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Biomarkers of Immunosuppressant Organ Toxicity after Transplantation - Status, Concepts and Misconceptions

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Abstract

Introduction—A major challenge in transplantation is improving long-term organ transplant and patient survival. Immunosuppressants protect the transplant organ from alloimmune reactions, but they also exhibit sometimes limiting side effects. The key to improving long-term outcome following transplantation is the selection of the correct immunosuppressive regimen for an individual patient to minimize toxicity while maintaining immunosuppressive efficacy.

Areas covered—Proteomics and metabolomics have the potential to develop sensitive and specific diagnostic tools for monitoring early changes in cell signal transduction, regulation and biochemical pathways. Here we review the steps required for the development of molecular markers from discovery, mechanistic and clinical qualification to regulatory approval, and present a critical discussion of the current status of molecular marker development as relevant for the management and individualization of immunosuppressive drug regimens.

Expert opinion—Although metabolomics and proteomics-based studies have yielded several candidate molecular markers, most published studies are poorly designed, statistically underpowered and/or often have not gone beyond the discovery stage. Most molecular marker candidates are still at an early stage. Due to the high complexity of and the resources required for diagnostic marker development, initiatives and consortia organized and supported by funding agencies and regulatory agencies will be critical.

1. Balancing Immunosuppression and Organ Toxicity- A Dilemma in Organ Transplantation

The calcineurin inhibitors cyclosporine and tacrolimus are the basis of most immunosuppressive protocols for the prevention of graft rejection following organ transplantation [1]. The predisposition of these agents to ultimately damage the organs they are intended to protect, especially the kidney, has always been recognized though largely tolerated due to their impressive ability to improve short-term outcomes. Over the last several decades calcineurin inhibitors are responsible for a significant improvement in short-term survival of transplant organs [2]. Although recent analyses have also indicated an

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increase of renal allograft half-lives, long-term results are still not acceptable [1]. Thus, so-called induction protocols and newer small molecule immunosuppressants such as mycophenolic acid and the proliferation signal inhibitors sirolimus and everolimus, both inhibitors of the mammalian target of rapamycin (mTOR) which allow for the reduction or avoidance of calcineurin inhibitor exposure are utilized more and more.

The risks associated with immunosuppressants include immunosuppression itself and organ toxicity. Immunosuppression may increase the prevalence of infections such as fungal and viral infections (CMV, BK virus and EBV), failure of immunization and risk of developing cancers. Calcineurin inhibitor-related toxicity was identified as one of the main reasons for the long-term failures. The most limiting side effects of calcineurin inhibitors are nephrotoxicity [3–5] and neurotoxicity [6,7]. Other adverse effects such as diabetes, hyperlipidemia and hypertension are likely to be responsible for the high cardiovascular risk of transplant patients. While cardiovascular complications are the major cause of death in kidney transplant patients [5], chronic renal allograft dysfunction is the principal cause of late renal allograft loss after the first year [5,8–10]].

1.1 Immunosuppressant-induced endothelial dysfunction and cardiovascular toxicity

The overall cardiovascular risk of transplant patients is multi-factorial with contributors including allograft endothelial dysfunction caused by alloimmune-dependent pathways, ischemia-reperfusion injury, metabolic alterations, chronic infections and direct endothelial activation by immunosuppressants [11]. There is an increase in oxidative stress related to endothelial dysfunction, inflammation and atherosclerosis in transplant patients that is a likely contributor to cardiovascular complications and chronic allograft failure [12]. A disturbance of the homeostatic balance between endothelium-derived relaxing factors such as nitric oxide and activating factors such as endothelin in transplant patients is generally accepted [13]. It was shown in an *in vitro* model testing the effects of immunosuppressants on human microvascular endothelial cells that immunosuppressants, dependent on dose and oxygenation state, modify endothelial activation [11]. In this model mycophenolate and methyl prednisolone caused relatively minor changes compared to cyclosporine, tacrolimus and sirolimus. The induction of oxidative stress was associated with changes in cell metabolism and apoptosis [11]. It was shown in rats that the negative effects of cyclosporine on endothelial function could be reversed by co-administration of anti-oxidants such as α -tocopherol and α -lipoic acid [14,15].

1.2 Nephrotoxicity

One of the major effects of cyclosporine on the kidney is tubular interstitial fibrosis associated with increased expression of TGF- β (*transforming growth factor- β*) [16]. There is evidence that cyclosporine nephrotoxicity is caused by indirect hemodynamic and/or direct effects on kidney cells. In addition to reduced nitric oxide [16] and increased endothelin concentrations, up-regulated expression of angiotensin II receptors [17] and increased calcium concentrations in smooth muscle cells resulting in greater sensitivity to vasoconstrictive stimuli [17] are likely to be involved in cyclosporine's negative effects on the kidney. On a cellular level, there is strong evidence that damage caused by radicals play a significant role in calcineurin inhibitor-induced kidney injury [18]. Studies showed that proximal tubular epithelial cells exposed to cyclosporine in rats accumulate intracellular reactive oxygen species and lipid peroxidation products and exhibit an altered glutathione redox state [19]. Cyclosporine is also known to induce apoptosis due to the increased expression of pro-apoptotic proteins (p53, Bax, Fas-L) and decreased expression of the survival gene Bcl-2 [20]. The role of a direct negative effect of calcineurin inhibitors on the kidney is further supported by evidence showing that transplant kidneys with high expression of the efflux transporter p-glycoprotein are less vulnerable to immunosuppressant

toxicity [21]. The proliferation signal inhibitors sirolimus and everolimus alone are markedly less nephrotoxic than calcineurin inhibitors. This led to the original belief that the different side effect spectra were an advantage of combining calcineurin inhibitors and proliferation signal inhibitors. However, pivotal phase III-clinical studies found that both proliferation inhibitors can enhance cyclosporine nephrotoxicity [22,23]. Changes of calcineurin inhibitor kidney tissue distribution and inhibition of glycolysis caused by proliferation inhibitors seem to be involved [24]. Although nephrotoxicity of immunosuppressants is a serious clinical problem, the underlying biochemical mechanisms are surprisingly still only incompletely understood.

1.3 Neurotoxicity

Neurotoxicity is one of the most significant clinical side effects of the immunosuppressive undecapeptide cyclosporine with a prevalence reported as high as 59% of transplant patients. The clinical symptoms of calcineurin inhibitor neurotoxicity consist of decreased responsiveness, hallucinations, delusions, seizures, cortical blindness, and stroke-like episodes and mimic those of mitochondrial encephalopathies [6,7,25]. Studies with isolated rat brain slices have shown that cyclosporine causes mitochondrial dysfunction resulting in inhibition of ATP synthesis and induction of oxygen radical formation [25,26].

1.4 Gastrointestinal toxicity

Although all immunosuppressants can cause gastrointestinal side effects, this is especially limiting in the case of mycophenolic acid. Mycophenolate-induced gastrointestinal toxicity including diarrhea, gastritis, anorexia, ulcerations, erosions (stomach and duodenum), necrosis, villous atrophy (duodenum) and enterocolitis similar to Crohn's disease, is a serious clinical problem in transplant patients [27,28]. The incidence of gastrointestinal intolerance limits the clinical use of and dosing of mycophenolate formulations and is frequently related to poor compliance as well as erratic absorption and thus may directly be associated with rejection episodes, the development of chronic rejection and ultimately poor graft survival. Surprisingly, although a clinically significant problem, as of today the biochemical mechanism of mycophenolate gastrointestinal toxicity remains largely unknown [27].

In promoting long-term survival, reducing immunosuppressant-induced toxicity may be as important as the reduction of the incidence and occurrence of acute rejection episodes [29]. The use of many immunosuppressant combinations make current, acute rejection rates clinically satisfactory, with the focus of interest in transplantation significantly shifted towards tolerability and long-term graft and patient survival [1]. This has led to several clinical studies aimed at reducing calcineurin inhibitor doses, or discontinuing calcineurin inhibitors or even starting *de novo* transplant patients on calcineurin inhibitor-free immunosuppressive protocols [1]. Success, however, has been limited. Studies without calcineurin inhibitors during the early post-transplant period reported that up to 40% of patients required treatment for acute rejection [30,31], indicating that the use of calcineurin inhibitors will remain critical in clinical transplantation medicine especially directly following transplantation. In cases of established nephrotoxicity it has been commonly believed that a switch to non-nephrotoxic immunosuppressants such as mycophenolic acid or the proliferation signal inhibitors, sirolimus and everolimus, allows for the reduction or even discontinuation of calcineurin inhibitors; so-called "calcineurin-inhibitor free" immunosuppressant long-term maintenance regimens [32,33]. However, in an effort to prevent calcineurin-induced nephrotoxicity, many studies detailing attempts to minimize or wean patients from these medications have shown that the potential reduction in toxicity is often offset by an increase in chronic rejection [34]. In fact, a retrospective analysis of 25,045 kidney transplant patients with good graft function indicated that withdrawal of

maintenance cyclosporine or tacrolimus or reduction of the dose of these agents below certain thresholds after the first year following the transplant to be associated with a significant risk of graft loss [35].

Because complete discontinuation of calcineurin inhibitors can increase damage of the transplant organ caused by chronic rejection at least in some patients, the discontinuation of calcineurin inhibitors may be considered a double-edge sword. In general, the primary problem with today's clinical management of transplant patients is that immunosuppressive drug regimens are driven mainly by clinical protocols and the individual patient is not a consideration until they demonstrate clinical symptoms. At this stage it is likely that irreversible damage already occurred, as discussed below. However, this clinical practice cannot be changed without the availability of more sensitive molecular diagnostic and monitoring strategies to guide individualization of immunosuppressive drug regimen preferably administered early after transplantation.

A good example of the issues associated with current clinical diagnostic strategies used in transplantation is the monitoring of transplant kidneys. As of today, serum creatinine concentrations are routinely used as a clinical marker for monitoring function of kidney allografts [36]. Once an elevation in serum creatinine concentrations is detected, a biopsy is then procured to differentiate between the possible diagnoses. A Banff-graded, two-core allograft biopsy remains the gold standard with which all novel diagnostic tools must be compared. However, even biopsies will not necessarily allow for conclusive diagnosis of the etiology of the observed histopathological changes with sufficient confidence. Lesions such as interstitial fibrosis and tubular atrophy, as well as glomerular injury are nonspecific responses to injury. Antibody-mediated endothelial activation, calcineurin inhibitor toxicity, recurrent disease, chronic inflammation, innate immune mechanisms as well as diabetes mellitus and hypertension have all been invoked as potential etiologies. Unfortunately, serum creatinine is not a sensitive biomarker. It has been shown that up to 30% of grafts with stable creatinine may have smoldering rejection and treatment of this chronic/subclinical rejection may result in improved graft function [37,38]. Since the procurement of a kidney biopsy is guided by a rise of creatinine levels in many centers, biopsies are usually taken at a late time point when the disease process has already caused significant damage and is already driven by secondary disease processes such as inflammation and fibrosis. At such a late stage it is difficult to determine the original trigger of the histopathological changes.

The key to reducing chronic damage caused by immunosuppressant toxicity, over-immunosuppression and under-immunosuppression is early detection. As aforementioned, the most common strategy to reduce the prevalence and severity of immunosuppressant toxicity has been the minimization or discontinuation of the doses of calcineurin inhibitors during long-term-maintenance immunosuppression. This is often performed without foreknowledge of the factors contributing to the chronic injury process in an individual kidney transplant patient and without guidance by an appropriate diagnostic strategy. This frequently results in reduction of the immunosuppressive efficacy of the drug regimen and creates a dilemma. As mentioned previously, a major factor contributing to transplant organ dysfunction is allograft immune response. Treatment to avoid damage by immunological responses requires enhanced immunosuppressive drug regimens. No non-invasive diagnostic tool that allows for differentiating between allograft dysfunction due to alloimmune response or immunosuppressant toxicity is currently available. Monitoring biochemical changes, and the detection of disease processes and immunosuppressant toxicity prior to significant histological or pathophysiological damage and while said process is still potentially reversible is an attractive concept.

Biomarkers are used to study, monitor and diagnose disease processes (please see definitions in Table 1). As indicated by the wide parameters of the definition, the measurement of biomarkers encompasses a large number of methodologies ranging from imaging technologies to gene arrays. In fact, clinical diagnostics must be considered as nothing less than the use of biomarkers [46]. Since metabolomics and proteomics are technologies that directly or indirectly assess molecular mechanisms, the use of the term “molecular marker” will be more focused here. It is important to note that the term molecular marker may refer to a single molecular entity as well as a panel of several molecular entities.

2. Molecular Marker Strategies- Why Are They Predictive?

Chronic disease processes and drug toxicities are characterized by silent and progressive courses and non-specific symptoms that, by current clinical diagnostic tools, often remain undetected in their early stages [47]. The quality of diagnostic tools is determined by their sensitivity and specificity. The sensitivity and specificity of chemical and biochemical molecular markers traditionally used in clinical diagnostics as well as preclinical and clinical drug development is sometimes poor. This relies on the fact that the following assumptions were often made: (A) one marker detects all disease processes/drug effects targeted against a specific organ, and (B) one marker fits all patient populations and age groups. Also, when these more traditional markers were established in the clinic, in many cases the mechanisms of diseases or drug effects were not well understood. Molecular markers did not have to undergo the rigorous validation and qualification procedures required by current regulatory guidelines and have historically been introduced as diagnostic tools to the clinic based on scientific consensus.

Poor sensitivity and specificity relate directly to poor predictive value. To better understand how molecular markers can be more predictive, it is important to look at the stages of kidney injury caused by a disease or drug. This is illustrated in Figure 1. The development of a disease process or drug injury can be divided into roughly three stages: a genetic, a biochemical and a symptomatic stage [52].

