR	Up-regulated: IGFBP7, VASN, EGF, LGALS3BP	Study collected sequential urines from the beginning after Tx. Analysed samples for rejection patterns were taken 7-11 d before biopsy
Mao *et al*[42]	U	C (bx) TCMR	22 27	C (bx) TCMR	14 10	SELDI-TOF-MS	Sensitivity 90% Specificity 71% (4-marker classifier)	None	All TCMR cases were subclinical rejections with grades ≥ Ⅰa
Metzger *et al*[43]	U	C (bx) Ⅰa Ⅰb	23 13 3	C (bx) Ⅰa Ⅰb	36 23 5	CE-MS LC-MS/MS	AUC 0.91 Sensitivity 93% Specificity 78% (14-marker classifier)	3 fragments of COL1A1, 1 fragment of COL3A1	Rejections in the training set were all subclinical. The validation set contained 10 clinical and 18 subclinical rejection cases. Confounder like ATI in biopsies, urinary tract infection and CMV infection were considered
O'Riordan *et al*[44]	U	C (st) AR	22 23	None		SELDI-TOF-MS	AUC 0.91 Sensitivity 91% Specificity 77% (2-marker classifier)	Up-regulated: SERPINA3 Downregulated: DEFB1	Study included healthy individuals
O'Riordan *et al*[45]	U	C (st) BL Ⅰa Ⅰb Ⅱa Ⅱb	22 3 6 4 7 3	None		SELDI-TOF MS LC-MS/MS	AUC 0.91 Sensitivity 91% Specificity 77% (2-marker classifier)	Up-regulated: SERPINA3 Downregulated: DEFB1	
Pisitkun *et al*[46]	U	C (bx) Ⅰa Ⅰb Ⅱa ATI	2 4 1 2 7	None		LC-MS/MS	NR	Numerous molecules	
Quintana *et al*[47]	U	C (st) a/cABMR IFTA	8 10 8	a/cABMR IFTA	8 6	MALDI-TOF-MS	IFTA *vs* cABMR AUC 1.0 Sensitivity 100% Specificity 100% (6-marker classifier)	None	Study included healthy individuals

Quintana *et al*[48]	U	C (st) a/cABMR IFTA	5 10 8	C (st) a/cABMR IFTA	9 11 8	LC-MS/MS	C *vs* IFTA/ABMR: AUC 0.82 IFTA *vs* ABMR 100% correct IFTA, 90% correct ABMR (2-markers)	Down-regulated: UMOD Differentiation between controls and IFTA/ABMR: KNG1	Study included healthy individuals Two unidentified peptides could differentiate between IFTA and ABMR, based on quantitative differences of the peptides (higher in ABMR)
Reichelt *et al*[49]	U	C (bx) Ⅰa Ⅰb Ⅱa Ⅱb	10 7 3 1 2	None		SELDI-TOF-MS	SAX2 protein chip: Sensitivity 90% Specificity 80% CM10 protein chip: Sensitivity 92% Specificity 85% (2-marker classifier)	None	
Schaub *et al*[13]	U	C (bx) Ⅰa Ⅰb Ⅱa ATI GL	22 7 8 3 5 5	None		SELDI-TOF-MS	Sensitivity 94% Specificity 82% (3-marker classifier)	Cleaved B2M Cleaved B2M	Study included healthy individuals. The clinical confounder CMV viremia was assessed. Longitudinal evaluation of urine proteome patterns differentiated between patients with stable course and rejection
Schaub *et al*[15]	U	C (bx) Ⅰa Ⅰb Ⅱa ATI GL	22 7 8 3 5 5	None		SELDI-TOF-MS, LC-MALDI-MS	NR		Study included healthy individuals. Study concentrated on cleavage mechanisms for b2-microglobulin
Sigdel *et al*[14]	U	C (bx) AR	10 10	None		LC-MALDI-MS/MS	NR	List of 73 candidates, incl. fragments of collagens, UMOD, B2M, PTGDS	Study included healthy individuals
Sigdel *et al*[50]	U	C (bx) AR	10 10	None		LC-MS/MS	AUC 0.84-0.97 for 3 single molecules (by ELISA)	Upregulated: SERPINF1 Down-regulated: UMOD, CD44	Study included healthy individuals and patients with native kidney disease (proteinuria)
Sigdel *et al*[51]	U	C (bx) Ⅰa-Ⅱb aABMR IFTA BKV	30 30 2 30 18	None		iTRAQ-LC-MS/MS	AUC 0.8 for 3 single molecules (by ELISA)	HLA-DRB1, KRT14, HIST1H4B, FGG, ACTB, FGB, FGA, KRT7, DPP4, cleaved B2M	In ELISA studies, FGG could also segregate AR from BKV-nephropathy Validation set for detection of FGG, HLA DRB1, FGB by ELISA included 44 stable transplant patients and 44 patients with rejection
Sigdel *et al*[52]	U	C (bx) ⩾ Ⅰa	20 20	None		iTRAQ-LC-MS/MS	NR	Enriched in exosomal fraction in AR: A2M, APOA2, APOM, CD5L, CLCA1, FGA, FGB, IGHM, DEFA5, PROS1, KIAA0753 Exclusively in the exosomal fraction in AR: CLCA1, PROS1, KIAA0753	Study concentrated on differences between the whole proteome in urine (non-fractionated) and the exosomal fraction
Stubendorff *et al*[53]	U	C (st) AR	16 16	C (st) AR	16 16	SELDI-TOF MS	Sensitivity 94% Specificity 44% (4-marker classifier) Sensitivity 80% Specificity 81% for 2 molecules (by ELISA)	Up-regulated: A1MG, HP	Results on longitudinally collected samples suggest that alpha-1-microglobulin and haptoglobin indicate upcoming AR early
Sui *et al*[54]	B	C (bx) AR CR	12 12 12	None		MALDI-TOF-MS	Recognition capability for AR 90%	None	Study included healthy individuals. Sample clean-up was performed with magnetic beads

