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Biomarkers in Transplantation- Proteomics and Metabolomics

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Abstract

Modern multi-analyte “omics” technologies allow for the identification of molecular signatures that confer significantly more information than measurement of a single parameter as typically used in current medical diagnostics. Proteomics and metabolomics bioanalytical assays capture a large set of proteins and metabolites in body fluids, cells or tissues and, complementing genomics, assess the phenome. Proteomics and metabolomics contribute to the development of novel predictive clinical biomarkers in transplantation in two ways: They can be used to generate a diagnostic fingerprint or they can be used to discover individual proteins and metabolites of diagnostic potential. Much fewer metabolomics than proteomics biomarker studies in transplant patients have been reported and, in contrast to proteomics discovery studies, new lead metabolite markers have yet to emerge.

Most clinical proteomics studies have been discovery studies. Several of these studies have assessed diagnostic sensitivity and specificity. Nevertheless, none of these newly discovered protein biomarkers has yet been implemented in clinical decision making in transplantation. The currently most advanced markers discovered in proteomics studies in transplant patients are the chemokines CXCL-9 and CXCL-10, which have successfully been validated in larger multi-center trials in kidney transplant patients. These chemokines can be measured using standard immunoassay platforms, which should facilitate clinical implementation. Based on the published evidence, it is reasonable to expect that these chemokine markers can help guiding and individualizing immunosuppressive regimens, may be able to predict acute and chronic T cell and anti-body mediated rejection and may be useful tools for risk stratification of kidney transplant patients.

Key Terms and Phrases

Biomarkers; transplantation; metabolomics; proteomics; validation; clinical implementation

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Conflicts of Interest

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Introduction

Today, clinical chemistry and biochemistry diagnostics in transplantation relies on a limited set of biomarkers, often only one parameter that is closely correlated with one functional aspect of the organ in question or with a specific disease process. However, there is nor will there ever be a single molecular marker that captures the function of a transplant organ in all its complexity. Modern bioanalytical “omics” technologies allow for the identification of molecular signatures that confer significantly more information than the measurement of a single parameter, just as a bar code contains more information than a single number. Proteomics and metabolomics complement genomics and assess the phenome¹.

Proteomics and metabolomics are multi-analyte technologies that in an ideal case can assess the complete set of proteins (proteome) or metabolites (molecules <1500 Da, metabolome). For detailed reviews of current metabolomics and proteomics technologies please see references^{1–11}. Proteomics and metabolomics strategies have extensively been used to develop new sensitive and specific biomarkers in transplantation¹². Among such biomarkers that can be used to monitor pathogenic processes and/or pharmacological responses to a therapeutic intervention, predictive and prognostic biomarkers are of interest. Whereas predictive biomarkers change in response to treatment, and predict a clinically relevant event or process, and may be used to stratify subsets of patients based on risk and likelihood to respond to treatment, prognostic biomarkers predict clinical outcome regardless of treatment¹².

Proteomics and metabolomics can contribute to the development of novel predictive clinical biomarkers in transplantation in two ways: They can be used in a non-targeted screening approach to generate a diagnostic fingerprint (non-targeted) or they can be used as discovery tools to identify individual proteins and metabolites of diagnostic potential for which then targeted and specific assays are developed and validated. It has to be noted that other than genome arrays, which capture the whole genome including important polymorphisms, current proteomics and metabolomics technologies capture only a part of the metabolome and proteome¹³. Thus results of proteomics and metabolomics analyses even of the same sample set may differ dependent on the analytical strategy chosen¹⁰. This may explain why discovery studies in the same patient population have yielded different proteomics or metabolomics markers. Another common challenge that especially non-targeted proteomics and metabolomics assays have in common are the differences in the abundance of proteins or metabolites in biological fluids that has been estimated to be more than 10 orders of magnitude^{11,14}.

Regulatory aspects

During biomarker discovery and development it has to be considered that before a biomarker can be implemented in clinical practice, in most cases, regulatory review may be required that includes a risk-benefit assessment based on the intended use of the molecular marker¹⁵. Regulatory agencies have published guidances that outline the biomarker development, review and approval process^{16–20}. Although such regulatory guidances were often written with genomics-based biomarkers in mind, they are also applicable for

proteomics and metabolomics markers. Based on such guidance, the biomarker development process can be divided in three stages: discovery, verification and qualification/clinical validation. These are followed by regulatory review and approval and finally clinical implementation. In the literature there is confusion regarding the term “validation”. While clinical validation is defined as a robust statistical evaluation as to whether or not a candidate biomarker fulfils the predictive requirements for use in a clinical setting¹², bioanalytical validation ensures that the analytical procedure(s) used to measure the biomarker meet(s) regulatory requirements.

