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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 tips: 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 summarises 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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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.

I. 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]. Particularly ABMR has gained an increasing recognition in the past decade. ABMR is a complicated form of rejection as it may become chronic and is difficult to treat. 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, 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, recognition of rejection is important not only in the early phase after transplantation but also in the entire long-term course on a continuous basis. Early diagnosis of any form of rejection is a pre-requisite 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 an increase in 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 help to 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. 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].

II. RATIONALE FOR A MULTI-MARKER APPROACH TO DIAGNOSE REJECTION

Rejection is a heterogeneous immunological process and therefore it is unlikely that a single marker or small number of markers can reflect all facets of rejection reliably. T cell-mediated rejection includes recognition and presentation of donor antigens by antigen-presenting cells to T cells, which become activated and then undergo

proliferation. Activated T cells can induce morphologically diverse and distinct patterns of immunological injury which are specified by the Banff classification criteria[7]. Primarily, the different entities of acute TCMR are based on the localization of the immunological attack such as the interstitium and tubuli (Banff grades borderline rejection, Ia and Ib) and the vasculature (Banff grades IIa/b and III). In most cases of vascular rejection, some degree of tubulointerstitial inflammation is present although pure cases of vascular rejection ('v-only') have been reported[8]. In ABMR, antigen-presenting cells activate T cells which in turn induce B cells to undergo plasma cell proliferation resulting in the production of donor-specific antibodies (HLA- and non-HLA). These antibodies bind to the peritubular and glomerular capillaries and to the endothelium of larger arteries. The antibody-mediated injury to these structures is mediated by local activation of complement factors however, non-complement-fixing antibodies may also play a role in some cases[9]. Patients may present with isolated findings of glomerulitis, peritubular capillaritis or intimal or transmural arteritis or a combination of these features[7].

Heterogeneity of rejection is not merely confined to the TCMR-/ABMR pathways and the localization of injury in the allograft. As a reflection of the severity of injury, the different forms of rejection may be subclinical i.e. without a concomitant decline in allograft function or clinical with accompanying graft dysfunction[10]. Morphologically, different severity grades are semi-quantitatively scored by the Banff classification[7]. As outlined above, rejection is a disease *process*. 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.

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]. 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 ß2-microglobulin or fragments of it have been reported as potential indicators of rejection[13,14]. Further analysis however showed that increased urinary ß2-microglobuline-derived peptides are similarly present in pure cases of tubular atrophy and interstitial fibrosis without any evidence of rejection[15-17].

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.

III. PROTEOME ANALYSIS

The proteome is the sum of all proteins and peptides present in a given individual at a given time point. Most of the contained peptides represent degradation products of proteins by proteolytic processes. Compared to the transcriptome or the metabolome, the proteome is the most functional compartment because it is subject to constant and sometimes drastic changes in response to external stimuli or alterations of the homeostasis[20].

For the most part, pathophysiology research and clinical analyses examine singular aspects of the proteome. Examples for this are the detection of specific proteins or peptides by ELISA or Western blotting techniques. This is a typical hypothesis-driven approach, which requires pre-existing knowledge on the targeted analyte for a certain disease condition. In contrast, proteomics is primarily a hypothesis-free, untargeted approach that attempts to explore the proteome in its entirety. By comparing the proteome of two or more distinct conditions (e.g. diseased and non-diseased) the differentially expressed proteins and peptides become evident.

Molecules identified in this way may be the starting point for diagnostic tests or may help to answer research questions in pathophysiology.

Technically, these "shotgun" proteomic technologies rely on the physicochemical properties of the proteomic compounds as compared to ELISA and Western blotting, which are based on immunological properties of the analyte and rely on the use of specific antibodies for their detection.

Rationale for proteomics

The rationale for biomarker research by proteomics is based on the hypothesis that at least one of the following conditions is true:

- Different proteins (in the sense of gene products) are found in case and control groups that represent group-specific features and give rise to case- and control-specific proteomic signatures.
- Proteins have undergone different modifications (protease cleavage, post-translational modifications) in case and control groups. In this case, the detected proteins or peptides are surrogate markers for disease-associated activity changes of enzymes or proteases.
- The proteins are detectable in both, case and control patients but are more prevalent in one of the groups. These quantitative differences can arise from altered production, degradation or release from cells by the disease process.