Wang *et al*[55]	B	C (bx) ⩾ I a TCMR ATI	19 14 28 10	C (bx) ⩾ I a	10 10	SELDI-TOF-MS	C *vs* subclinical ⩾ I a Sensitivity 100% Specificity 90% (3-marker classifier) C *vs* TCMR Sensitivity 90% Specificity 90% (7-marker classifier) AR *vs* subclinical Sensitivity 100% Specificity 100% (4-marker classifier)	None	⩾ I a refers to subclinical rejections only. All (non-graded) TCMR cases were clinical rejections
Wittke *et al*[56]	U	C (bx) I a I b II a II b UTI	29 11 6 1 1 10	C (bx) I a I b UTI	10 6 3 7	CE-MS, LC-MS/MS	Sensitivity 67% Specificity 80% (17-marker classifier)	COL4A5	Transplant patients with urinary tract infection were included, with biopsy-confirmed absence of rejection. Of the rejection cases, 13 were subclinical and 6 clinical
Wu *et al*[57]	B	C (st) I b II a II b III	8 1 2 1 1	None		iTRAQ-2D LC-MS/MS	NR	Numerous molecules belonging to different pathways: *e.g.*, inflammatory response, complement, defence response, protein maturation and processing, humoral immune response	
Yang *et al*[58]	U	C (bx) TCMR aABMR ATI	36 30 25 10	C (bx) TCMR aABMR	14 10 10	SELDI-TOF-MS	C *vs* TCMR/ABMR Sensitivity 100% Specificity 78% (3-marker classifier) ABMR *vs* TCMR Sensitivity 80% Specificity 95% (5-marker classifier)	None	
Zhang *et al*[59]	U	C (bx) CR/(AR)	41 90	None		MALDI-TOF-MS MALDI-MS/MS	Different classifier combinations: Sensitivity 73%-88% Specificity 53%-62%	Up-regulated: B2M, SERPINA1. Down-regulated: PSAP	Study included healthy individuals and patients with native kidney disease (nephrotic syndrome). Saposin B was high in transplant patients with stable course over 280 d and low in patients with subsequent graft failure
Ziegler *et al*[60]	B	C I a I b	48 10 7	None		SELDI-TOF-MS MALDI-MS/MS	Sensitivity 100% Specificity 94% for 2 molecules (by ELISA)	Out of 22 candidates decreased: APOA1, SERPINA3	Two patients with TCMR had also signs of additional ABMR. The 2 markers for rejection were not informative in samples collected a few days before the rejection