A genetic predisposition may increase the risk for an individual to develop a disease, modify the efficacy or tolerability of a drug, or influence its tissue distribution and pharmacokinetics; however, in most cases, other factors, such as diseases, drugs, nutritional status and/or environmental factors, will also be required to trigger a pathologic biochemical process.

Though changes in gene expression, protein expression and biochemical profiles occur during the biochemical stage, the cells and organs are still able to compensate. At this stage, an injury process should be detectable if sufficiently sensitive assays are available. If no notable histological damage occurs during the biochemical phase, the disease process may be fully reversible with an appropriate therapeutic intervention available. Biochemical changes on a cellular, organ or systemic level can no longer be compensated for during the symptomatic stage. This leads to pathophysiological and histological changes that define the symptoms of the injury process. Most established outcome metrics presently used during preclinical and clinical drug development detect injury processes in their symptomatic stage. Monitoring biochemical changes and detecting an injury process before detectable histological or pathophysiological damage occurs is an attractive concept. If the cause-effect relationships between protein expression, biochemical changes, the symptoms of a disease and a drug effect or toxicity are known, detecting specific changes in protein and cell biochemistry patterns has the potential to predict development of the symptomatic injury.

Technologies such as genomics/transcriptomics, proteomics and biochemical profiling (metabolomics) could develop molecular marker strategies that allow for monitoring early

changes in cell signal transduction, regulation and biochemistry with high sensitivity and specificity and, therefore, to detect an injury process at a much earlier stage than by the currently established clinical diagnostic markers.

3. Genotype, Phenotype and Systems Biology

The relationship between genomics, transcriptomics, proteomics and metabolomics is illustrated in Figure 2. Instead of focusing on genes, proteins or metabolites alone, systems biology is the study of an organism viewed as an integrated and interacting network of genes, proteins and biochemical reactions. Proteomics and metabolomics complement genetics are considered phenotypic molecular markers and are still less established than genetic screening technologies. In the past, the potential to monitor changes in mRNA expression and micro-arrays as diagnostic tools rather than metabolomics or proteomics approaches has been explored. Conclusions are drawn based on screening gene expression. In most cases, there has been utilization of non-targeted gene chips covering the complete human genome including important single nucleotide polymorphisms (SNPs) and targeted gene arrays containing a limited array of genes and their SNPs. It is often assumed that changes of mRNA concentrations correspond with changes in the number of functional proteins and that, accordingly, these are associated with changes in signal transduction and cell biochemistry that then predict and/or ultimately will result in pathophysiological and histological changes. Therefore, downstream confirmation through analysis of protein concentrations and/or metabolites is usually a requirement [54]. Metabolic profiling technologies are not as well developed as gene array technologies and generate a lower throughput. However, the correlation between transcriptomics and proteomic or metabolic changes is poorer than expected and in many cases the chain of assumptions mentioned above may be invalid. This is due to the inability to guarantee that more or less of mRNA also leads to an increase or reduction of the expression of a protein. Even if protein concentrations follow the changes in mRNA expression, concentrations may not correlate with activities. Reasons include changes in gene splicing, translational modifications, reaction with oxygen radicals and allosteric regulation by substrates, products and other inhibitors and activators. Also worthy of consideration is that changes in cell biochemistry protein patterns directly causes pathophysiological changes and histological damage of an organ or the system; therefore, these are usually more closely associated with a disease process or drug toxicity than genes and mRNA [55]. Another potentially significant limitation is that diagnostics based on gene chips or arrays will, in many cases, require a biopsy. While proteins and metabolite patterns can be evaluated or quantified in tissue samples and biopsies; organ function can also be assessed in body fluids such as plasma procured using minimally invasive or non-invasive sampling procedures. It seems reasonable to assume that biochemical and protein changes in cells and organs are, to a certain extent, seen in bodily fluids. Cells either directly or indirectly (*via* extracellular fluid) communicate with body fluids *via* excretion, trans-membrane diffusion and transport and after cell death, release proteins into body fluids [56]. Certain fluids such as urine (kidney), bile (liver), and cerebrospinal fluid (CNS) mainly reflect changes in specific organs and are considered “proximal fluids”. A proximal fluid is defined as a biofluid closer to, or in direct contact with, the site of disease or drug effect. This is in contrast to the measurement of molecular markers in blood, plasma or serum reflecting changes in the systemic compartment [57]. Once in systemic circulation, these molecular markers are quickly diluted and eventually mix with metabolites, proteins and peptides from other sources potentially complicating the location of an injury.

4. Strategies for the Discovery of Phenotypical Molecular Markers

Modern analytical technologies such as those based on nuclear magnetic resonance spectroscopy (NMR), mass spectrometry, and anti-body based multiplexing platforms or chips facilitate identification of patterns that confer significantly more information than the measurement of a single parameter, in the way that a bar code contains more information than a single number. Well qualified molecular marker patterns will yield more detailed and mechanistically relevant information translating into good specificity. The better the specificity of a molecular marker pattern the greater is the reduction of non-specific background noise usually resulting in better sensitivity.

The steps involved in the development of a molecular marker are:

- discovery (for example using non-targeted screening technologies such as genomics, proteomics and metabolomics),
- candidate marker identification,
- development and validation of targeted analytical assays,
- mechanistic and clinical qualification,
- establishment of sensitivity and specificity in pre-clinical and/or clinical trials and
- review and approval by regulatory agencies.

Metabolomics and proteomics strategies can be non-targeted or targeted [58]. For comprehensive reviews of current metabolomics and proteomics technologies please see references [51,53,59–67].

5. Molecular Marker Development- Qualification, Validation and Regulatory Aspects

The subsequent steps following candidate marker or a candidate marker panel identification are the development and validation of targeted analytical assays and qualification. A representative work flow is shown in Figure 3.

5.1. Qualification

Today, technologies can measure hundreds and thousands of proteins and metabolites. Interpreting the complex information generated can present a challenge. The qualification process bridges the results of molecular marker measurements, symptomatic drug effects and disease outcomes. Qualification has been differentiated from validation. While validation focuses on the reliability and performance characteristics of the analytical assay used to measure molecular markers [50,69,70], qualification is defined as, “a graded, fit-for purpose evidentiary process linking a biomarker with biology and clinical endpoints” [71]. As indicated by this definition, there are two key aspects to the qualification of a molecular marker:

- a. to mechanistically link the molecular marker to the biochemical process underlying a disease or drug effect. This has also been referred to as “biomarker verification”.
- b. to establish a link between the molecular marker and clinical end points.

The most important first step of the qualification of a molecular marker is a clear understanding of its scientific, preclinical, clinical and regulatory use. This will have a critical impact on the extent and depth of the required work. There are three basic strategies

available for establishing a mechanistic link between the molecular marker and the biochemical process underlying a disease or drug effect:

- a. leverage of pre-existing knowledge,
- b. application of biostatistical strategies, and
- c. experiments identifying the underlying molecular mechanisms leading to changes in the molecular marker.

In most cases, a thorough literature analysis and/or data mining approach already provides substantial information. The next step is usually a gap analysis providing the basis for a qualification plan and the tools to map out which further *in vitro* and *in vivo* studies will be required. Experiments supporting a mechanistic qualification strategy may include, but are not limited to, the assessment of dose- and time-dependency, recovery, gene knock-outs, knock-downs, and gene silencing. The weakest of the three mechanistic qualification strategies, is the reliance on solely biostatistical evaluations such as algorithms available in several current biomarker discovery software packages. Most biostatistical methods establish associations and correlations, which may suggest but rarely prove cause-effect relationships. Establishing cause-effect relationships between a drug or disease effect and the molecular marker lies at the core of a robust mechanistic qualification strategy. Even the discovery of a molecular marker in clinical trials does not necessarily mean that it is clinically relevant, and a proper qualification may require a bed-to-benchtop approach. Often only cell and animal studies will allow for a systematic in-depth mechanistic evaluation. Also, patient populations, especially transplant patients, are often complex with many confounding factors. Once a mechanistic link is established by studying drug effects in the laboratory, molecular marker qualification studies in healthy volunteers as translational proof of concept are usually of significant value.

The second critical component of a comprehensive molecular marker qualification is to show that a molecular marker is linked to and/or is a valid predictor of a disease process or drug effect in humans. In addition to sensitivity and specificity, a rigorous clinical qualification should also include the assessment of time- and dose-dependency. The extent and rigor of these studies will depend on the goal of the molecular marker qualification [72] - if the marker is just exploratory and will be used for confirmatory purposes, or if it will be used to support regulatory claims. In the latter case, studies must go beyond proof of concept in terms of statistical power considerations, documentation, monitoring and regulatory compliance. Receiver operating characteristic (ROC) curves for the definition of sensitivity and specificity [73] are basic metrics used to assess biomarker performance [42]. In general, area-under-the-ROC curves (AUC_{ROC}) ≤ 0.5 are not considered useful and indicate the molecular marker's inability to discriminate between treatment, disease or the control group. While in the ROC analyses of pre-clinical animal studies histology is often used as the gold standard endpoint, clinical trials utilize established outcome parameters and clinical endpoints. Nevertheless, it is important for the quality of the ROC analysis that the reference outcome parameters to be precise and non-biased.

5.2. Validation

The success of translating a molecular marker from the discovery stage into pre-clinical testing and clinical development will greatly depend on the availability of robust, precise and sensitive assays that are simple and/or automated and that allow for the measurement of a larger number of samples [74].

If a molecular marker plays an important role during the pre-clinical development of an immunosuppressant, quantification of the molecular marker may need to comply with the rules of good laboratory practices (GLP). Validation of analytical assays for the

quantification of metabolic and protein molecular markers may have to follow applicable regulatory guidances and standards [57,75,76] including but not limited to, FDA [77], Clinical and Laboratory Standard Institute [78], and EMEA/ICH [79].

5.3. Regulatory Aspects

Regulatory review includes a risk-benefit assessment based on the intended use of the molecular markers and the qualification data and assay validation which includes stability testing. Regulatory agencies classify biomarkers as exploratory, probable valid and known valid [42,70,71,80]. Molecular markers can be developed for two purposes: to support development of a specific drug (context-specific) or as a clinical diagnostic tool. In principal, the regulatory paths are different, although there is overlap, but will converge if a context-specific marker will also be used as a clinical diagnostic tool to manage patients on a specific drug.

In the United States, the use of molecular marker data in regulatory review and decisions is currently based on the FDA guidance “Providing Clinical Evidence of Effectiveness for Human Drug and Biological Products” [81]. If appropriately qualified, molecular markers can support primary outcomes; they may help to understand and monitor mechanisms of toxicity, drug interactions, disease-drug interactions and the effects of genotypes, gender and age. They can also be used to stratify patient populations and to guide subgroup analyses in order to bridge safety and efficacy data between different populations such as adults to pediatric patients and among different ethnic groups. Molecular markers will also require regulatory review and approval if they are developed into commercial clinical diagnostic tests [57].

6. The Current Status of Molecular Marker Discovery and Development in Transplantation

6.1. Cardiovascular molecular markers

The cause for the increased cardiovascular risk in transplant patients is multifactorial and the effects of immunosuppressants on endothelial function is one of the major contributors. Imaging and function tests are important cardiovascular biomarkers. However, so-called soluble biomarkers have the advantage that they only require a simple blood draw and are therefore more suitable for routine monitoring. In the case of cardiovascular molecular markers, blood, plasma or serum must be considered proximal matrices in which most molecular cardiovascular markers are measured. The effects of immunosuppressants on endothelial pathways have been studied (for a summary please see [82]), however, there are currently no specific markers that can indicate immunosuppressant-induced endothelial toxicity in transplant patients. Transplant patients may benefit from the availability of sensitive and predictive molecular markers in several ways [83]: (a) identification of asymptomatic patients at risk, (b) identification of non-traditional risks, (c) determination of cardiovascular tolerability and safety of immunosuppressive drug regimen, and (d) longitudinal monitoring of cardiovascular risk and disease development. Two major groups of molecular markers for cardiovascular disease are currently explored: inflammatory markers and endothelial dysfunction markers. Cardiovascular risk markers that have been studied in transplant patients are summarized in Table 2. However, comprehensive and systematic studies assessing the value of molecular markers to predict, detect and monitor immunosuppressant endothelial toxicity and cardiovascular risk as well as to individualize immunosuppressive drug regimens in transplant patients are lacking.