Sample matrix

In biomarker research, easily accessible matrices like blood or urine are preferred because procurement of tissue is more invasive. Proteomics on tissue or isolated cells is technically feasible and has been performed in research studies but is less common in biomarker studies. Blood has a high dynamic range of protein concentrations, necessitating depletion of the most abundant proteins to enhance the sensitivity of detection. 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 proteome from other compounds, followed by the separation, ionization and mass detection of the protein and peptide compounds.

Sample preparation. Before mass spectrometry analysis a sample usually needs processing to remove insoluble materials like cell debris and interfering salt and lipids. These pre-analytical steps may include centrifugation, ultrafiltration and immunodepletion of abundant proteins that increase background noise and may impede detection of lesser abundant proteins and peptides. 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 subsequent mass detection[23].

Protein ionization. There are many different mass spectrometry methods but they all share common principles. Proteins and peptides are transformed 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 (ESI), matrix-assisted laser desorption/ionization (MALDI) and surface enhanced laser desorption-ionization (SELDI) are the main ionization techniques used in clinical proteomics. In MALDI, samples are spotted onto a plate, mixed with matrix, dried and analyzed under high vacuum. MALDI is often performed without prior separation of the proteins. In SELDI, the principle of MALDI is combined with selective surface binding to functionalized matrices. Different chip surfaces for hydrophobic, ionic or affinity binding of the proteins are commercially available. Before analysis, the sample is spotted on the functionalized chip matrix, and all non-adsorbed molecules are washed away. In ESI, the separated LC or CE effluent is ionized on-line, meaning that the effluent coming from the separation device is directly ionized in a high-voltage field, which results in desolvatization.

MALDI results in single charged ions and easily interpretable spectra. In contrast, ESI generally multiplies charged ions resulting in more complex spectra that are however, richer in information. This is because ESI has a higher ionization efficiency and consequently, a better linear response between the detected ions and the proteins/peptides in the original sample[24].

Protein mass detection. The next element of the mass spectrometry chain is the detector. Many different concepts exist, mostly in respect to how an ionic signal is amplified. "Time of flight" (TOF), 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 protein/peptide abundance in a sample. Advanced methods have been developed to compare the protein/peptide abundance between different samples.

'Isobaric Tags for Relative and Absolute Quantification' (iTRAQ) is a label-based technique. Each sample is labelled with a specific isobaric tag and subsequently, all

samples are analysed in a single MS run. Upon collision in MS/MS, corresponding proteins and peptides from different samples will break similarly but will release different reporter ions based on the specific isobaric tag. By comparing the sample-specific tags the abundance of proteins/peptides in different samples can be estimated in a relative fashion. Nevertheless, this approach typically requires trypsin digestion of the samples and is cost intensive[25].

Multiple reaction monitoring (MRM) is an isotope label-based technique[26]. MRM requires a triple quadrupole mass spectrometer that is capable of three subsequent collision-induced fragmentation steps. It selectively determines the fragment spectrum for a known peptide of interest and precisely measures the abundance of each fragment in a subsequent step. For quantitative analyses, a known quantity of the same but isotope-labelled peptide is added to the sample before the MS run. The labelled peptide has the same amino acid sequence as the unlabelled native peptide, resulting in the same fragmentation ions but in a different mass due to the isotope label. This enables absolute quantification of the peptide of interest, by comparing the signal intensities of corresponding labelled and unlabelled ions. MRM has a low detection limit and is therefore useful for sensitive, absolute quantification of known protein/peptide markers.

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 define proteomic markers for disease conditions. 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. The linear sequence of the peptide is then deduced by the mass differences between the fragments that exactly correspond to the masses of the sequentially removed amino acids[27].

Construction of multi-marker diagnostic models.