Samples and study design

One of the most important factors that determines success or failure of a proteomics/metabolomics-based biomarker development and clinical implementation is the sample^{10,11,21}. However, in the vast majority of the relevant published literature, sample collection, handling and storage procedures are neither appropriately described nor validated¹². For example, for urine samples it makes a difference if spot urine is used or urine is collected over a certain time period. Again, it will make a difference if first void and midstream spot urine is collected. Non-targeted proteomics and metabolomics assays are able to detect many hundreds of proteins and metabolites with a wide range of different physico-chemical properties and stabilities so that variability in sample handling and sample quality can introduce artifacts and bias^{10,11,21}. This is even more important taking into consideration that proteomics and metabolomics biomarker discovery studies are often based only on a limited number of samples, typically between $n=10-50$ /study group, so that outliers may have a significant effect. Another problem with most proteomics and metabolomics discovery studies is that often single samples from a variety of patients are included in such studies. A potential problem with such an approach is that a disease or pharmacodynamics/toxicodynamic drug effect often changes its molecular signature as said processes progress over time. Also, transplant patients are a highly complex patient population with many confounding factors such as other diseases and multiple medications. However, very few published proteomics/metabolomics discovery studies have made an attempt to control or correct for such potentially interfering factors. It is reasonable to expect that the collection of longitudinal sample sets during controlled prospective studies will yield more valid information than single samples that have more or less randomly been collected from certain patient populations.

Predictive biomarkers in transplantation based on non-targeted proteomics

Most proteomics studies in transplant patients have focused on kidney transplant patients and the urine proteome. Such studies have typically used mass spectrometry-based assays. Many of the earlier studies used surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF)¹¹. One of the problems with this technology is that the time-of-flight mass spectrometer does often not have sufficient resolution to allow for identification of protein and peptide marker candidates¹¹. Such studies have typically characterized marker proteins based only on their mass/charge. A more recent example for such a study is reference²². However, verification of marker protein identity is desirable. An example for a proteomics study that identified specific proteins, in this case a urinary protein

biomarker pattern including β_2 -microglobulin, neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule 1 (KIM-1) and clusterin to detect and diagnose chronic allograft nephropathy, using high-resolution mass spectrometry in combination with immunological assays is reference²³.

A non-targeted urine proteomics test based on two-dimensional capillary electrophoresis/mass spectrometry (CE-MS) is commercially offered for the clinical diagnosis of chronic kidney diseases²⁴. This technology has also successfully been used in clinical trials in transplant patients^{25,26}. The data from one of these studies indicated that urine samples from patients with subclinical acute T-cell-mediated tubule-interstitial rejection could be distinguished from non-rejection controls mainly based on altered collagen α (I) and α (III) chain fragments suggesting an involvement of matrix metalloproteinase-8 (MMP-8)²⁵. Nevertheless, this CE-MS proteomics assay has mainly been studied in chronic kidney disease patients and its value specifically for transplant patients still remains to be evaluated. Other proteomics approaches and signatures in the blood of cardiac^{27–29} and kidney transplant patients³⁰ have been reported. Based on the literature, the proteomics panel in kidney transplant patient to diagnose early acute rejection has been submitted as a Voluntary Exploratory Data Submission to the United States Food and Drug Administration and a regulatory clinical trial is in progress³¹.

Predictive biomarkers in transplantation based on targeted proteomics, proteins and peptides