Patient group definitions: C (bx): Control patients with biopsy-confirmed absence of rejection; C (st): Control patients without biopsy to exclude rejection; AR: Acute rejection without further histologic grading; CR: Chronic rejection without further histologic grading; TCMR: T cell-mediated without further histologic grading; ABMR: Antibody-mediated rejection with prefix "a" (acute) and "c" (chronic); BL: Borderline rejection (suspicious for rejection); IFTA: Interstitial fibrosis and tubular atrophy; BKV: BK virus nephropathy; ATI: Acute tubular injury; GL: *De novo* or recurrent glomerulopathy; UTI: Urinary tract infection with biopsy-confirmed absence of rejection; I a, I b: T cell-mediated tubulointerstitial (rejection specified as "mild" (a) and "severe" (b); II a, II b: T cell-mediated vascular rejection specified as "mild" (a) and "severe" (b); III: T cell-mediated vascular rejection with transmural arteritis; CMV: Cytomegalovirus; AUC: Area under the curve; CE: Capillary electrophoresis; iTRAQ: Isobaric Tags for Relative and Absolute Quantification; LC: Liquid chromatography; MALDI: Matrix-assisted laser desorption ionization; MS: Mass spectrometry; MS/MS: Tandem mass spectrometry; SELDI: Surface-enhanced laser desorption ionization; TOF: Time of flight; B/U: Examined matrix (blood: B, urine: U); *n*: Number of patients in each category; NR: Not reported.

the remaining studies, no clear Banff descriptors were provided leaving it open whether TCMR or ABMR was present and which severity grades and subtypes of rejection were observed. Apparently, almost all studies concentrated on acute rejection. Cases with chronic TCMR were included in the study of Jahnukainen *et al*[39], patients with chronic active ABMR were reported by Quintana *et al*[47,48]. One study examined chronic rejection without detailed scoring with regard to TCMR and ABMR[59].

In any proteomic marker discovery study the selection of appropriate comparators (controls) is an

important issue because definition of proteome patterns specific for the disease condition - in this case rejection - is deduced by comparison to samples without the disease condition. Thirteen studies used samples from clinically stable transplant patients without confirming absence of rejection by biopsy. This implies that these patients could have had subclinical rejection (*i.e.*, typical histological rejection findings without concomitant impaired allograft function). It has been shown that subclinical rejection produces proteomic patterns which are similar to clinical rejection and three studies have examined subclinical TCMR so far[42,43,56].

Another important point to consider is the delimitation of confounding conditions. For example, it is well known that acute tubular injury is present in a substantial proportion of patients with acute rejection[43]. If no measures are taken to differentiate the proteomic signature of rejection from acute tubular injury, the proteomic profile for rejection might lack specificity as tubular injury is a non-specific finding which is also related to drug-toxicity and ischemic/reperfusion injury. In fact, some of the studies included control samples with acute tubular injury[13,15,46,55,58]. Likewise, infection could be a confounder, as inflammatory pathways are activated in both, infection and rejection. To this end, BK virus nephropathy, urinary tract infection and CMV have been taken into account in some studies[13,39,43,51]. Another important confounder may be concurrent IFTA present in biopsies with ABMR as compared to biopsies showing IFTA without rejection which was addressed in the studies from Quintana *et al*[47,48].

Sample size numbers varied considerably in the studies, with two to ninety rejection samples for the trainings set, and with seven to twenty-eight for the validation of the discovered proteomic markers. There is certainly no simple rule of thumb to determine the necessary sample size. As discussed in the second chapter, rejection is a heterogeneous condition. Variability can probably be reduced by applying stringent histomorphological and clinical criteria to define the disease condition, nevertheless training sets for rejection should be large enough to cover the whole spectrum of the rejection type studied. In addition, controls/comparator groups without rejection should be of sufficient size to cover the whole spectrum of confounding conditions. Eventually, measures like area under the curve (AUC), sensitivity, specificity, negative and positive predictive values will give information about the performance of the defined marker set for rejection. Some of the studies reported exceptionally optimistic performance values, however, performance derived from cross-validation within the training set inherently carries overfitting of proteomics data and validation with external samples can correct for this limitation.