In many cases, average levels of single proteins or peptides are significantly different between case and control groups but large overlap of values is observed when individual samples are compared with each other[28]. To construct classifiers with as little overlap as possible between case and control groups, biomarkers are often combined into multi-marker sets[29]. 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 specificity and on the complexity of the disease of interest.

The algorithms to integrate multiple discriminative proteins into a biomarker model are difficult to understand for laypersons. Basically, they 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. In our experience, the most frequently used algorithms are "Support Vector Machine" (SVM), adaptive boosting, random forest and neural networks.

IV. 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 1). 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[16,17,30–35]. 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 1-4.

The remaining 29 studies[15,34,36–62] are listed in Table 1-4. Five studies reported a prospective study design[38,42,46,47,63], 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. Eight studies described longitudinal aspects with regard to sample collection[40], profiling of sequential samples or comparison of proteome patterns before and after rejection[36,38,42,46,51,55,62] and to the assessment of graft survival[61].

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[52,55,62,64].

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*.[41] mRNA expression in biopsies was examined in parallel to the urinary proteome. O'Riordan *et al*[46] stained biopsies to confirm the identified urinary proteomic marker ß-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[36,47–49,60,64,65]; in one study[65] a few patients were reported to have mixed rejection (TCMR+ABMR). In 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*[40], patients with chronic active ABMR were reported by Quintana *et al*[48,49]. One study examined chronic rejection without detailed scoring with regard to TCMR and ABMR[61].

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[43,44,58].

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[44]. If no measures are taken to differentiate the proteomic signature of rejection from acute tubular injury e.g. by including appropriate controls with 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 samples with acute tubular injury[47,51,57,60]. 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[13,40,41,58,64]. 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*[48,49].

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 marker sets. 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, ß2-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.

V. Conclusion and Perspectives

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:

Study design

- sufficient number of patients with biopsy-confirmed absence of rejection, representing the whole spectrum of transplanted patients
- rigorous histological and serological classification of patients with rejection, with a sufficient number of cases for each rejection type
- inclusion of important and frequent confounding conditions which may be concurrently present in patients with and without rejection (either in the biopsy or clinically)
- 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

- emphasis on early markers which can detect incipient, subclinical stages of rejection (this will require longitudinal sample collections)
- development of markers which can indicate response to the rejection therapy (this will require longitudinal observation)
- prospective, randomized studies with and without non-invasive monitoring to determine the costs and benefits

Technical aspects

- uniform sample collection protocols, sample preparation and analyses, especially if proteomic markers should find wide application
- development of simplified test systems which can be applied outside highly specialized laboratories (provided the number of proteomic markers is not too high)
- 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
- identification of confounders that reduce the sensitivity or specificity of the proteome markers