Non-targeted proteomics fingerprints can only be of diagnostic value if the assay is at least somewhat quantitative. The more targeted a protein assay is, the more sensitive and quantitative it typically is³². Genomics and proteomics approaches have been used to identify protein biomarker candidates, which then have been further studied using targeted, quantitative protein assays. Numerous protein biomarker candidates have been studied in transplant patients. For comprehensive reviews and overview tables, please see references^{9,33–35}. While most of these protein markers have not yet progressed beyond the discovery/early proof-of-concept stage, several promising candidates have emerged¹². Among these the most advanced marker candidates are the CXC-receptor 3 chemokines CXCL-9 and CXCL-10 in the urine of kidney transplant patients. CXCL-9 and CXCL-10 are secreted by leukocytes in the kidney graft and are inflammation markers³⁶. As such they have been shown to be early markers of kidney graft dysfunction including, but not limited to, kidney graft inflammation associated with subclinical tubulitis³⁷, subclinical rejection³⁸ and subclinical BK virus infections³⁹. The sensitivity and specificity of CXCL-9 and CXCL-10 exceeded that of creatinine concentrations in serum³⁷. In addition, CXCL-9 was clinically qualified/validated in a multicenter observational study in 280 adult and pediatric kidney transplant patients⁴⁰. The results showed that low urinary CXCL-9 protein concentrations collected 6 months after transplantation from stable allograft recipients classified individuals least likely to develop acute rejection or a reduction in estimated glomerular filtration rate between 6 and 24 months. In a prospective study of non-sensitized stable living donor kidney transplant patients randomized to stay on or to be withdrawn from tacrolimus, high urinary CXCL-9 levels predated clinical detection of acute rejection by a

median of 15 days⁴¹. It was also found that the combination of urinary CXCL-10 levels normalized to urine creatinine with donor-specific antibody monitoring significantly improved the non-invasive diagnosis of antibody-mediated rejection and may allow for the stratification of patients at high risk for graft loss⁴². Other protein markers that have shown promise in clinical validation trials are urinary CCL-2 (predictor of interstitial fibrosis and atrophy and kidney graft loss)⁴³, urinary β 2-microglobulin (chronic allograft nephropathy)⁴⁴ and serum aminoacylase-1 (long term outcome in patients with delayed kidney graft function)⁴⁵. In addition, urinary kidney dysfunction markers that have originally been discovered in patients with acute kidney injury such as urinary neutrophil gelatinase-associated lipocalin (NGAL), IL-18 and kidney injury molecule-1 (KIM-1) have been studied in kidney transplant patients, but none of these seem as advanced in their development as CXCL-9 and CXCL-10 in this specific patient population³⁵.

Among others, a discovery study identified a panel of cytokines, chemokines and immune-receptors (IL-6, IL-2R, CCL-2, CCL-5, CCL-8, CXCL-9 and CXCL-10) in serum and plasma as markers of acute liver transplant rejection⁴⁶. In addition, protein markers of native kidney function recovery after liver transplantation⁴⁷, chronic kidney dysfunction⁴⁸ and liver fibrosis markers after hepatitis C virus reoccurrence in liver transplant patients⁴⁹ have been studied. It was also found that after liver transplantation in patients with recurrent hepatitis plasma CXCL-10 could differentiate patients with slow from those with rapid progression of fibrosis⁵⁰. Moreover, in a biomarker discovery study in lung transplant patients, CXCL-8, CXCL-10, CCL-2, CCL-3, CCL-4 CCL-7, and CCL-3 in bronchial lavage fluid could differentiate between different phenotypes of chronic lung allograft dysfunction⁵¹. A few clinical targeted proteomics/protein biomarker studies in heart transplant patients have also been described (for a review, please see reference⁹). Nevertheless, based on the current literature, targeted proteomics/protein biomarkers identified in studies in liver, heart, and lung transplant patients have not yet progressed beyond the discovery/proof-of-concept stages.

Predictive biomarkers in transplantation based on metabolomics

Markedly less metabolomics than proteomics biomarker studies in transplant patients have been reported. The metabolome is a challenge as, other than the genome or proteome, it is a partially open system that is constantly in flux and can be affected by food, the environment and gut microorganisms (the microbiome)⁵². Bioanalytical metabolomics assays are challenging as the metabolome covers a wide range of compounds with very different physico-chemical properties, and wide coverage requires the combination of multiple assays^{13,53}. Thus, of 4229 confirmed and highly probable serum metabolites, 3247 were found to be lipids and phospholipids⁵³. Surprisingly, lipids and phospholipids have received relatively little attention in metabolomics studies in transplant patients yet, although several of these are bioactive and do not communicate with the environment, in contrast to their usually less disease and organ-specific water soluble counterparts. Metabolomics studies have assessed the biochemical effects of immunosuppressants and their combinations on the kidney, the assessment of donor organ quality, storage and ischemia/reperfusion injury as well as relevant therapeutic interventions, and kidney transplant function in patients. These studies have been reviewed in reference⁵⁴. More recent studies include the effect of

calcineurin inhibitors⁵⁵ and allo-immune response on urine metabolite profiles after kidney transplantation^{56,57} as well as the metabolic profiling of transplant liver biopsies⁵⁸. Moreover, metabolomics studies in chronic kidney diseases such as^{59–63} may also be of potential interest for monitoring kidney function in transplant patients.