Various molecules have been discovered in the different studies and only a few were independently reported by different research groups, like fragments of collagens, β2-microglobulin, alpha-1-antichymotrypsin and uromodulin. The large variability in the reported markers for rejection is probably not primarily related to differences in the rejection characteristics of the examined patients. As outlined in chapter Ⅲ, "proteome analysis", the use of different MS methods will inevitably result in capturing diverse peptides and proteins. This issue is certainly relevant once efforts are undertaken to implement such tests into the clinical routine.

An important aspect is the biological significance of the identified molecules and the identification of the modulated processes which are involved. Combining all proteins from the studies mentioned above resulted in eighty-nine non-redundant molecules. These were subjected to a systematic analysis of biological contextualization using the pathway- and enzyme reaction-related Reactome information resource (Figure 3). Based on the known molecular associations a physical interaction graph was constructed (Figure 4). The analyses were performed without prior knowledge of disease areas or other information that might lead to bias. Reactome analysis using ClueGO (PMID: 19237447) showed processes related to platelet degranulation, keratan sulfate degradation, lipid digestion, mobilization and transport, antigen presentation and interferon gamma signalling to be directly associated with the input proteins. If the molecules involved worked in a synchronized manner some degree of physical association should be expected. To test this, the proteins were clustered using MiMI (PMID: 18812364), which connects molecules based on prior knowledge observed in other studies such as protein-protein interactions. This analysis allows expanding the molecular network to connect a maximum number of input proteins using gap-filling, or bridging, proteins. What is evident from the analysis (Figure 4) is that indeed a majority of molecules form a large network that is bound together by an additional 35 entries, which can serve as an entry point for further investigations. To this end, several of these gene ontology pathways have also been deduced from microarray analysis of transplant biopsies with rejection[61].

CONCLUSION

In summary, the studies published so far convincingly show that proteomics is capable of discovering molecular mechanisms of renal allograft rejection and of defining molecular markers which can aid to detect rejection early and reliably. To bring proteomics further forward into clinical application in kidney transplantation the limitations of previous studies should be used as challenges for future trials in the discovery and/or validation of rejection markers. Points to consider include but are not limited to:

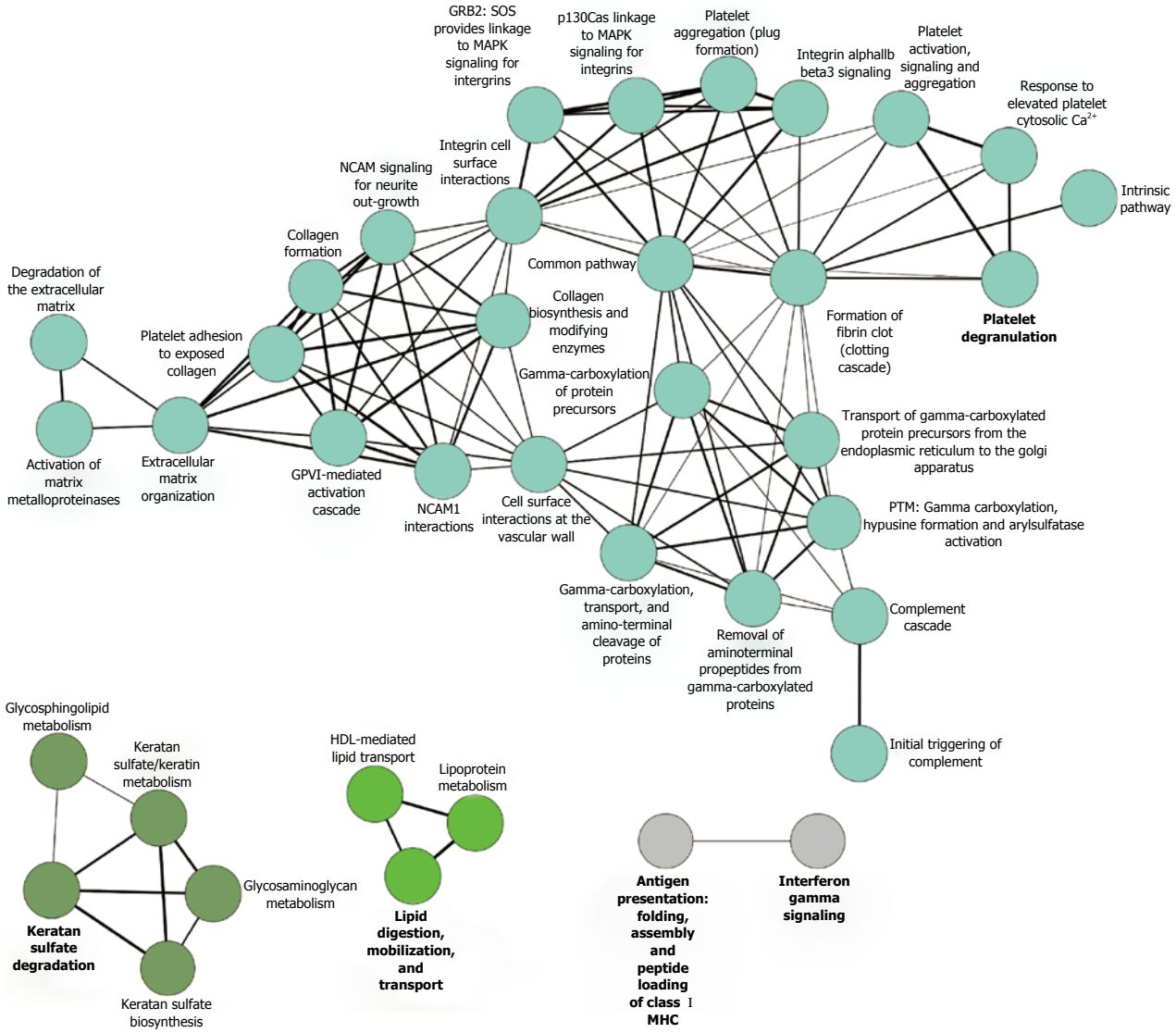

Figure 3 Reactome graph, showing the functional association of renal allograft rejection molecules. Literature-derived proteins associated with acute and chronic rejection (n = 89, concatenated from the proteomic studies listed in Table 1) were analyzed by functional Reactome group-clustering using CytoScape's ClueGO plug-in (CytoScape v2.8.3, ClueGO v1.5). Enriched Reactome-terms are represented as circles, and lines denote the relationship between these terms as functional groups. Line thickness and font-size are directly correlated with the statistical significance of terms and relationships (all with $P < 0.05$ after Bonferroni-adjustment for multiple testing correction). MAPK: Mitogen-activated protein kinase; GRB2: Growth factor receptor-bound protein 2; NCAM: Neural cell adhesion molecule.

Study design: (1) Sufficient number of patients with biopsy-confirmed absence of rejection, representing the whole spectrum of transplanted patients; (2) Rigorous histological and serological classification of patients with rejection, with a sufficient number of cases for each rejection type; (3) Inclusion of important and frequent confounding conditions which may be concurrently present in patients with and without rejection (either in the biopsy or clinically); and (4) Besides validation on selected samples as done so far in some studies, prospective in-place validation under everyday clinical conditions to determine the practical value of non-invasive tests for rejection.

Endpoints: (1) Emphasis on early markers which can detect incipient, subclinical stages of rejection (this will require longitudinal sample collections); (2) Development of markers which can indicate response to the rejection therapy (this will require longitudinal observation); and (3) Prospective, randomized studies with and without non-invasive monitoring to determine the costs and benefits.

Technical aspects: (1) Uniform sample collection protocols, sample preparation and analyses, especially if proteomic markers should find wide application; (2) Development of simplified test systems which can be applied outside highly specialized laboratories (provided the number of proteomic markers is not too high); (3) Reliable measures for the test system (AUC, sensitivity, specificity, negative and positive predictive values, thresholds of the test), all derived from independent validation studies and measures for reproducibility/variability; and (4) Identification of confounders that reduce the sensitivity or specificity of the proteome markers.