6.2. Nephrotoxicity

Interest has mainly focused on urine as a diagnostic matrix. The reasons are obvious: urine can easily and non-invasively be collected and it is a proximal fluid that is in direct contact with the kidney. However, unanswered questions remain: the metabolite and protein contents can be influenced by the collection method (first void or spot urine) and molecular marker concentrations often need to be normalized to reduce the influence of differences in dilution. Although there is consensus that normalization of urinary molecular marker concentrations based on urinary creatinine in patients with disease processes or drug effects that affect release and handling of creatinine by the kidney will give misleading results [99], interestingly, there has been very little discussion about solutions for this critical problem

6.2.1. Metabolomics—Several studies have focused on the effects of immunosuppressants alone and in combination on kidney tissue metabolite patterns and the metabolite patterns in blood and urine [100–102]. While most of these studies have been purely descriptive and show urine metabolite pattern changes typical for primary proximal tubular injury, recently a series of systematic studies has been published that also included first qualification steps [24,52,68,103,104]. After treatment of rats with calcineurin inhibitors and their combination with sirolimus for 28 days, glomerular filtration rates were significantly reduced. The decrease of glomerular filtration rates was associated with significant changes in urine metabolite patterns that correlated with the reduction in glomerular filtration rates. The changes of metabolite patterns in urine were associated with a combination of changes in glomerular filtration, changes in secretion/absorption by tubulus cells and changes in kidney cell metabolism [24]. Based on these results, a combinatorial metabolite marker for monitoring immunosuppressant-induced kidney dysfunction in rats treated with calcineurin inhibitors was proposed [52]: markers of glomerular filtration (creatinine), reabsorption (glucose), tubulus cell metabolism (citrate, oxoglutarate, lactate), active secretion and kidney amino acylase activity (hippurate), as well as oxidative stress (isoprostanes), and the release of metabolites protective against the protein-precipitating effect of uric acid (trimethyl amine-N-oxide). An association between immunosuppressant-induced changes in kidney metabolism and urine metabolite patterns was confirmed by proteomics studies that were conducted to mechanistically explain and qualify the urinary metabolite pattern changes [104]. The changes in expression of several enzymes compared to untreated controls explained several of the changes in metabolite patterns observed in urine. The extent of changes in glomerular filtration rates after 28 days was predicted by the extent of metabolite pattern changes in urine after 6 days, even though glomerular filtration rates at that time were not different from baseline, and histological changes were not detectable [24]. In this study after 6 days of treatment, urine metabolite patterns were similar to those reported for agents causing oxidative damage, while pattern changes after 28 days were typical for agents that cause S3 tubular damage [24]. These results matched the histologies showing specific damage of the proximal *tubuli*. These studies suggested the following mechanism causing the characteristic changes in urine metabolite patterns: calcineurin inhibitors directly and/or indirectly (*via* endothelial dysfunction) derail mitochondrial oxidation causing oxygen radical formation, inhibition of Krebs cycle and decline of energy production. The proximal tubulus cell tries to compensate by activating anaerobic glycolysis and importing Krebs cycle intermediates from urine *via* the NaDC1 and NaDC3 transporters [24,104]. In an open label, placebo-controlled, crossover study the time-dependent toxicodynamic effects of a single oral cyclosporine dose (5 mg/kg) on the kidney was assessed in thirteen healthy individuals [68]. In plasma and urine samples, 15-F_{2t}-isoprostane concentrations using HPLC-MS and metabolite profiles using ¹H-NMR spectroscopy were analyzed. The increase in urinary 15-F_{2t}-isoprostane concentrations observed 4 hours after administration of cyclosporine indicated an increase in oxidative stress. 15-F_{2t}-isoprostaglandine concentrations were in average 2.9-fold higher

after cyclosporine than after placebo. Unsupervised metabolome analysis using principal components and partial least square fit analyses revealed significant changes in urine metabolites typically associated with negative effects on proximal tubulus cells. The major metabolites that differed between the 4h- urine samples after cyclosporine and the placebo were citrate, hippurate, lactate, TMAO, creatinine and phenylalanine (see Figure 3) indicating that analysis of urinary metabolites was a sensitive enough marker for detection of the effects of a single cyclosporine dose already shortly after drug administration and that the results in rats at least translate into healthy humans. Creatinine concentrations in serum remained unchanged [68]. A decrease in citrate concentrations in urine kidney transplant patients had also been reported by others [105]. The results of the study by Klawitter et al. [24] also suggested that changes in urine metabolite patterns reflected the negative effects of immunosuppressants on kidneys with better sensitivity and specificity than metabolite changes in blood. However, it has been reported that immunosuppressants alone and in combination may lead to changes of metabolite patterns in the blood of rats [106] and transplant patients [107–109].

Le Moyec et al. [110] found that the most relevant ¹H-NMR signals for evaluating renal function after transplantation were those arising from citrate, trimethylamine-N-oxide, alanine, and lactate when compared to creatinine. The respective variations of these metabolites in urine were associated with cyclosporine toxicity and rejection. Several other clinical studies have shown that rejection of a kidney transplant leads to changes in urine metabolite patterns [111–114]. However, no attempt has been made to further develop and qualify these urinary metabolite markers and no study that assesses if urine metabolite patterns can differentiate between kidney injury caused by allo-immune response or immunosuppressant toxicity have been described.

6.2.2. Proteomics and protein markers in urine—The effect of kidney injury on urine, kidney biopsies and plasma proteomes has been studied in animal models [115] and in multiple clinical trials using non-targeted proteomics [51]. These studies, however, have focused mostly on transplant kidney injury caused by allo-immune reactions and not specifically on immunosuppressant toxicity or on markers that allow for differentiating between immunosuppressant toxicity and allo-immune reaction-mediated injury.

The concept of targeted protein molecular markers of kidney dysfunction in urine is attractive. The United States FDA and European Medicines Agency (EMA) recently approved a set of seven urinary proteins as molecular markers of nephrotoxicity that were submitted by the Predictive Safety Testing Consortium (PSTC) in collaboration with multiple pharmaceutical companies to the Voluntary Exploratory Data Submission (VXDS) committee of the United States FDA [116–119]. These markers are urinary total protein, albumin, β 2-microglobulin, cystatin C, kidney injury molecule-1 (KIM-1), clusterin and trefoil factor-3 and are for regulatory use in certain preclinical settings [116–119]. Table 3 lists promising urinary molecular marker candidates that have emerged over recent years. As indicated, several of these have been studied in transplant patient populations. However, their utility in the individualized management of transplant patients and in monitoring immunosuppressant toxicity still needs to be established in prospective studies. It is important to note that not all of these molecular markers may be useful for all types of kidney injury depending on the mechanistic reason why they are changing and depending on the time point relative to the start the injury process when the samples are collected. Thus, a marker of inflammation may not respond when the primary target of a drug toxicity is the proximal tubule, but may be changed at a later time point once this injury has triggered an inflammatory response. However, this has the advantage that the analysis of a panel of several diverse markers may allow for differential diagnosis of a disease process or drug

effect and may allow for monitoring time-dependent changes such as progression of injury and/or its recovery.

6.3. Markers of gastrointestinal toxicity

Candidate gastrointestinal injury markers that have been described in transplant patients are summarized in Table 4. However, none of these has been used to assess gastrointestinal toxicity of immunosuppressants. It can be expected that sensitive assays that allow for identifying transplant patients who will tolerate immunosuppressants or their combinations only after a few doses and before gastrointestinal symptoms develop will be of clinical importance. In addition to functional tests such as the sucrose or the ^{51}Cr EDTA absorption tests, the following candidate markers have shown promise: Determination of the plasma citrulline level is a reliable marker for assessing the mass of functional intestinal tissue. Citrulline is an amino acid formed almost exclusively in enterocytes and not present in food proteins [165]. Clinically relevant are decreased citrulline levels as they reflect a lack of functional mass of enterocytes. Calprotectin is measured in feces and is a member of the S-100 protein family. Calprotectin is present in squamous epithelial cells (not in normal cells), neutrophils and macrophages [179]. Inflammation stimulates its excretion into gut. Other potentially useful markers include adipsin, C-reactive protein (inflammation marker in Crohn's disease) and lathosterol in feces (bile mal absorption when mucosa is dysfunctional) [179]. As aforementioned, the potential value of these markers in transplant patients remains to be evaluated.

No "soluble" molecular markers for immunosuppressant-induced neurotoxicity in transplant patients have been described.

Recent studies have indicated the potential value of urinary cell micro-RNAs for the sensitive diagnosis of renal allograft rejection and BK virus infections [162–164]. If this approach will be able to sensitively and specifically detect immunosuppressant toxicity has not yet been studied.

7. Expert Opinion

Patients are all unique and this variability is more complex for the transplant patient as pharmac- and toxicodynamic responses to drugs are determined by the genomes of both the recipient and the organ donor. Immunological mismatches between recipient and donor, immunological processes, underlying diseases, and potential infections such as viral infections each add a further level of complexity. It is surprising that there have been only sporadic efforts to develop molecular marker-based strategies using modern analytical technologies for diagnostics, monitoring and prediction of drug response for the long-term management of transplant patients, given the critical importance of individualizing immunosuppressive drug regimens early after transplantation in order to ensure long-term transplant organ and patient survival.

Metabolomics and proteomics-based discovery studies have yielded several candidate molecular markers and marker panels, however, the development of which into clinical diagnostic tools must be considered still in its early stages. Several of the urine protein kidney dysfunction markers in Table 3 seem furthest along on their way to become novel clinical tools for the management of transplant patients.

Overall, the challenges of developing sensitive and specific molecular marker strategies for the management of transplant patients are the same as those of molecular markers in other disease areas. Transplantation will benefit from the progress of the development of such markers in other disciplines such as cardiovascular risk and gastrointestinal function

markers. An ideal molecular marker (A) is an early indicator of active damage (is sensitive), (B) is quantitative and correlates with the severity of damage, (C) can be easily measured in an easily accessible matrix, (D) has good stability, (E) can be quantified using a validated, robust, reproducible, sensitive and specific high-throughput assay, (F) is the result of a well-known molecular mechanism (is well qualified), (G) can be translated across species, (H) discriminates between different molecular effects (is specific), and (I) can be utilized to localize injury.

As aforementioned, it is reasonable to expect that these criteria can be fulfilled by a molecular marker panel rather than a single molecular marker. A realistic and powerful approach is the development of “combinatorial biomarkers”. Those are molecular marker panels that typically consist of 3–10 individual parameters [46]. Specific combinatorial biomarker panels generally confer significantly more information than a single measurement and, thus, can be expected to have better specificity and sensitivity.

In terms of biomarker discovery and qualification and determination of sensitivity and specificity, it is critical to take time-dependency of protein and biochemical changes into account. While a symptomatic injury is the end-stage and, although the extent may change, its protein and biochemical signature often remains unchanged over longer time periods. In contrast, during the earlier biochemical stage, protein and biochemical patterns can change quickly as the injury progresses. This may include compensatory mechanisms, secondary mechanisms such as oxygen radical formation and damage, changes in cell function and regulation, and the triggering of additional processes such as immune reactions and inflammation. Different stages during the development of a biochemical injury may be characterized by different sets of markers and this time-dependency and its underlying mechanistic dynamics need to be understood. Accordingly, the timing of sample collection has to be a critical consideration. It may also be necessary to develop different sets of molecular markers for different stages of a disease process.

Another problem is the current practice to determine sensitivity and specificity by comparison with a current gold standard outcome parameter or established clinical end point. In the case of development of molecular markers, this means the assessment of the extent to which a certain molecular marker pattern will be successful in predicting the development of a certain symptomatic disease end-stage such as transplant kidney dysfunction. However, today's disease classifications are often symptom-based and that these end-stage injuries may alternately be caused by distinct underlying biochemical mechanisms that ultimately cause the same symptoms can be problematic. As aforementioned, several of these distinct and alternate biochemical processes may not even be known yet and may eventually require new classification of the symptomatic disease process. Symptomatic injuries caused by different drug toxicities and diseases will ultimately involve the same pathobiochemical and pathological mechanisms such as mitochondrial dysfunction, the formation of oxygen radicals, necrosis, apoptosis, inflammation and immune reactions. This means that the further a pathological process has progressed the more difficult it will be to find specific molecular marker changes. Current practices and guidances require a specific molecular marker or marker pattern caused by an early biochemical disease process to be qualified based on a gold standard, typically a late disease stage. This lacks specificity as this stage is mainly caused and driven by common disease processes such as oxidative stress and inflammation. One of the problems with the gold standard outcome being less specific than the molecular marker is that there is no 1:1 relationship between a molecular marker and the predicted clinical outcome. Several molecular marker patterns caused by distinct biochemical disease processes that ultimately lead to the same symptoms will be valid predictors of a single clinical outcome. Such a scenario leads to good specificity- a specific marker pattern will be able to reliably predict a

certain clinical outcome. However, sensitivity will be poor since the same outcomes caused by other biochemical processes will be missed. Following current practices and guidances this may lead to the rejection of a valid highly specific molecular marker. Paradoxically, a less predictive and specific molecular marker that is a surrogate for later and more common disease processes may be acceptable.

In many cases there is poor consensus for defining a clinical endpoint against which a molecular marker needs to be qualified. For example, there are more than 30 different definitions of acute renal failure, or now acute kidney injury, in the published literature [49,180]. It will be difficult to establish sensitivity and specificity if the gold standard outcome against which a molecular marker will be qualified is of poor quality. Kidney transplantation represents a good example. The histology of a kidney biopsy is considered the current gold standard. However, since the procedure is invasive and involves certain risks, kidney biopsies are often procured at a late stage and in many cases the histological findings are inconclusive and do not allow for determination of the original disease mechanism that triggered the processes leading to kidney injury and the observed histopathological changes. Overall, this raises the question of whether or not establishing the quality and acceptance of a molecular marker by determining specificity and sensitivity is a valid approach.

Poor study design, population heterogeneity, under-powered studies, insufficient analysis of confounding factors and co-variables as well as methodological weaknesses in data collection and analysis as well as lack of quality control have led to the reporting of numerous candidate molecular marker sets for a given disease that show little or no overlap between studies. This lack of replication has undermined confidence that truly disease-associated clinically useful molecular markers can be found [181]. Another problem is that most studies are limited to molecular marker discovery, but very few groups have taken the step of qualifying their candidate molecular markers and demonstrating their clinical usefulness in prospective and appropriately powered trials. The field of transplantation is no exception. This is not surprising given the complexity of the molecular marker qualification process. A full biomarker qualification is a highly integrated and comprehensive project that requires extensive inter-disciplinary expertise, collaborations and resources. Communication tools and infrastructures such as initiatives driven and supported by funding agencies, consortia, and accessible databases will be critical [182].

Overall, it is reasonable to expect the development of novel sensitive and specific diagnostic tools to guide dosing and individualization of a patient's immunosuppressive drug regimen to be of equal merit as the development of better and safer immunosuppressive drugs. Ideally, the development of new immunosuppressants and context-specific, molecular marker-based monitoring tools should go hand-in-hand so that by the time a novel immunosuppressant receives marketing approval, an approved and tested molecular marker strategy for selection of patients, who adequately respond to the drug and tolerate the drug well, and for management of patients, who receive this drug, are available.