Targeted approaches using validated, quantitative LC-MS/MS assays indicated that the transmethylation pathway intermediates S-adenosyl methionine and S-adenosyl homocysteine as well as the arachidonic acid bioactive lipid pathway metabolites (18-HEPE and 12-HETE) may be predictive markers of kidney transplant rejection^{64,645}.

Overall, none of these metabolomics studies has resulted in biomarkers that have progressed beyond the discovery stage.

Clinical biomarker validation

A major bottleneck in the development of promising proteomics/metabolomics biomarkers is progression into the clinical validation stage. Clinical validation of a biomarker corresponds to phase III of a drug development and is in most cases beyond the resources of a single research group. However, the availability of proteomics/metabolomics databases and sample banks from well-controlled prospective clinical trials can greatly facilitate and speed up the clinical validation process. A good example is the clinical validation of CLCX-9 using samples collected during the United States National Institutes of Health NIH Clinical Trials in Organ Transplantation-01 study³⁹.

Clinical implementation

Although, as aforementioned, several promising predictive proteomics/protein biomarkers have emerged and have successfully been qualified in larger transplant patient sample sets and clinical trials, none of those have successfully been implemented in clinical practice yet.

First recommendations for guidelines how to appropriately collect and handle proteomics samples have been developed⁶⁶. Nevertheless, these are mostly intended to guide the collection of research samples. Complex sampling procedures are more difficult to establish in clinical routine environment²¹. Diagnostic proteomics, metabolomics and targeted multi-analyte assays will mostly be laboratory-developed tests. However, most guidelines for bioanalytical assay validation have been written having drug compounds in mind. The validation and quality control of multi-analyte assays for the qualitative and quantitative analysis of endogenous compounds is challenging, especially in the case of non-targeted assays⁶⁷. First regulatory guidance addressing this issue, such as reference⁶⁸, are emerging. Biomarker assays used for medical decisions should be fully validated. Acceptance criteria should be defined based on the pre-study validation results in the context of mechanistic as well as clinical considerations for each individual biomarker or component of a metabolite or protein molecular marker multiplexing assay⁶⁷. Especially for more complex proteomics and metabolomics assays, laboratory-to-laboratory variability will be an important issue since small changes in the sample processing procedure (e.g. removal of high-abundance proteins for proteomics analysis), instrumentation, analytical method and data processing algorithms can lead to differences among laboratories. Another, in comparison trivial yet

unresolved problem, is the normalization of marker levels in urine samples to compensate for differences in urine concentration in individual samples. Urine is an attractive matrix since in direct contact with the kidney, a so-called proximal matrix. However, the commonly used normalization strategy based on creatinine urine concentrations may become misleading in certain cases, as kidney injury may also affect creatinine concentrations in urine¹¹. So far no consensus has been reached how to effectively address this problem.

In most cases “predictive” means that a biomarker is more sensitive and specific than established clinical markers such as creatinine concentrations in serum or transplant organ biopsies. Such predictive biomarkers show a clinically relevant change preceding the increase of established, less sensitive clinical markers. One of the thresholds for their clinical use is that many clinicians do often feel uncomfortable to base decisions on such new, predictive biomarkers and are unsure about the potential therapeutic consequences of the results⁶⁹. It is reasonable to expect that this will be the case the more complex and the more specific, sensitive and predictive a new biomarker is. For the most advanced markers, clinical studies in transplant patients have not yet gone beyond assessment of their diagnostic sensitivity and specificity; the value of such markers and their potential advantage in guiding therapeutic decisions and in improving outcomes after transplantation still needs to be established in prospective studies.

Conclusions

While metabolomics and targeted metabolite markers are still in the discovery stage, proteomics discovery studies have yielded several promising targeted protein markers, foremost urinary chemokines such as CXCL-9 and CXCL-10 and urinary protein kidney injury markers such as NGAL and KIM-1. Based on evidence in the literature, these may help guiding and individualizing immunosuppressive regimens, will be able to predict and monitor allo-immune responses and may be useful tools for risk stratification of transplant patients. Moreover, these chemokines and protein kidney injury markers can be measured using standard immunoassay platforms, which should facilitate clinical implementation. For these markers further steps towards implementation in clinical practice are justified.

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