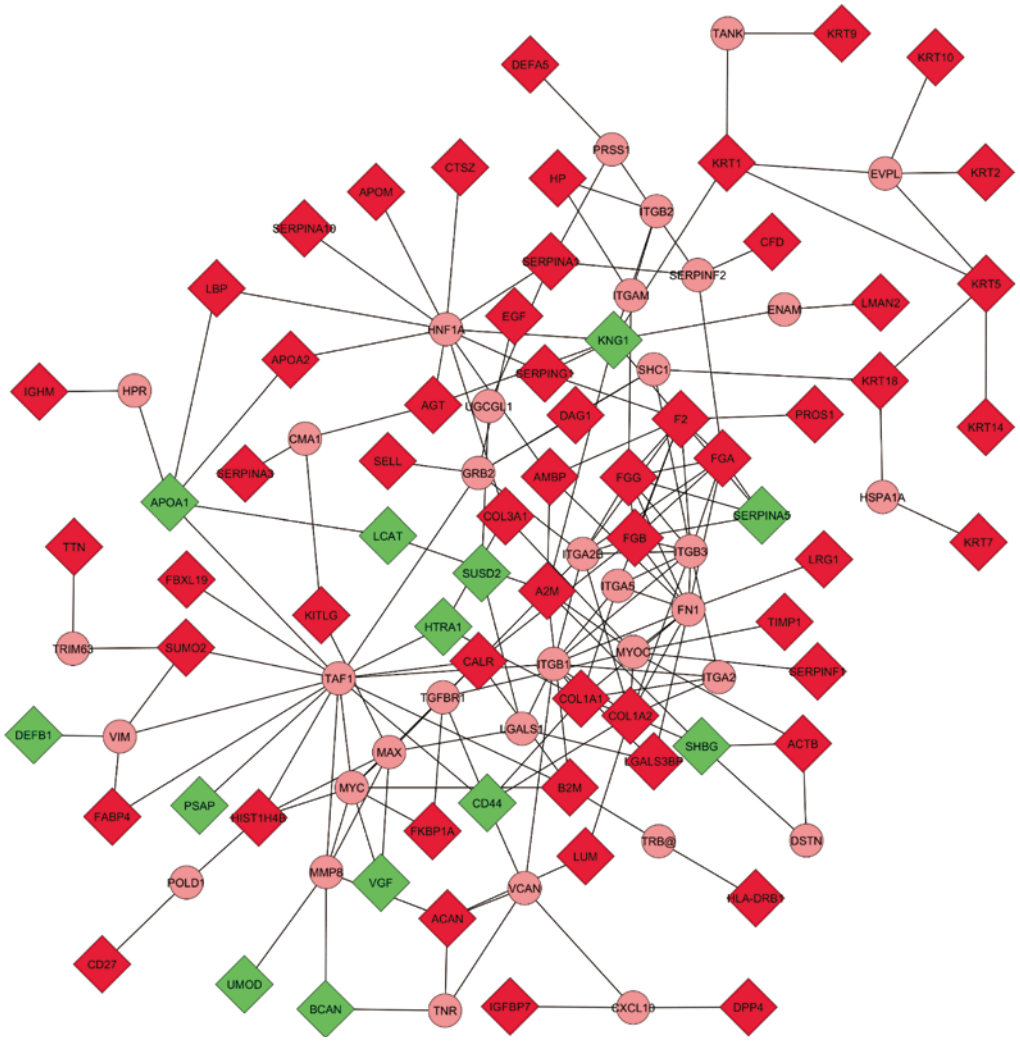

Figure 4 Expanded molecular interaction model. Physical interaction representation of molecules involved in renal allograft rejection. The concatenated list of literature-derived proteins associated with acute and chronic rejection was subjected to interactome-mapping using CytoScape's Michigan Molecular Interactor (MiMI) plug-in (CytoScape v2.8.2, MiMI v3.1). Known protein-protein interactions with up to two additional bridging molecules to maximize the interconnectivity were used to generate the map shown, which contains 68 of the 89 differentially expressed molecules and 35 additional bridging proteins. Input molecules are depicted as rectangles, and bridging molecules as circles. Each line between proteins represents a direct known association. Included literature-derived proteins associated with acute and chronic renal allograft rejection in the network (Rectangles; Green: Down-regulated; Red: Up-regulated; *n* = 68); Included additional bridging proteins for maximum interconnectivity (circles; *n* = 35); Excluded literature-derived proteins associated with acute and chronic renal allograft rejection not connected to the network (not shown; *n* = 21). A2M: Alpha-2-macroglobulin; ACAN: Aggrecan core protein; ACTB: Actin, cytoplasmic 1; AGT: Angiotensinogen; AMBP: Alpha-1-microglobulin; APOA1: Apolipoprotein A1; APOA2: Apolipoprotein A-2; APOM: Apolipoprotein M; B2M: Beta-2-microglobulin; BCAN: Brevican core protein; CALR: Calreticulin-3; CD27: CD27 antigen; CFD: Complement factor D; COL1A1: Collagen alpha-1(Ⅰ) chain; COL1A2: Collagen alpha-2(Ⅰ) chain; COL3A1: Collagen alpha-1(Ⅲ) chain; CTSZ: Cathepsin Z; DAG1: Dystroglycan; DEFA5: Defensin-5; DEFB1: β-defensin 1; DPP4: Dipeptidyl peptidase 4; EGF: Pro-epidermal growth factor; F2: Prothrombin; FABP4: Fatty acid-binding protein, adipocyte; FBXL19: F-box/LRR-repeat protein 19; FGA: Fibrinogen alpha chain; FGB: Fibrinogen beta chain; FGG: Fibrinogen gamma chain; FKBP1A: Peptidyl-prolyl cis-trans isomerase FKBP1A; HIST1H4B: Histone H4; HLA-DRB1: HLA-DRB1 protein; HP: Haptoglobin; HTRA1: Serine protease HTRA1; IGFBP7: Insulin-like growth factor-binding protein 7; IGHM: Ig mu chain C region; KITLG: Kit ligand; KNG1: Kininogen-1; KRT: Keratin, type Ⅱ cytoskeletal; KRT9: Keratin, type I cytoskeletal 9; LBP: LPS-binding protein; LCAT: Phosphatidylcholine-sterol