HIGHLIGHTS BOX

- Organ toxicity of immunosuppressants negatively affects graft and patient survival, contributes to chronic allograft injury and thus limits long-term outcomes after kidney transplantation.
- Clinical diagnostic tools based on molecular markers have the potential to detect immunosuppressant toxicity earlier and with better specificity than current clinical markers such as creatinine in serum.

- Molecular markers will allow for individualization of immunosuppressive drug regimens and a positive effect on long-term outcomes can be expected.
- The development of molecular markers into diagnostic tools is an extensive effort that includes the following steps: discovery, mechanistic qualification (verification), clinical qualification and regulatory review and approval
- Several molecular markers for monitoring kidney function, cardiovascular toxicity and gastrointestinal toxicity have been described. However, none of these has progressed into clinical practice yet
- Kidney dysfunction protein markers in urine are the molecular markers that are furthest along in their development.
- Due to its complexity, the development of molecular markers into clinical tools to improve long-term outcomes after transplantation should be driven and supported by funding agencies, regulatory agencies and consortia with academia and industry

References

1. Womer KL, Kaplan B. Recent Developments in kidney transplantation– a critical assessment. *Am J Transplant.* 2009; 9:1265–71. [PubMed: 19459815]
2. Campistol JM, Grinyo JM. Exploring treatment options in renal transplantation: The problems of chronic allograft dysfunction and drug-related nephrotoxicity. *Transplantation.* 2001; 71:SS42–51. [PubMed: 11583488]
3. Dunn CJ, Wagstaff AJ, Perry CM, Plosker GL, Goa KL. Cyclosporine: an updated review of the pharmacokinetic properties, clinical efficacy and tolerability of microemulsion-based formulation (Neoral) in organ transplantation. *Drugs.* 2001; 61:1957–2016. [PubMed: 11708766]
4. Kahan BD. Cyclosporine. *N Engl J Med.* 1989; 321:1725–38. [PubMed: 2687689]
5. Kahan BD. Potential therapeutic interventions to avoid or treat chronic allograft dysfunction. *Transplantation.* 2001; 71:SS52–7. [PubMed: 11583490]
6. Bechstein WO. Neurotoxicity of calcineurin inhibitors: impact and clinical management. *Transplant Int.* 2000; 13:313–6.
7. Gijtenbeek JM, van den Bent MJ, Vecht CJ. Cyclosporine neurotoxicity: a review. *J Neurol.* 1999; 246:339–46. [PubMed: 10399863]
8. Nankivell BJ, Borrows RJ, Fung CLS, O'Connell PJ, Allen RDM, Chapman JR. The natural history of chronic allograft rejection. *New Engl J Med.* 2003; 349:2326–33. [PubMed: 14668458]
- 9*. Nankivell BJ, Borrow RJ, Fung CLS, O'Connell PJ, Chapman JR, Allen RD. Calcineurin inhibitor nephrotoxicity: longitudinal assessment by protocol histology. *Transplantation.* 2004; 78:557–65. This study highlights the clinical importance of immunosuppressant-induced nephrotoxicity and showed that pathologic changes of CsA nephrotoxicity were virtually universal by 10 years after transplantation. [PubMed: 15446315]
10. Nankivell BJ, Chapman JR. Chronic allograft nephropathy: current concepts and future directions. *Transplantation.* 2006; 81:643–54. [PubMed: 16534463]
- 11*. Trapp A, Weis M. The impact of immunosuppression on endothelial function. *J Cardiovasc Pharmacol.* 2005; 45:81–7. A systematic in vitro study assessing the molecular mechanisms of immunosuppressant-induced effects on endothelial cells. [PubMed: 15613984]
12. Moreno JM, Ruiz MC, Ruiz N, et al. Modulation factors of oxidative status in stable renal transplantation. *Transplant Proc.* 2005; 37:1428–30. [PubMed: 15866626]
13. Tepperman E, Ramzy D, Prodger J, et al. Vascular effects of immunosuppression. *Can J Surg.* 2010; 53:57–63. [PubMed: 20100415]
14. Lexis LA, Fenning A, Brown L, et al. Antioxidant supplementation enhances erythrocyte antioxidant status and attenuates cyclosporine-induced vascular dysfunction. *Am J Transplant.* 2006; 6:41–9. [PubMed: 16433755]

15. Louhelainen M, Merasto S, Finckenberg P, et al. Lipoic acid supplementation prevents cyclosporine-induced hypertension and nephrotoxicity in spontaneously hypertensive rats. *J Hypertens*. 2006; 24:947–56. [PubMed: 16612258]
16. Vaziri ND, Ni Z, Zhang YP, Ruzics EP, et al. Depressed renal and vascular nitric oxide synthase expression in cyclosporine-induced hypertension. *Kidney Int*. 1998; 54:482–91. [PubMed: 9690215]
17. Avdonin PV, Cottet-Maire F, Afanasjeva GV, et al. Cyclosporine A up-regulates angiotensin II receptors and calcium responses in human vascular smooth muscle cells. *Kidney Int*. 1999; 55:2407–14. [PubMed: 10354289]
18. Josephine A, Amudha G, Veena CK, et al. Oxidative and nitrosative stress mediated renal cellular damage induced by cyclosporine A: role of sulphated polysaccharides. *Biol Pharm Bull*. 2007; 30:1254–9. [PubMed: 17603163]
19. Wang C, Salahudeen AK. Lipid peroxidation accompanies cyclosporine nephrotoxicity: effects of vitamin E. *Kidney Int*. 1995; 47:927–34. [PubMed: 7752594]
20. Shihab FS, Bennett WM, Yi H, Andoh TF. Effect of pirfenidone on apoptosis-regulatory genes in chronic cyclosporine nephrotoxicity. *Transplantation*. 2005; 79:419–26. [PubMed: 15729167]
21. Hauser IA, Schaeffeler E, Gauer S, et al. ABCB1 genotype of the donor but not of the recipient is a major risk factor for cyclosporine-related nephrotoxicity after renal transplantation. *J Am Soc Nephrol*. 2005; 16:1501–11. [PubMed: 15772250]
22. Kahan BD, for the Rapamune US Study Group. Efficacy of sirolimus compared with azathioprine for reduction of acute renal allograft rejection: a randomized multicenter study. *Lancet*. 2000; 356:194–202. [PubMed: 10963197]
23. Kahan BD, Kaplan B, Lorber MI, et al. RAD in de novo renal transplantation: comparison of three doses on the incidence and severity of acute rejection. *Transplantation*. 2001; 71:1400–6. [PubMed: 11391226]
24. Klawitter J, Bendrick-Pearl J, Rudolph B, et al. Urine metabolites reflect time-dependent effects of cyclosporine and sirolimus on rat kidney function. *Chem Res Toxicol*. 2009; 22:118–28. [PubMed: 19099400]
25. Serkova N, Christians U. Transplantation: Toxicokinetics and mechanisms of toxicity of cyclosporine and macrolides. *Curr Opin Invest Drugs*. 2003; 4:1287–96.
26. Christians U, Gottschalk S, Miljus J, et al. Alterations in glucose metabolism by cyclosporine in rat brain slices link to oxidative stress: interactions with mTOR inhibitors. *Br J Pharmacol*. 2004; 143:388–96. [PubMed: 15339861]
27. Arns W. Noninfectious gastrointestinal (GI) complications of mycophenolic acid therapy: A consequence of local GI toxicity? *Transplant Proc*. 2007; 39:88–93. [PubMed: 17275481]
28. Simmons WD, Rayhill SC, Sollinger HW. Preliminary risk-benefit assessment of mycophenolate mofetil in transplant rejection. *Drug Safety*. 1997; 17:75–92. [PubMed: 9285199]
29. DeMattos AM, Olyaei AJ, Bennett WM. Nephrotoxicity of immunosuppressive drugs: long-term consequences and challenges for the future. *Am J Kidney Dis*. 2000; 35:333–46. [PubMed: 10676738]
30. Kreis H, Cisterne JM, Land W, et al. Sirolimus in association with mycophenolate mofetil induction for the prevention of acute graft rejection in renal allograft recipients. *Transplantation*. 2000; 69:1252–60. [PubMed: 10798738]
31. Groth CG, Bäckman L, Morales JM, et al. Sirolimus (rapamycin)-based therapy in human renal transplantation: similar efficacy and different toxicity compared with cyclosporine. *Transplantation*. 1999; 67:1036–42. [PubMed: 10221490]
32. Golshayan D, Pascual M. Minimization of calcineurin inhibitors to improve long-term outcomes in kidney transplantation. *Transplant Immunol*. 2008; 20:21–8.
33. Pretagostini R, Cinti P, Lai Q, et al. Minimization of immunosuppressive therapy and immunological monitoring of kidney transplant recipients with long-term allograft survival. *Transplant Immunol*. 2008; 20:3–5.
34. Han WK, Bonventre JV. Biologic markers for the early detection of acute kidney injury. *Curr Opin Crit Care*. 2004; 10:476–82. [PubMed: 15616389]

- 35*. Opelz G, Döhler B. Discontinuing maintenance immunosuppression after the first year posttransplant. *Transplantation*. 2008; 86:371–6. This registry analysis shows that the reduction of calcineurin inhibitors doses or complete discontinuation during long-term management of transplant patients can adversely affect long-term outcomes. [PubMed: 18698238]
36. Berl T. American Society of Nephrology Renal Research Report. *J Am Soc Nephrol*. 2005; 16:1886–1903. [PubMed: 15888557]
37. Nankivell BJ, Chapman JR. The significance of subclinical rejection and the value of protocol biopsies. *Am J Transplant*. 2006; 6:2006–12. [PubMed: 16796717]
- 38*. Chapman JR, O'Connell PJ, Nankivell BJ. Chronic renal allograft dysfunction. *J Am Soc Nephrol*. 2005; 16:3015–26. An important review that discusses and explains the risks of overlooking transplant kidney dysfunction during its earlier stages as it is common with current clinical monitoring strategies. Late identification of chronic allograft dysfunction in individual patients has meant that strategies for intervening to prevent chronic renal allograft dysfunction and subsequent graft loss tend to be “too little and far too late.”. [PubMed: 16120819]
39. Puntman VO. How-to-guide on biomarkers: biomarker definitions, validation and applications with examples from cardiovascular disease. *Postgrad Med J*. 2009; 85:538–45. [PubMed: 19789193]
40. Biomarkers Definitions Working Group. Biomarker and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther*. 2001; 69:89–95. [PubMed: 11240971]
41. Vasan RS. Biomarkers of cardiovascular disease: molecular basis and practical considerations. *Circulation*. 2006; 113:2335–62. [PubMed: 16702488]
42. Goodsaid FM, Frueh FW, Mattes W. Strategic paths for biomarker qualification. *Toxicology*. 2008; 245:219–23. [PubMed: 18280028]
43. Holmes E, Wilson ID, Nicholson JK. Metabolic phenotyping in health and disease. *Cell*. 2008; 134:714–7. [PubMed: 18775301]
44. Nicholson JK. Global systems biology, personalized medicine and molecular epidemiology. *Mol Syst Biol*. 2006; 2:52. [PubMed: 17016518]
45. Peng J, Gygi SP. Proteomics: The move to mixtures. *J Mass Spectrom*. 2001; 36:1083–91. [PubMed: 11747101]
46. Koop R. Combinatorial biomarkers: from early toxicology assays to patient population profiling. *Drug Discov Today*. 2005; 10:781–8. [PubMed: 15922936]
47. Stojnev S, Pejic M, Dolicanin Z, et al. Challenges of genomics and proteomics in nephrology. *Ren Failure*. 2009; 31:765–72.
48. Musso CG, Michelángelo H, Vilas M, Reynaldi J, Martinez B, Algranati L, Macías Núñez JF. Creatinine reabsorption by the aged kidney. *Int Urol Nephrol*. 2009; 41:727–31. [PubMed: 19115077]
- 49*. Vaidya VS, Ferguson MA, Bonventre JV. Biomarkers of acute kidney injury. *Annu Rev Pharmacol Toxicol*. 2008; 48:463–8. A review explaining in detail the potential clinical utility of novel urinary protein markers for the diagnosis and monitoring of acute clinical injury (see Table 4) and their potential clinical impact. [PubMed: 17937594]
50. Müller PY, Dieterle F. Tissue-specific, non-invasive toxicity biomarkers: translation from preclinical safety assessment to clinical safety monitoring. *Expert Opin Drug Metab Toxicol*. 2009; 5:1023–38. [PubMed: 19611406]
51. Christians, U.; McCrery, S.; Klawitter, J.; Klawitter, J. The role of proteomics in the study of kidney diseases and in the development of diagnostic tools. In: Edelstein, CL., editor. *Biomarkers of Kidney Disease*. San Diego: Elsevier; 2011.
52. Christians U, Klawitter J, Bendrick-Pearl J, Schöning W, Schmitz V. Toxicodynamic therapeutic drug monitoring of immunosuppressants: promises, reality and challenges. *Ther Drug Monit*. 2008; 30:151–8. [PubMed: 18367974]
53. Xu EY, Schaefer WH, Xu Q. Metabolomics in pharmaceutical research and development: metabolites, mechanisms and pathways. *Curr Opin Drug Discov Devel*. 2009; 12:40–52.
54. Devarajan P. Emerging urinary biomarkers in the diagnosis of acute kidney injury. *Expert Opin Med Diagn*. 2008; 2:387–98. [PubMed: 19079800]