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 5). 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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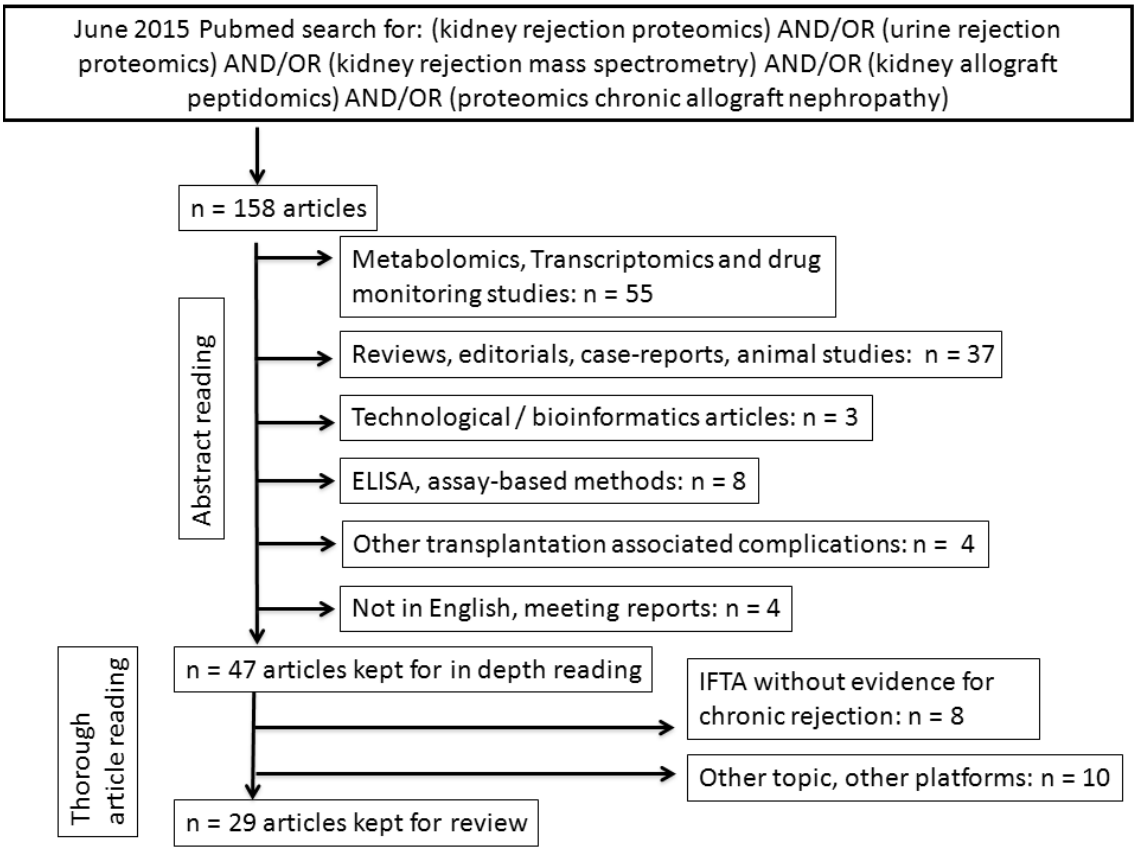
Figure 1

Search strategy for proteomic studies in the field of renal allograft rejection.

(IFTA; interstitial fibrosis and tubular atrophy).



Tables 1-4. Proteomic studies on renal allograft rejection

B/U; examined matrix (blood: B, urine: U), n; number of patients in each category, n.r.; not reported.

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); Ia, Ib; T cell-mediated tubulointerstitial (rejection specified as 'mild' (a) and 'severe' (b). IIa, IIb; T cell-mediated vascular rejection specified as 'mild' (a) and 'severe' (b), III; T cell-mediated vascular rejection with transmural arteritis, 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.

CMV; cytomegalovirus, AUC; area under the curve, CE; capillary electrophoresis, iTRAQ; Isobaric Tags for Relative and Absolute Quantification', iTRAQ, 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.

Abbreviated gene names of proteins and peptides are explained in the Supplemental 1.

Table 1

Author	B/U	Training set	n	Validation set	n	Proteomic method	Performance	Identified molecules	Remarks
Akkina *et al* [36]	U	C (bx) BL IIa aABMR	13 1 1 1	none		iTRAQ-MALDI-MS/MS	n.r.	none	Study included healthy individuals. Study concentrates on longitudinal stability of peptides in rejecting and non-rejecting patients.
Clarke *et al* [37]	U	C (st) AR	15 15	none		SELDI-TOF-MS	Accuracy 91% Sensitivity 83% Specificity 100% (2-marker classifier)	none	
Freue *et al* [38]	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* [39]	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* [40]	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* [41]	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* [42]	U	C (st) BL Ia IIa	6 1 4 1	none		2D LC-MS/MS	n.r.	Up-regulated: IGFBP7, VASN, EGF, LGALS3BP	Study collected sequential urines from the beginning after Tx. Analysed samples for rejection patterns were taken 7-11 days before biopsy.
Mao *et al* [43]	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* [44]	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	The training set contained 10 clinical and 18 subclinical rejection cases. Rejections in the validation set were all subclinical. Confounder like ATI in biopsies, urinary tract infection and CMV infection were considered
O'Riordan *et al* [45]	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