acyltransferase; LGALS3BP: Galectin-3-binding protein; LMAN2: Vesicular integral-membrane protein VIP36; LRG1: Leucine-rich alpha-2-glycoprotein; LUM: Lumican; PROS1: Vitamin K-dependent protein S; PSAP: Saposin B; SELL: L-selectin; SERPINA1: Alpha-1-antitrypsin; SERPINA10: Protein Z-dependent protease; SERPINA3: Alpha-1-anti-chymotrypsin; SERPINA5: Serine protease inhibitor; SERPINF1: Pigment epithelium-derived factor; SERPING1: Plasma protease C1 inhibitor; SHBG: Sex hormone-binding globulin; SUMO2: Small ubiquitin-related modifier 2; SUSD2: Sushi domain-containing protein 2; TIMP1: Metalloproteinase inhibitor 1; TTN: Titin; UMOD: Uromodulin; VGF: Neurosecretory protein VGF; CMA1: Chymase; CXCL10: C-X-C motif chemokine 10; DSTN: Destrin; ENAM: Enamelin; EVPL: Envoplakin; FN1: Fibronectin; GRB2: Growth factor receptor-bound protein 2; HNF1A: Hepatocyte nuclear factor 1-alpha; HPR: Haptoglobin-related protein; HSPA1A: Heat shock 70 kDa protein 1A; ITGA2: Integrin alpha-2; ITGA2B: Integrin alpha-Ⅱb; ITGA5: Integrin alpha-5; ITGAM: Integrin alpha-M; ITGB1: Integrin beta-1; ITGB2: Integrin beta-2; ITGB3: Integrin beta-3; LGALS1: Galectin-1; MAX: Protein max; MMP8: Neutrophil collagenase; MYC: Myc proto-oncogene protein; MYOC: Myocilin; POLD1: DNA polymerase delta catalytic subunit; PRSS1: Trypsin-1; SERPINF2: Alpha-2-antiplasmin; SHC1: SHC-transforming protein 1; TAF1: Transcription initiation factor TFIID subunit 1; TANK: TRAF family member-associated NF-kappa-B activator; TGFBR1: TGF-beta receptor type-1; TNR: Tenascin-R; TRB@: T-cell receptor beta; TRIM63: E3 ubiquitin-protein ligase TRIM63; UGCGL1: UDP-glucose:glycoprotein glucosyltransferase 1; VCAN: Versican core protein; VIM: Vimentin; AFM: Afamin; CD5L: CD5 antigen-like; CLCA1: Calcium-activated chloride channel regulator 1; CLEC14A: C-type lectin domain family 14 member A; DPEP1: Dipeptidase; FAM151A: Protein FAM151A; FAM3C: Protein FAM3C; GGT6: Gamma-glutamyltransferase 6; GLB1: Beta-galactosidase; HAVCR2: Hepatitis A virus cellular receptor 2; KIAA0753: Uncharacterized protein KIAA0753; LGALS9B: Galectin-9B; MBL: Mannose-binding lectin; MMP-7: Matrilysin; MRC2: C-type mannose receptor 2; PGA4: Pepsin A-4; PI16: Peptidase inhibitor 16; RTN4RL2: Reticulon-4 receptor-like 2; SERPINA2P: Putative alpha-1-antitrypsin-related protein; SHISA5: Protein shisa-5; VASN: Vasorin.