55. Schnackenberg LK. Global metabolic profiling and its role in systems biology to advance personalized medicine in the 21st century. *Expert Rev Mol Diagn.* 2007; 7:247–59. [PubMed: 17489732]
56. Anderson NL, Polanski M, Pieper R, et al. The human plasma proteome: a nonredundant list developed by combination of four separate sources. *Mol Cell Proteomics.* 2004; 3:311–26. [PubMed: 14718574]
- 57*. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat Biotechnol.* 2006; 24:971–83. This is a comprehensive review of the principles of protein marker development as clinical diagnostic tools and the regulatory approval process. [PubMed: 16900146]
58. Nobeli I, Thornton JM. A bioinformatician's view of the metabolome. *Bioessays.* 2006; 28:534–45. [PubMed: 16615085]
59. Christians, U.; Albuissou, J.; Klawitter, J.; Klawitter, J. The role of metabolomics in the study of kidney diseases and in the development of diagnostic tools. In: Edelstein, CL., editor. *Biomarkers of Kidney Disease*. San Diego: Elsevier; 2011.
60. Oldiges M, Lütz S, Pflug S, et al. Metabolomics: current state and evolving methodologies and tools. *Appl Microbiol Biotechnol.* 2007; 76:495–511. [PubMed: 17665194]
61. Dettmer K, Aronov PA, Hammock BD. Mass spectrometry-based metabolomics. *Mass Spectrom Rev.* 2007; 26:51–78. [PubMed: 16921475]
62. Gowda GA, Zhang S, Gu H, et al. Metabolomics-based methods for early disease diagnostics. *Expert Rev Mol Diagn.* 2008; 8:617–33. [PubMed: 18785810]
63. Want EJ, Nordström A, Morita H, et al. From exogenous to endogenous: the inevitable imprint of mass spectrometry in metabolomics. *J Proteome Res.* 2007; 6:459–68. [PubMed: 17269703]
64. Domon B, Aebersold R. Mass spectrometry in protein analysis. *Science.* 2006; 312:212–7. [PubMed: 16614208]
65. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature.* 2003; 422:198–207. [PubMed: 12634793]
66. de Hoog CL, Mann M. Proteomics. *Annu Rev Genomics Hum Genet.* 2004; 5:267–93. [PubMed: 15485350]
67. Decramer S, Gonzalez de Peredo A, Breuil B, et al. Urine in clinical proteomics. *Mol Cell Proteomics.* 2008; 7:1850–62. [PubMed: 18667409]
- 68*. Klawitter J, Haschke M, Kahle C, et al. Toxicodynamic effects of ciclosporin are reflected by metabolite profiles in the urine of healthy individuals after a single dose. *Br J Clin Pharmacol.* 2010; 70:241–51. A placebo-controlled clinical trial in healthy individuals showing that ¹H-NMR-based metabolic profiling in urine is sensitive enough to detect the negative effect of a single 5 mg/kg cyclosporine dose kidneys already within the first hours after oral administration. [PubMed: 20653677]
69. Lee JW, Figeys D, Vasilescu J. Biomarker assay translation from discovery to clinical studies in cancer drug development: quantification of emerging protein biomarkers. *Adv Cancer Res.* 2007; 96:269–98. [PubMed: 17161683]
- 70*. Burckart GJ, Amur S, Goodsaid FM, et al. Qualification of biomarkers for drug development in organ transplantation. *Am J Transplant.* 2008; 8:267–70. This review critically discusses the development of biomarkers and regulatory requirements in the field of organ transplantation. [PubMed: 18093264]
- 71*. Wagner JA, Williams SA, Webster CJ. Biomarkers and surrogate end points for fit-for-purpose development and regulatory evaluation of new drugs. *Clin Pharmacol Ther.* 2007; 81:104–7. A critical review and discussion of biomarkers in drug development and the related regulatory aspects and challenges. [PubMed: 17186007]
- 72*. Lesko LJ, Atkinson AJ Jr. Use of biomarkers and surrogate endpoints in drug development and regulatory decision making: criteria, validation, strategies. *Annu Rev Pharmacol Toxicol.* 2001; 41:347–66. This article discusses the FDA's positions and perspectives regarding biomarker development, qualification and approval. [PubMed: 11264461]
73. Fawcett T. Introduction to ROC analysis. *Pattern Recognition Letters.* 2006; 27:861–74.

74. Lee JW, Weiner RS, Sailstad JM, et al. Method validation and measurement of biomarkers in nonclinical and clinical samples in drug development: a conference report. *Pharm Res.* 2005; 22:499–511. [PubMed: 15846456]
75. Hortin GL. Can mass spectrometric protein profiling meet desired standards of clinical laboratory practice? *Clin Chem.* 2005; 51:3–5. [PubMed: 15613702]
76. Rodriguez H, Tezak Z, Mesri M, et al. Analytical validation of protein-based multiplex assays: a workshop report by the NCI-FDA interagency oncology task force on molecular diagnostics. *Clin Chem.* 56:237–43. [PubMed: 20007859]
77. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research and Center for Veterinary Medicine. Guidance for the Industry. Bioanalytical Method Validation. 2001 [last accessed 29 August 2010]. Version May 2001. <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM070107.pdf>
78. Clinical and Laboratory Standard Institute. [last accessed 29 August 2010]. www.clsi.org
79. European Agency for the Evaluation of Medicinal Products. ICH topic Q2 (R1). Validation of analytical procedures: text and methodology. Note for guidance on analytical procedures: Text and methodology. 1995 [last accessed 29 August 2010]. CPMP ICH/381/95. http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002662.pdf
80. United States Food and Drug Administration, Center for Drug Evaluation and Research. Guidance for the Industry. Pharmacogenomic data submissions. March 2005 [last accessed 29 August 2010]. <http://www.fda.gov/downloads/RegulatoryInformation/Guidances/ucm126957.pdf>
81. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research. Guidance for Industry: Providing Clinical Evidence of Effectiveness for Human Drug and Biological Products. 1998 [last accessed 29 August 2010]. Version May 1998. <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078749.pdf>
82. Nickel T, Schlichting L, Weis M. Drugs modulating endothelial function in transplantation. *Transplantation.* 2006; 82 (1S):S41–6. [PubMed: 16829796]
83. Rubin C, Nolin TD, Himmelfarb J. Are biomarkers useful for assessing cardiovascular risk in patients with chronic kidney disease. *Curr Opin Nephrol Hypertens.* 2007; 16:506–11. [PubMed: 18089962]
84. Gallego, MJ.; Zoja, C.; Morigi, M., et al. Cyclosporine enhances leukocyte adhesion to vascular endothelium under physiologic flow conditions; *Am J Kidney Dis.* 1996. p. 23-31.
85. Al-Massarani G, Vacher-Coponat H, Paul P, et al. Impact of immunosuppressive treatment on endothelial biomarkers after kidney transplantation. *Am J Transplant.* 2008; 8:2360–7. [PubMed: 18925903]
86. Yilmaz MI, Saglam M, Carrero JJ, et al. Normalization of endothelial dysfunction following renal transplantation is accompanied by a reduction of circulating visfatin/NAMPT. A novel marker of endothelial damage? *Clin Transplant.* 2009; 23:241–8. [PubMed: 19402217]
87. Edwards BS, Hunt SA, Fowler MB, Valentine HA, Anderson LM, Lerman A. Effect of cyclosporine on plasma endothelin levels in humans after cardiac transplantation. *Am J Cardiol.* 1991; 67:782–784. [PubMed: 2006635]
88. Ramzy D, Rao V, Tumati LC, Xu N, Miriuka S, Delgado D, Ross HJ. Role of endothelin-1 and nitric oxide bioavailability in transplant-related vascular injury: comparative effects of rapamycin and cyclosporine. *Circulation.* 2006; 4;114(1 Suppl):I214–9.
89. Müller TF, Vogl M, Neumann MC, Lange H, Grimm M, Müller MM. Noninvasive monitoring using serum amyloid A and serum neopterin in cardiac transplantation. *Clin Chim Acta.* 1998; 276:63–74. [PubMed: 9760020]
90. Maury CP, Teppo AM, Ahonen J. Posttransplantation monitoring of high-density lipoprotein-associated serum amyloid A protein: a new diagnostic aid in the detection of renal allograft rejection. *Uremia Invest.* 1985; 9:277–80. [PubMed: 3915928]

91. Aukrust P, Halvorsen B, Yndestad A, Ueland T, Øie E, Otterdal K, Gullestad L, Damås JK. Chemokines and cardiovascular risk. *Arterioscler Thromb Vasc Biol.* 2008; 28:1909–19. [PubMed: 18669888]
92. Sahin G, Akay OM, Kus E, Bal C, Yalcin AU, Gulbas Z. Effects of immunosuppressive drugs on platelet aggregation and soluble P-selectin levels in renal transplant patients. *Ren Fail.* 2009; 31:111–7. [PubMed: 19212907]
93. Aharinejad S, Krenn K, Zuckermann A, et al. Matrix metalloproteases and their tissue inhibitor in cardiac transplantation. *Eur J Cardiothorac Surg.* 2007; 32:48–51. [PubMed: 17482473]
94. Caglar K, Yilmaz MI, Saglam M, et al. Endothelial dysfunction and fetuin A levels before and after kidney transplantation. *Transplantation.* 2007; 83:392–7. [PubMed: 17318070]
95. Yilmaz MI, Saglam M, Caglar K, et al. Endothelial functions improve with decrease in asymmetric dimethylarginine (ADMA) levels after renal transplantation. *Transplantation.* 2005; 27(80):1660–6. [PubMed: 16378057]
96. Nouri-Majalan N, Masoumi R, Nafisi R, Nogh H, Ghafari A, Moghaddasi S. Relationship between serum homocysteine and other parameters in renal transplant patients. *Transplant Proc.* 2009; 41:2826–8. [PubMed: 19765447]
97. Lexis LA, Fenning A, Brown L, Fassett RG, Coombes JS. Antioxidant supplementation enhances erythrocyte antioxidant status and attenuates cyclosporine-induced vascular dysfunction. *Am J Transplant.* 2006; 6:41–9. [PubMed: 16433755]
98. Vural A, Yilmaz MI, Caglar K, et al. Assessment of oxidative stress in the early posttransplant period: comparison of cyclosporine A and tacrolimus-based regimens. *Am J Nephrol.* 2005; 25:250–5. [PubMed: 15925859]
99. Conti M, Moutereau S, Esmilaire L, et al. Should kidney tubular markers be adjusted for urine creatinine? The example of urinary cystatin C. *Clin Chem Lab Med.* 2009; 47:1553–6. [PubMed: 19877765]
100. Wishart DS. Metabolomics: the principles and potential applications to transplantation. *Am J Transplant.* 2005; 5:2814–20. [PubMed: 16302993]
101. Wishart DS. Metabolomics: a complementary tool in renal transplantation. *Contrib Nephrol.* 2008; 160:76–87. [PubMed: 18401163]
102. Lenz EM, Bright J, Knight R, et al. Cyclosporin A-induced changes in endogenous metabolites in rat urine: a metabolomic investigation using high field ¹H NMR spectroscopy, HPLC-TOF/MS and chemometrics. *J Pharm Biomed Anal.* 2004; 35:599–608. [PubMed: 15137985]
103. Schmitz V, Klawitter J, Bendrick-Peart J, et al. Metabolic profiles in urine reflect nephrotoxicity of sirolimus and cyclosporine following rat kidney transplantation. *Nephron.* 2009; 111:e80–91. [PubMed: 19293597]
- 104*. Klawitter J, Klawitter J, Kushner E, et al. Association of immunosuppressant-induced protein changes in the rat kidney with changes in urine metabolite patterns: A proteo-metabonomic study. *J Proteome Res.* 2010; 9:865–75. This study links the changes in urinary metabolite patterns after exposure of rats to cyclosporine and/or sirolimus for 28 days to changes in protein expression in the kidney and shows the usefulness of a proteo-metabonomic approach for mechanistic molecular marker qualification. [PubMed: 19994912]
105. Stapenhorst L, Sassen R, Beck B, et al. Hypocitraturia as a risk factor for nephrocalcinosis after kidney transplantation. *Pediatr Nephrol.* 2005; 20:652–6. [PubMed: 15782300]
106. Serkova NJ, Christians U. Biomarkers for toxicodynamic monitoring of immunosuppressants: NMR-based quantitative metabolomics of the blood. *Ther Drug Monit.* 2005; 27:733–7. [PubMed: 16404806]
107. Kanaby M, Akcay A, Huddam B, et al. Influence of cyclosporine and tacrolimus on serum uric acid levels in stable kidney transplant recipients. *Transplant Proc.* 2005; 37:3119–20. [PubMed: 16213325]
108. Perico N, Codreanu I, Caruso M, et al. Hyperuricemia in kidney transplantation. *Contrib Nephrol.* 2005; 147:124–31. [PubMed: 15604612]
109. Armstrong KA, Johnson DW, Campbell SB, et al. Does uric acid have a pathogenic role in graft dysfunction and hypertension in renal transplant patients? *Transplantation.* 2005; 80:1565–71. [PubMed: 16371927]