Table 2

Author	B/U	Training set	n	Validation set	n	Proteomic method	Performance	Identified molecules	Remarks
O'Riordan *et al* [46]	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* [47]	U	C (bx) Ia Ib IIa ATI	2 4 1 2 7	none		LC-MS/MS	n.r.	Numerous molecules	
Quintana *et al* [48]	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* [49]	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: KNG	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* [50]	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* [51]	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	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	n.r.	Cleaved B2M	Study included healthy individuals. Study concentrated on cleavage mechanisms for ß2-microglobulin.
Sigdel *et al* [14]	U	C (bx) AR	10 10	none	LC-MALDI-MS/MS	n.r.	List of 73 candiates, incl. fragments of collagens, UMOD, B2M, PTGDS	Study included healthy individuals

Table 3

Author	B/U	Training set	n	Validation set	n	Proteomic method	Performance	Identified molecules	Remarks
Sigdel *et al* [52]	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* [64]	U	C (bx) Ia-IIb aABMR IFTA BKV	30 30 2 30 18	none		iTRAC-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* [54]	U	C (bx) ≥Ia	20 20	none		iTRAC-LC-MS/MS	n.r.	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* [55]	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* [56]	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* [57]	B	C (bx) ≥Ia* TCMR ATI	19 14 28 10	C (bx) ≥Ia*	10 10	SELDI-TOF-MS	C vs. subclinical ≥Ia 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	≥Ia* refers to subclinical rejections. All (non-graded) TCMR cases were clinical rejections.
Wittke *et al* [58]	U	C (bx) Ia Ib IIa IIb UTI	29 11 6 1 1 10	C (bx) Ia Ib UTI	10 6 3 7	CE-MS, LC-MS/MS	Sensitivity 67% Specificity 80% (17-marker classifier)	coll4A5	Transplant patients with urinary tract infection were included, with biopsy-confirmed absence of rejection. Of the rejection cases, 13 were subclinical and 6 clinical.

Table 4

Author	B/U	Training set	n	Validation set	n	Proteomic method	Performance	Identified molecules	Remarks
Wu *et al* [63]	B	C (st) Ib IIa IIb III	8 1 2 1 1	none		iTRAQ-2D LC-MS/MS	n.r.	Numerous molecules belonging to different pathways: e.g. inflammatory response, complement, defense response, protein maturation and processing, humoral immune response	
Yang *et al* [60]	U	C (bx) TCMR aABMR ATI	36 30 25 10	C (bx) TCMR aABMR	14 10 10	SELDI-TOF-MS	C vs. TCMR/aABMR Sensitivity 100% Specificity 78% (3-marker classifier) ABMR vs. TCMR Sensitivity 80% Specificity 95% (5-marker classifier)	none	
Zhang *et al* [61]	U	C (bx) CR/(AR)	41 90	none		MALDI-TOF-MS, MALDI-MS/MS	Different combinations of 1-6 classifiers: 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 healthy persons and transplant patients with stable course over 280 d and low in patients with subsequent graft failure.

Ziegler *et al* [62]	B	C Ia Ib	48 10 7	none	SELDI-TOF-MS, MALDI-MS/MS	Sensitivity 100% Specificity 94% for 2 molecules (by ELISA)	Out of 22 candiates 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.

Table 5. Ongoing proteomic studies on rejection in renal transplant patients. 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.

Study Identifier & Title	Aim	Institution/PI	single/ multi-centre	patients	study start	estimated primary completion	status of the study
NCT01515605 Molecular Biological and Moleculargenetic 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	n.r.	1000	Jan 2011	Mar 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	Phase III in-place validation of a pre-defined, published urinary peptide panel for acute TCMR against the current standard allograft biopsy [44]	Hannover Medical School, Germany	multi	600	Oct 2011	Dec 2015	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 & IFTA	Northwestern University, Chicago, Illinois, US	single	250	Apr 2010	Apr 2016	recruiting
NCT01289717 Discovery and Validation of Proteogenomic Biomarker Panels in a Prospective Serial Blood & 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, US	multi	307	Mar 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 US	single	50	April 2015	Dec 2016	recruiting

PI; principal investigator site, AR; acute rejection, IFTA; interstitial fibrosis and tubular atrophy, n.r.; not reported. Abbreviated gene names of proteins are explained in the Supplemental 1.