Table 2 Ongoing proteomic studies on rejection in renal transplant patients

Study identifier and title	Aim	Institution/PI	Single/ multi-centre	Patients	Study start	Estimated primary completion	Status of the study
NCT01515605 Molecular biological and molecular genetic monitoring of therapy after kidney transplantation	Analysis of GATA3, GATA4, GAPDH, TRPC3, TRPC6, granzyme B, perforin, FOXP3, ISG15, Mx1, MMP-3, MMP-9 in blood cells, proteomic analysis of urine, tissue analysis in a longitudinal fashion. Correlation of these parameters to the outcome	Odense University Hospital, Denmark	NR	1000	January 2011	March 2014	Unknown
NCT01315067 Non-invasive diagnosis of acute rejection in renal transplant patients using mass spectrometry of urine samples - a multicentre diagnostic phase Ⅲ trial[62]	Phase Ⅲ in-place validation of a pre-defined, published urinary peptide panel for acute TCMR against the current standard allograft biopsy[43]	Hannover Medical School, Germany	Multi	600	October 2011	December 2015	Active, not recruiting
NCT01531257 Proteogenomic monitoring and assessment of kidney transplant recipients	Validation of a set of candidate molecules by urine proteomics, gene expression analysis of blood cells and graft biopsies in a longitudinal fashion with respect to AR and IFTA	Northwestern University, Chicago, Illinois, United States	Single	250	April 2010	April 2016	Recruiting
NCT01289717 Discovery and validation of proteogenomic biomarker panels in a prospective serial blood and urine monitoring study of kidney transplant recipients - transplant proteogenomics	Discovery and validation of candidate molecules by urine proteomics, gene expression analysis of blood cells and allograft biopsies in a longitudinal fashion with respect to AR and IFTA	National Institute of Allergy and Infectious Diseases; Northwestern University, Chicago, Illinois, United States	Multi	307	March 2011	June 2016	Active, not recruiting
NCT02463253 Correlation of molecular biomarkers with biopsy findings and outcomes in renal transplant recipients	Analysis of proteogenomic and proteomic biomarkers in relation to the biopsy diagnosis of acute rejection in a longitudinal fashion	University of California, Sacramento, California, United States	Single	50	April 2015	December 2016	Recruiting

All studies are prospective, observational cohort studies in adult patients. Preliminary reports have not been published yet. Except study NCT 01315067, all studies collect samples in a longitudinal fashion and examine additional markers obtained by genomic analysis of blood cells. PI: Principal investigator site; AR: Acute rejection; IFTA: Interstitial fibrosis and tubular atrophy; NR: Not reported.

Some of these goals may be not too far away on the horizon. Currently, a few ongoing studies might address some of the discussed issues (Table 2). All studies are prospective, observational cohort studies and all except one collect samples in a longitudinal fashion. Results are expected in 2015 and 2016. These studies will hopefully clarify which role proteomic markers for rejection might have in the future care of kidney transplant patients.

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