110. Le Moyec L, Pruna A, Eugène M, et al. Proton nuclear magnetic resonance spectroscopy of urine and plasma in renal transplantation follow-up. *Nephron*. 1993; 65:433–9. [PubMed: 8289995]
111. Foxall PJ, Mellotte GJ, Bending MR, et al. NMR spectroscopy as a novel approach to the monitoring of renal transplant function. *Kidney Int*. 1993; 43:234–45. [PubMed: 8433564]
112. Knoflach A, Binswanger U. Serum hippuric acid concentration in renal allograft rejection, ureter obstruction, and tubular necrosis. *Transpl Int*. 1994; 7:17–21. [PubMed: 8117397]
113. Rush D, Somorjai R, Deslauriers R, et al. Subclinical rejection--a potential surrogate marker for chronic rejection--may be diagnosed by protocol biopsy or urine spectroscopy. *Ann Transplant*. 2000; 5:44–9. [PubMed: 11217206]
114. Wang NJ, Zhou Y, Zhu TY, et al. Prediction of acute cellular renal allograft rejection by urinary metabolomics using MALDI-FTMS. *J Proteome Res*. 2008; 7:3597–601. [PubMed: 18620448]
115. Voshol H, Brendlen N, Müller D, et al. Evaluation of biomarker discovery approaches to detect protein biomarkers of acute renal allograft rejection. *J Proteome Res*. 2005; 4:1192–9. [PubMed: 16083269]
116. European Medicines Agency, Committee for Medicinal Products for Human Use. Final conclusions on the pilot joint EMEA/FDA VXDS experience on qualification of nephrotoxicity biomarkers. January 2009 [last accessed 31 August 2010]. http://www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_procedural_guideline/2009/10/WC500004205.pdf
117. Vaidya VS, Ozer JS, Dieterle F, et al. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. *Nat Biotechnol*. 2010; 28:478–85. [PubMed: 20458318]
118. Dieterle F, Perentes E, Cordier A, et al. Urinary clusterin, cystatin C, beta2-microglobulin and total protein as markers to detect drug-induced kidney injury. *Nat Biotechnol*. 2010; 28:463–9. [PubMed: 20458316]
119. Bonventre JV, Vaidya VS, Schmodder R, et al. Next-generation biomarkers for detecting kidney toxicity. *Nat Biotechnol*. 2010; 28:436–40. [PubMed: 20458311]
120. Betton GR, Kenne K, Somers R, Marr A. Protein biomarkers of nephrotoxicity; a review and findings with cyclosporin A, a signal transduction kinase inhibitor and N-phenylanthranilic acid. *Cancer Biomark*. 2005; 1:59–67. [PubMed: 17192032]
121. Aicher L, Wahl D, Arce A, Grenet O, Steiner S. New insights into cyclosporine A nephrotoxicity by proteome analysis. *Electrophoresis*. 1998; 19:1998–2003. [PubMed: 9740060]
122. Aicher L, Meier G, Norcross AJ, Jakubowski J, Varela MC, Cordier A, Steiner S. Decrease in kidney calbindin-D 28kDa as a possible mechanism mediating cyclosporine A- and FK-506-induced calciuria and tubular mineralization. *Biochem Pharmacol*. 1997; 53:723–31. [PubMed: 9113092]
123. Shlipak MG, Sarnak MJ, Katz R, et al. Cystatin C and the risk of death and cardiovascular events among elderly persons. *N Engl J Med*. 2005; 352:2049–60. [PubMed: 15901858]
124. Conti M, Moutereau S, Zater M, et al. Urinary cystatin C as a specific marker of tubular dysfunction. *Clin Chem Lab Med*. 2006; 44:288–91. [PubMed: 16519600]
125. Le Bricon T, Thervet E, Benlakehal M, Bousquet B, Legendre C, Erlich D. Changes in plasma cystatin C after renal transplantation and acute rejection in adults. *Clin Chem*. 1999; 45:2243–9. [PubMed: 10585359]
126. Yang GP, Lau LF. Cyr61, product of a growth factor-inducible immediate early gene, is associated with the extracellular matrix and the cell surface. *Cell Growth Differ*. 1991; 2:351–7. [PubMed: 1782153]
127. Muramatsu Y, Tsujie M, Kohda Y, et al. Early detection of cysteine rich protein 61 (CYR61, CCN1) in urine following renal ischemic reperfusion injury. *Kidney Int*. 2002; 62:1601–10. [PubMed: 12371960]
128. Sundberg AG, Nilsson R, Appelkvist EL, Dallner G. Immuno-histochemical localization of alpha and pi class glutathione transferases in normal human tissues. *Pharmacol Toxicol*. 1993; 72:321–31. [PubMed: 8372055]

129. Sundberg AG, Appelkvist EL, Bäckman L, Dallner G. Urinary pi-class glutathione transferase as an indicator of tubular damage in the human kidney. *Nephron*. 1994; 67:308–16. [PubMed: 7936021]
130. Bäckman L, Appelkvist EL, Ringdén O, Dallner G. Glutathione transferase in the urine: a marker for post-transplant tubular lesions. *Kidney Int*. 1988; 33:571–7. [PubMed: 3283401]
131. Harrison DJ, Kharbanda R, Cunningham DS, McLellan LI, Hayes JD. Distribution of glutathione S-transferase isoenzymes in human kidney: basis for possible markers of renal injury. *J Clin Pathol*. 1989; 42:624–8. [PubMed: 2738168]
132. Eijkenboom JJ, van Eijk LT, Pickkers P, Peters WH, Wetzels JF, van der Hoeven HG. Small increases in the urinary excretion of glutathione S-transferase A1 and P1 after cardiac surgery are not associated with clinically relevant renal injury. *Intensive Care Med*. 2005; 31:664–7. [PubMed: 15812628]
133. Pelsers MM. Fatty acid-binding protein as marker for renal injury. *Scand J Clin Lab Invest*. 2008; 241 (supplement):73–7.
134. Ichimura T, Asseldonk EJ, Humphreys BD, Gunaratnam L, Duffield JS, Bonventre JV. Kidney injury molecule-1 is a phosphatidylserine receptor that confers a phagocytic phenotype on epithelial cells. *J Clin Invest*. 2008; 118:1657–68. [PubMed: 18414680]
135. Vaidya VS, Ramirez V, Ichimura T, Bobadilla NA, Bonventre JV. Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury. *Am J Physiol Renal Physiol*. 2006; 290:F517–29. [PubMed: 16174863]
136. Han WK, Bailly V, Abichandani R, Thadhani R, Bonventre JV. Kidney Injury Molecule-1 (KIM-1): a novel biomarker for human renal proximal tubule injury. *Kidney Int*. 2002; 62:237–44. [PubMed: 12081583]
137. Sánchez-Pozos K, Lee-Montiel F, Pérez-Villalva R, et al. Polymerized type I collagen reduces chronic cyclosporine nephrotoxicity. *Nephrol Dial Transplant*. 2010; 25:2150–8. [PubMed: 20139407]
138. Fernandes I, Zhang Y, Qi Y, et al. Impact of reduced nephron mass on cyclosporine- and/or sirolimus-induced nephrotoxicity. *Transplantation*. 2009; 88:1323–31. [PubMed: 20029328]
139. Pérez-Rojas J, Blanco JA, Cruz C, et al. Mineralocorticoid receptor blockade confers renoprotection in preexisting chronic cyclosporine nephrotoxicity. *Am J Physiol Renal Physiol*. 2007; 292:F131–9. [PubMed: 16835406]
140. Hong ME, Hong JC, Stepkowski S, Kahan BD. Correlation between cyclosporine-induced nephrotoxicity in reduced nephron mass and expression of kidney injury molecule-1 and aquaporin-2 gene. *Transplant Proc*. 2005; 37:4254–8. [PubMed: 16387092]
141. Maatman RG, van de Westerlo EM, van Kuppevelt TH, Veerkamp JH. Molecular identification of the liver- and the heart-type fatty acid-binding proteins in human and rat kidney. Use of the reverse transcriptase polymerase chain reaction. *Biochem J*. 1992; 288:285–90. [PubMed: 1280113]
142. Kamijo A, Sugaya T, Hikawa A, et al. Clinical evaluation of urinary excretion of liver-type fatty acid binding protein as a marker for monitoring chronic kidney disease: a multi-center trial. *J Lab Clin Med*. 2005; 145:125–33. [PubMed: 15871303]
143. Davey PG, Cowley DM, Geddes AM, Terry J. Clinical evaluation of beta 2-microglobulin, muramidase, and alanine aminopeptidase as markers of gentamicin nephrotoxicity. *Contrib Nephrol*. 1984; 42:100–6. [PubMed: 6152413]
144. Tolkoff-Rubin NE, Rubin RH, Bonventre JV. Non-invasive renal diagnostic studies. *Clin Lab Med*. 1988; 8:507–26. [PubMed: 2901937]
145. Miyata T, Jadoul M, Kurokawa K, Van Ypersele de Strihou C. Beta-2 microglobulin in renal disease. *J Am Soc Nephrol*. 1998; 9:1723–35. [PubMed: 9727382]
146. Duraj FF, Bäckman L, Dati F, Ringdén O. Serum levels of alpha-1 microglobulin and beta-2 microglobulin in bone marrow transplant recipients treated with cyclosporin A. *Transpl Int*. 1991; 4:146–50. [PubMed: 1720317]
147. Bäckman L, Ringdén O, Björkhem I, Lindbäck B. Increased serum beta 2 microglobulin during rejection, cyclosporine-induced nephrotoxicity, and cytomegalovirus infection in renal transplant recipients. *Transplantation*. 1986; 42:368–71. [PubMed: 3020748]

148. Palmieri L, Ronca G, Cioni L, Puccini R. Enzymuria as a marker of renal injury and disease: studies of N-acetyl-beta-glucosaminidase, alanine aminopeptidase and lysozyme in patients with renal disease. *Contrib Nephrol.* 1984; 42:123–9. [PubMed: 6152415]
149. Diener U, Knoll E, Ratge D, Langer B, Wisser H. Urinary excretion of alanine-aminopeptidase and N-acetyl-beta-D-glucosaminidase during sequential combination chemotherapy. *J Clin Chem Clin Biochem.* 1982; 20:615–9. [PubMed: 6183389]
150. Liangos O, Perianayagam MC, Vaidya VS, et al. Urinary N-acetyl-beta-(D)-glucosaminidase activity and kidney injury molecule-1 level are associated with adverse outcomes in acute renal failure. *J Am Soc Nephrol.* 2007; 18:904–12. [PubMed: 17267747]
151. Gruber SA, Gallichio M, Rosano TG, Kaplan SS, et al. Comparative pharmacokinetics and renal effects of cyclosporin A and cyclosporin G in renal allograft recipients. *J Clin Pharmacol.* 1997; 37:575–86. [PubMed: 9243350]
152. Marchewka Z, KuŹniar J, Długosz A. Enzymatic markers of cyclosporine nephrotoxicity in patients after renal transplantation. *Int Urol Nephrol.* 1999; 31:727–34. [PubMed: 10755366]
153. Bornstein B, Arenas J, Morales JM, Praga M, Rodicio JL, Martinez A, Valdivieso L. Cyclosporine nephrotoxicity and rejection crisis: diagnosis by urinary enzyme excretion. *Nephron.* 1996; 72:402–6. [PubMed: 8852487]
154. Haase M, Bellomo R, Devarajan P, Schlattmann P, Haase-Fielitz A. NGAL Meta-analysis Investigator Group. Accuracy of neutrophil gelatinase-associated lipocalin (NGAL) in diagnosis and prognosis in acute kidney injury: a systematic review and meta-analysis. *Am J Kidney Dis.* 2009; 54:1012–24. [PubMed: 19850388]
155. Mishra J, Dent C, Tarabishi R, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *Lancet.* 2005; 365(9466):1231–8. [PubMed: 15811456]
156. Mishra J, Ma Q, Prada A, et al. Identification of neutrophil gelatinase-associated lipocalin as a novel early urinary biomarker for ischemic renal injury. *J Am Soc Nephrol.* 2003; 14:2534–43. [PubMed: 14514731]
157. Wasilewska A, Zoch-Zwierz W, Taranta-Janusz K, Michaluk-Skutnik J. Neutrophil gelatinase-associated lipocalin (NGAL): a new marker of cyclosporine nephrotoxicity? *Pediatr Nephrol.* 2010; 25:889–97. [PubMed: 20072790]
158. Bernard AM, Vyskocil AA, Mahieu P, Lauwerys RR. Assessment of urinary retinol-binding protein as an index of proximal tubular injury. *Clin Chem.* 1987; 33:775–9. [PubMed: 3297418]
159. Camara NO, Matos AC, Rodrigues DA, Pereira AB, Pacheco-Silva A. Urinary retinol binding protein is a good marker of progressive cyclosporine nephrotoxicity after heart transplant. *Transplant Proc.* 2001; 33:2129–31. [PubMed: 11377474]
160. Câmara NO, Matos AC, Rodrigues DA, Pereira AB, Pacheco-Silva A. Early detection of heart transplant patients with increased risk of ciclosporin nephrotoxicity. *Lancet.* 2001; 357:856–7. [PubMed: 11265958]
161. McDonough AA, Biemesderfer D. Does membrane trafficking play a role in regulating the sodium/hydrogen exchanger isoform 3 in the proximal tubule? *Curr Opin Nephrol Hypertens.* 2003; 12:533–41. [PubMed: 12920402]
162. Dadhania D, Snopkowski C, Ding R, et al. Validation of noninvasive diagnosis of BK virus nephropathy and identification of prognostic biomarkers. *Transplantation.* 2010; 90:189–97. [PubMed: 20526237]
163. Hartono C, Muthukumar T, Suthanthiran M. Noninvasive diagnosis of acute rejection of renal allografts. *Curr Opin Organ Transplant.* 2010; 15:35–41. [PubMed: 19935064]
164. Anglicheau D, Sharma VK, Ding R, et al. MicroRNA expression profiles predictive of human renal allograft status. *Proc Natl Acad Sci USA.* 2009; 106:5330–5. [PubMed: 19289845]
165. Crenn P, Messing B, Cynober L. Citrulline as a biomarker of intestinal failure due to enterocyte mass reduction. *Clin Nutr.* 2008; 27:328–39. [PubMed: 18440672]
166. Ruiz P, Tryphonopoulos P, Island E, et al. Citrulline evaluation in bowel transplantation. *Transplant Proc.* 2010; 42:54–6. [PubMed: 20172280]

167. David AI, Selvaggi G, Ruiz P, et al. Blood citrulline level is an exclusionary marker for significant acute rejection after intestinal transplantation. *Transplantation*. 2007; 84:1077–81. [PubMed: 17998860]
168. Gondolesi G, Ghirardo S, Raymond K, et al. The value of plasma citrulline to predict mucosal injury in intestinal allografts. *Am J Transplant*. 2006; 6:2786–90. [PubMed: 16952300]
169. David AI, Gaynor JJ, Zis PP, et al. An association of lower serum citrulline levels within 30 days of acute rejection in patients following small intestine transplantation. *Transplant Proc*. 2006; 38:1731–2. [PubMed: 16908264]
170. Pappas PA, Tzakis GA, Gaynor JJ, et al. An analysis of the association between serum citrulline and acute rejection among 26 recipients of intestinal transplant. *Am J Transplant*. 2004; 4:1124–32. [PubMed: 15196071]
171. Cagnola H, Scaravonati R, Cabanne A, et al. Evaluation of calprotectin level in intestinal content as an early marker for graft rejection. *Transplant Proc*. 2010; 42:57–61. [PubMed: 20172281]
172. Sindhi R, AshokKumar C, Mazariegos G, et al. Immune monitoring in small bowel transplantation. *Curr Opin Organ Transplant*. 2010; 15:349–56. [PubMed: 20489630]
173. Akpinar E, Vargas J, Kato T, et al. Fecal calprotectin level measurements in small bowel allograft monitoring: a pilot study. *Transplantation*. 2008; 85:1281–6. [PubMed: 18475184]
174. Sudan D, Vargas L, Sun Y, Bok L, Dijkstra G, Langnas A. Calprotectin: a novel noninvasive marker for intestinal allograft monitoring. *Ann Surg*. 2007; 246:311–5. [PubMed: 17667511]
175. Pakarinen MP, Kuusanmäki P, Lauronen J, Paavonen T, Halttunen J. Effects of ileum transplantation and chronic rejection on absorption and synthesis of cholesterol in pigs. *Pediatr Surg Int*. 2003; 19:656–61. [PubMed: 14574608]
176. Nikkilä K, Miettinen TA, Höckerstedt KV, Isoniemi H. Sterol parameters as markers of liver function in primary biliary cirrhosis before and after liver transplantation. *Transpl Int*. 2005; 18:221–5. [PubMed: 15691276]
177. Ritchie BK, Brewster DR, Davidson GP, Tran CD, McNeil Y, Hawkes JS, Butler RN. 13C-sucrose breath test: novel use of a noninvasive biomarker of environmental gut health. *Pediatrics*. 2009; 124:620–6. [PubMed: 19581263]
178. Johansson JE, Ekman T. Gut mucosa barrier preservation by orally administered IgA-IgG to patients undergoing bone marrow transplantation: a randomised pilot study. *Bone Marrow Transplant*. 1999; 24:35–9. [PubMed: 10435732]
179. Merrick AB, Bruno ME. Genomic and proteomic profiling for biomarkers and signature profiles of toxicity. *Curr Opin Mol Therapeut*. 2004; 6:600–7.
180. Kellum JA, Levin N, Bouman C, Lameire N. Developing a consensus classification system for acute renal failure. *Curr Opin Crit Care*. 2002; 8:509–14. [PubMed: 12454534]
181. Lee BT, Liew L, Lim J, et al. Candidate List of yoUr Biomarker (CLUB): A web-based platform to aid cancer biomarker research. *Biomark Insights*. 2008; 3:65–71. [PubMed: 19578495]
182. Sistare F, Dieterle F, Troth S, et al. Towards consensus practices to qualify safety biomarkers for use in early drug development. *Nat Biotechnol*. 2010; 28:446–54. [PubMed: 20458314]

Figure 1.

Time-Dependency of Kidney Tubular Epithelium Injury and Molecular Markers in Urine (based on references [49,50] and reproduced from [51] with permission from Elsevier).

After and during an injury such as drug toxicity, a disease process or ischemia/reperfusion injury, cell function will be affected first. This may include absorption from and excretion into urine as well as cell metabolism. The extent of the resulting urine metabolite pattern changes will depend on the intensity of the injury and how many cells/tubuli are affected. Depending on the type of injury (acute or chronic), sooner or later damage to the cells will lead to changes in protein patterns in urine. So-called repair proteins will be formed and also the pattern of proteins excreted into urine may change. As increasing numbers of cells die by necrosis and/or apoptosis, the biochemical phase of injury will progress towards the symptomatic phase. These cells will release at least some of their contents such as metabolites, proteins, RNA and DNA into the urine. Cell death will also trigger secondary reactions such as inflammation and fibrosis. From this point on, there may no longer be a possibility of complete recovery. The injury results in histological changes and kidney function will be reduced. Currently established diagnostic markers such as creatinine concentrations in serum and blood urea nitrogen will not significantly change until the symptomatic phase.

Figure 2.
The Systems Approach to Assessment and Monitoring Diseases and Drug Effects Using
“omics” Technologies. (Based on reference [53])

Figure 3.

Representative Flow of a Molecular Marker Discovery (based on [68] and reproduced from [59] with permission from Elsevier. This figure shows the workflow of a non-targeted metabolome analysis as used in a cross-over, two-period clinical study to compare the effect of a single oral 5 mg/kg cyclosporine dose (Neoral, Novartis, Basel, Switzerland) to placebo (Neoral formulation without cyclosporine) on the kidney in thirteen healthy individuals [68]. Metabolome profiling started with the acquisition of a set of ^1H -NMR spectra in urine. The spectra were then reduced to histograms ("binning") which represent the area under the curve in a certain spectral region. This created an ensemble of XY-tables (spectral region versus integral), the so-called bucket tables. The spectra were analyzed using a principal components analysis (PCA) and partial least squares fit analysis (PLS) (AMIX software, Bruker, Rheinstetten, Germany). In the PCA, the principal components are constructed in such a way that the first explains most of the variance in the ensemble, the second explains the second most, and so on. The clustering analysis of the scores plots, the PC_1 versus the PC_2 , was used to determine if groups of spectra differed from each other. Thus, hidden phenomena that were not obvious from the usual spectral dimension could be discovered. The spectral regions that caused the separation were identified in the loading plots, which form the link back to the spectral dimension. The compounds under the signals that were responsible for the separation of the effects of drug and placebo identified using of 2D-NMR.

Table 1

Definitions

Based on references [39–45]

Biomarker	<p>A characteristic objectively measured and evaluated as an indicator of normal biological processes or pharmacological responses to a therapeutic intervention.</p> <p><u>Type 0 biomarker</u>: a marker of the natural history of a disease that correlates longitudinally with known clinical indices</p> <p><u>Type I biomarker</u>: a marker that captures the effects of a therapeutic intervention in with its mechanism of action</p> <p><u>Context independent</u>: developed for general clinical and pre-clinical testing</p> <p><u>Context specific</u>: developed in association with a drug development program and, accordingly, to study and monitor the effects of specific drugs</p> <p><u>Antecedent</u>: Identifying the risk of developing an illness</p> <p><u>Screening</u>: screening for subclinical disease</p> <p><u>Diagnostic</u>: recognizing overt disease</p> <p><u>Staging</u>: categorizing disease severity</p> <p><u>Monitoring</u>: assessing disease progression, therapeutic efficacy and adverse effects</p> <p><u>Prognostic</u>: predicting future disease course/response to therapy</p>
Clinical end point	<p>A characteristic or variable that reflects how a patient feels, functions or survives.</p> <p><u>Intermediate (non-ultimate) end point</u>: a true clinical endpoint, a symptom or measure of function but not the ultimate end-point of the disease</p> <p><u>Ultimate end point</u>: survival or the rate of other or irreversible morbid events</p>
Surrogate end point	A biomarker intended to substitute for a clinical endpoint aiming to predict clinical benefit or harm or lack of benefit or lack of harm on the basis of epidemiological, therapeutic, pathophysiological or other scientific evidence.
Metabolome	A quantitative descriptor of all endogenous low-molecular-weight components in a biological sample such as urine or plasma. Each cell type and biological fluid has a characteristic set of metabolites that reflects the organism under a particular set of environmental conditions and that fluctuates according to physiological demands. The metabolome can be divided into the primary metabolome (as controlled by the host genome) and the co-metabolome (dependent on the microbiome).
Metabonome	Theoretical combinations, sums and products of the interactions of multiple metabolomes (primary, symbiotic, parasitic, environmental, and co-metabolic) in a complex systems.
Metabolomics	The comprehensive quantitative analysis of all the metabolites of an organism or a specific biological sample.
Metabonomics	The quantitative measurement over time of the metabolic responses of an individual or population to a disease, drug treatment or other challenge.
Microbiolome	The consortium of microorganisms, bacteria, protozoa, and fungi that live commensally or symbiotically with a host.
Xenometaolome	Characteristic profile of non-endogenous compounds such as drugs, their metabolites and their excipients, dietary components, herbal medicines and environmental exposure.
Proteome	The expressed protein and peptide complement of a cell, organ or organism, including all isoforms and post-translational variants.
Proteomics	The systematic analysis of proteins for their identity, quantity and function.

Table 2
Most Promising Candidate Molecular Markers of Endothelial Inflammation and Toxicity (all in plasma or serum)

Molecular Markers	Description	Transplantation	References
C-reactive protein	<ul style="list-style-type: none"> C-reactive protein (CRP) is the best characterized of the currently available inflammatory biomarkers and has emerged as a potential marker for cardiovascular risk. Composed of 5–23-kDa subunits, CRP is a circulating pentraxin that plays a major role in the human innate immune response. 	CRP has been included in several clinical studies to assess cardiovascular risk in transplant patients. In none of these studies were the endothelial effects induced by immunosuppressants differentiated from other endothelial stress factors after transplantation.	[83]
cellular adhesion molecule 1 (ICAM-1)	Leukocyte adhesion molecule	The effects of cyclosporine on endothelial ICAM-1 have been studied in vitro and in animal models, but there is no clinical study assessing ICAM-1 as a clinical marker of immunosuppressant-induced vascular effects.	[84]
Soluble adhesion molecule (sVCAM-1)	Leukocyte adhesion molecule	This marker was used to compare the impact of two immunosuppressive regimens (CsA/Aza vs. Tac/MMF) in 52 kidney transplant patients, both before graft and 3, 6, 9 and 12 months after transplantation, in reference to 50 healthy controls. Endothelial effects induced by immunosuppressants were not differentiated from the other endothelial stress factors after transplantation.	[85]
Visfatin	<ul style="list-style-type: none"> Visfatin, also known as nicotinamide phosphoribosyl transferase (NAMPT), is expressed in endothelial cells. It is an independent predictor of levels of soluble vascular cell adhesion molecule (sVCAM)-1, a marker of endothelial damage. 	Fifty-eight living donor kidney transplant non-diabetic recipients, 31 on cyclosporine and 27 on tacrolimus immunosuppression, were studied longitudinally. Endothelial function improved during the first month after transplantation, and the extent of improvement correlated with reductions in circulating visfatin, adiponectin and hsCRP levels. Visfatin was the strongest predictor of brachial artery flow mediated dilatation both before and after kidney transplantation.	[86]
Endothelin-1 (ET-1)	<ul style="list-style-type: none"> Secretion of ET-1 results in long-lasting vasoconstriction, increased blood pressure and, in turn, overproduction of free radicals. Dysregulation of the endothelin system is an important factor in the pathogenesis of several diseases including atherosclerosis, hypertension. 	Most investigators have found an increase in endothelin-1 levels during treatment with cyclosporine, although this is not a consistent finding. The effect of cyclosporine on endothelin-1 release and levels in rat has extensively been studied.	[86–88]
Serum amyloid A (SAA)	Acute phase reactant	SAA has been discussed as a rejection marker of heart and kidney allografts, but has not systematically been studied as a marker to evaluate the effects of immunosuppressants on endothelial cells.	[89,90]
Interleukins (IL): IL-6, IL-8, IL-18	<ul style="list-style-type: none"> Pro-inflammatory cytokines IL-18 is an inflammatory marker produced by macrophages that stimulates release of interferon gamma by T-cells 	The potential value of cytokines as cardiovascular risk markers after transplantation and to monitor the cardiovascular effects of immunosuppressants has not yet systematically been studied.	[91]
P-selectin	<ul style="list-style-type: none"> P-selectin belongs to the family of selectin adhesion molecules 	In a study, to assess the effects of various immunosuppressive drugs on platelet function of renal transplant patients, soluble P-selectin levels were measured in 40 kidney transplant patients. Soluble P-selectin levels were appreciably higher in cyclosporine-treated patients, and statistically	[92]

Molecular Markers	Description	Transplantation	References
	<ul style="list-style-type: none"> It is expressed by platelets and endothelial cells on stimulation. This pattern of expression may indicate an involvement of this molecule in inflammation and coagulation. 	significant differences were observed compared with those of tacrolimus-treated patients ($p < 0.05$), hypertensive subjects ($p < 0.01$), and healthy subjects ($p < 0.05$).	
Matrix metalloproteinase-9	<ul style="list-style-type: none"> Member of the metallo proteinase family Breaks down collagen fragments Degradation of the matrix by MMP-9 at the endothelial layer promotes recruitment of monocyte-derived cells into the sub-endothelial space. 	Endomyocardial biopsies and serum samples were obtained from 66 recipients at 1, 2, 3, 4, 7, 12, 24, and 52 weeks post-transplant during the routine follow-up protocol, and MMP-1, MMP-8, MMP-9, and tissue inhibitor of metalloproteinases (TIMP)-1 serum concentrations were measured by enzyme-linked immunosorbent assay (ELISA). Immunosuppression comprised cyclosporine (CyA; $n=46$) or tacrolimus (TAC; $n=20$) with mycophenolate mofetil and steroids. Early increase in MMP and TIMP serum levels following cardiac transplantation indicates involvement of these molecules in the reaction of the transplant to ischemia-reperfusion or early immunologic adaptation processes of the host. Endothelial effects induced by immunosuppressants were not differentiated from the other endothelial stress factors after transplantation.	[93]
Fetuin-A	<ul style="list-style-type: none"> circulating levels of fetuin-A, a well-described inhibitor of calcification, regulate the cell-dependent process of osteogenesis. low circulating fetuin-A levels are associated with a greater prevalence and/or severity of vascular calcification. 	Fetuin A was studied as a marker to evaluate the risk of vascular calcifications in cyclosporine ($n=21$) and tacrolimus-treated kidney transplant patients ($n=21$). A positive correlation between fetuin-A levels and brachial artery endothelium-dependent vasodilatation. Endothelial effects induced by immunosuppressants were not differentiated from the other endothelial stress factors after transplantation.	[94]
Asymmetric dimethyl arginine (ADMA)	<ul style="list-style-type: none"> ADMA is an endogenous inhibitor of nitric oxide (NO) synthase. By competitively displacing L-arginine from the substrate binding site of NO synthase, ADMA interferes with many of the physiological functions of NO, like endothelium-dependent vasodilatation and leukocyte adhesion. 	The levels of ADMA and CRP were studied in 11 kidney transplant patients receiving cyclosporine and 16 kidney transplant patients receiving tacrolimus-based immunosuppression before and after transplantation. The results indicated that ADMA is associated with brachial artery endothelium-dependent vasodilatation in chronic kidney disease both before and after kidney transplantation. Endothelial functions improve at the very beginning of the post-transplantation period with accompanying reduction in ADMA and CRP levels. Endothelial effects induced by immunosuppressants were not differentiated from the other endothelial stress factors after transplantation.	[95]
Homocysteine	<ul style="list-style-type: none"> Homocysteine is a sulfhydryl-containing amino acid derived from dietary methionine. Multiple mechanisms relate hyperhomocystinemia to vascular risk, including endothelial dysfunction, platelet activation, a pro-inflammatory response, and accelerates oxidation of LDL-C 	The effects of homocysteinemia was evaluated in a cross-sectional study based on 47 patients (30 males, 17 females) who received unrelated living donor renal transplants. Serum homocystein concentrations correlated with higher cyclosporine trough levels and obesity. Hyperhomocysteinemia was more common among patients taking MMF than azathioprine, but had no effect on intrarenal resistive index or carotid intima-media thickness.	[96]
Isoprostanes	<ul style="list-style-type: none"> $F_{2\alpha}$ isoprostane detection is one of the best characterized stable oxidative stress markers. since calcineurin inhibitors cause oxidative stress, oxidative stress markers may be directly linked to their toxicodynamic mechanism. 	The effects of immunosuppressants on isoprostane formation has mostly been studied in urine.	[68]

Molecular Markers	Description	Transplantation	References
Erythrocyte antioxidant status	The parameters measured in erythrocytes included several or all of the following: glutathione, methemoglobin, superoxide dismutase, catalase, glutathione peroxidase, glucose-6-phosphate dehydrogenase, alpha-tocopherol and malondialdehyde.	Cyclosporine induces endothelial and smooth muscle dysfunction via an increase of the concentrations of reactive oxygen species. The erythrocyte antioxidant status has been used as a parameter to assess the efficacy of anti-oxidant therapies mainly in rat models but also in clinical trials.	[97,98]
Circulating endothelial cells	<ul style="list-style-type: none"> • Circulating endothelial cells are mature cells that are shed from the vessel wall in response to injury • Given the extremely low number of circulating endothelial cells in healthy individuals, an increase in circulating endothelial cells indicates the presence of endothelial damage. • Increased circulating endothelial cell levels have been reported in cardiovascular disease with prognostic implications. 	This marker was used to compare the impact of two immunosuppressive regimens (CsA/Aza vs. Tac/MMF) in 52 kidney transplant patients, both before graft and 3, 6, 9 and 12 months after transplantation, in reference to 50 healthy controls. Endothelial effects induced by immunosuppressants were not differentiated from the other endothelial stress factors after transplantation.	[85]
Endothelial microparticles	<ul style="list-style-type: none"> • Endothelial microparticles are microvesicles (0.1 to 1.5 µm) released from the membrane of activated or apoptotic endothelial cells. • Endothelial microparticles express endothelial specific surface markers reflecting their cell origin and state of activation. • Endothelial microparticles are not only a reflection of endothelial dysfunction, but may also induce or enhance preexisting vascular dysfunction, as shown by their ability to impair nitric oxide (NO) release from vascular endothelial cells. • Endothelial microparticle levels correlated with the severity of endothelial dysfunction as assessed by angiography. 	This marker was used to compare the impact of two immunosuppressive regimens (CsA/Aza vs. Tac/MMF) in 52 kidney transplant patients, both before graft and 3, 6, 9 and 12 months after transplantation, in reference to 50 healthy controls. Endothelial effects induced by immunosuppressants were not differentiated from the other endothelial stress factors after transplantation.	[85]

Table 3
Most Promising Candidate Molecular Markers of Immunosuppressant Kidney Toxicity in Urine

Molecular Markers	Description	Transplantation	References
Calbindin	<ul style="list-style-type: none"> Calbindin D is a vitamin D-dependent calcium-binding protein of 28 kDa that is found predominantly in the epithelial cells of the distal tubules of the kidney. Nephrotoxic drugs and diseases involving the distal tubule have been shown to change calbindin concentrations in urine. 	Increased calbindin concentrations after exposure to cyclosporine or tacrolimus were found in rat urine, in the rat kidney and in an anecdotal study in humans.	[104,120–122]
Cystatin C	<ul style="list-style-type: none"> 13 kDa extracellular inhibitor of cysteine proteases. Serum concentrations are independent of gender, muscle mass and age. Is freely filtered, reabsorbed and catabolized by the proximal tubulus. There is no active excretion. Urinary cystatin C concentrations are elevated in patients with tubular injury 	A study in 30 kidney transplant showed that cystatin C concentrations in plasma increased as a result of injury caused by alloimmune reactions and/or nephrotoxicity.	[123–125]
Cystein-Rich Protein (Cyr61)	<ul style="list-style-type: none"> Is a heparin binding protein that is secreted and associated with cell surfaces and extracellular matrix. Was found to be secreted in the straight proximal tubulus only a few hours after injury It must be considered a limitation that urinary concentrations were found to decrease over time although kidney injury was progressing. 	Is a promising molecular marker for nephrotoxicity, but has not yet been studied in immunosuppressant-induced toxicity.	[126–127]
α -glutathione-S-transferase (α -GST)	<ul style="list-style-type: none"> Cytosolic enzyme in the proximal tubule The appearance of α-GST is due to leakage of cytosolic content into the urine, dying cells or due to shedding of viable or apoptotic cells into the urine. 	Was tested in clinical trial with up to 69 kidney transplant patients. Interestingly, these studies suggested that α -GST and π -GST can differentiate between immunosuppressant nephrotoxicity and acute rejection.	[128–130]
π -glutathione-S-transferase (π -GST)	<ul style="list-style-type: none"> Cytosolic enzyme in the distal tubule and collection duct. Is released into the urine likely <i>via</i> the same mechanisms as α-GST. Has been used together with α-GST to differentiate between proximal and distal tubule damage. 	<i>vide supra</i>	[128–132]
Heart-type fatty acid- binding protein	<ul style="list-style-type: none"> belongs to the family of 15 kDa cytoplasmic fatty acid-binding proteins Is an injury marker of the distal tubule 	Has been listed as a marker of nephrotoxicity and as a marker of kidney function after renal transplantation in reference [119]	[119,133]
Kidney injury molecule-1 (KIM-1)	<ul style="list-style-type: none"> A type 1 trans-membrane protein not detected in normal kidney tissue 	Cyclosporine increases KIM-1 expression. Interestingly, most studies did not utilize urinary KIM-1 concentrations, but measured mRNA in kidney tissue [134–136]. Although a promising marker for immunosuppressant-induced	[119,134–140]

Molecular Markers	Description	Transplantation	References
Liver-type Fatty Acid Binding Protein (L-FABP)	<ul style="list-style-type: none"> Is expressed at very high levels in case of dedifferentiated proximal tubulus cells, after ischemic or toxic injury and in case of renal cell carcinoma A soluble form of cleaved KIM-1 can then be detected in urine 	nephrotoxicity, most studies are in rats and there are no clinical studies in transplant patients studying KIM-1 as a potential marker of immunosuppressant nephrotoxicity.	[119,141,142]
	<ul style="list-style-type: none"> Liver fatty acid binding protein is a 14-kDa protein that is normally expressed in the kidney proximal convoluted and straight tubuli Increased urinary L-FABP concentrations were found in patients with acute kidney injury, non-diabetic chronic kidney disease, early diabetic nephropathy, idiopathic focal glomerulosclerosis [and polycystic kidney disease. A challenge is that due to its size L-FABP can be filtrated, but is mainly taken up by the proximal tubulus. There is some evidence that plasma may not affect urine concentrations. 	Has been listed as a marker of nephrotoxicity and as a marker of kidney function after renal transplantation in reference [119]	
	<ul style="list-style-type: none"> It is the 11.8 kDa light chain of the MHC I molecule expressed on the surfaces of nucleated cells Its monomeric form is filtrated and re-absorbed in the proximal tubulus Has been shown to be an early marker of tubular dysfunction 	The effect of cyclosporine of β_2 -microglobulin concentrations in urine was studied in 77 bone marrow transplant [143] and 83 kidney transplant patients [144]. The latter study showed that β_2 -microglobulin concentrations in urine were unable to distinguish between alloimmune injury, cyclosporine nephrotoxicity and kidney injury caused by CMV infection.	
	<ul style="list-style-type: none"> NAG (> 130 kDa) is a proximal tubule lysosomal enzyme. sensitivity, subtle alterations in the epithelial cells in the brush border of the proximal result in shedding of the enzyme into urine Increased NAG concentrations in urine have been found after exposure to nephrotoxic drugs, in patients with delayed renal allograft function, with acute kidney injury, with chronic glomerular disease, with diabetic nephropathy and following cardio-pulmonary bypass. 	NAG has extensively been used as a non-invasive urinary marker of cyclosporine-induced kidney injury in rat studies. Several studies in kidney transplant patients (all with n< 40) have shown that it is a sensitive indicator of kidney dysfunction, but alone cannot distinguish between immunosuppressant toxicity and alloimmune injury.	
	<ul style="list-style-type: none"> NGAL is a lysosomal enzyme that seems to play a role in apoptosis, triggers nephrogenesis by stimulating the conversion of mesenchymal cells into kidney epithelia and in the kidney is mainly located in the proximal tubulus. Its size is about 25kD and it is protease resistant. It is filtered by the kidney and its plasma/urine concentration relationship will require further clarification. There is evidence that NGAL may be useful as a sensitive and predictive marker of ischemia/reperfusion, acute kidney injury, nephrotoxicity and chronic kidney disease. 	Serum and urinary NGAL was used as a nephrotoxicity marker in 19 children with steroid-dependent nephrotic syndrome. Both serum NGAL and urinary NGAL concentrations increased during the course of cyclosporine treatment. However, based on the serum and urinary NGAL/creatinine receiver operating characteristic curve and area under the curve (AUC) analysis, it remains uncertain whether urinary NGAL is a good predictor of cyclosporine nephropathy [154].	
Neutrophil gelatinase-associated lipocalin (NGAL)	<ul style="list-style-type: none"> A 21 kDa protein that is synthesized in the kidney and is involved in vitamin A transport 	A study in 36 heart transplant patients suggested that retinol binding protein is a predictive marker of cyclosporine nephrotoxicity [158].	[144,158–160]

Molecular Markers	Description	Transplantation	References
	<ul style="list-style-type: none"> It is freely filtrated and reabsorbed in the proximal tubulus Plasma and urine concentrations may be associated and vitamin A deficiency may cause false negatives 		
Sodium/hydrogen exchanger isoform 3	<ul style="list-style-type: none"> Located in the proximal tubule and Henle's loop The sodium/hydrogen exchanger continuously reabsorbs the bulk of the filtered sodium, controlling salt delivery to the distal nephron which is critical for tubuloglomerular feedback autoregulation and for fine control of salt excretion in the distal nephron 	Has been listed as a marker of nephrotoxicity and as a marker of kidney function after renal transplantation in references [119]	[119,161]
Micro RNAs in urine	<ul style="list-style-type: none"> Micro RNAs can be determined in cells released into urine. Urinary micro RNAs reflect changes in the kidney 	Urinary micro RNAs have shown promise in the diagnosis of acute rejection and viral infections, however, immunosuppressant-induced nephrotoxicity has not yet been studied.	[162–164]
Urinary metabolite marker panel:	A combination of markers of glomerular filtration (creatinine), reabsorption (glucose), tubulus cell metabolism (citrate, oxoglutarate, lactate), active secretion and kidney amino acylase activity (hippurate), as well as oxidative stress (isoprostanes), and the release of metabolites protective against the protein-precipitating effect of uric acid (trimethyl amine-N-oxide)	This molecular marker panel was found to be sensitive in rat studies [24,103,104] and translated into healthy individuals [68]. In this study it was shown that metabolite patterns in urine changed already within the first 4 hours after a single oral 5 mg/kg cyclosporine (Neoral) dose. Clinical trials in kidney transplant patients are currently in progress.	[24,68,103,104]
<ul style="list-style-type: none"> creatinine citrate oxoglutarate lactate hippurate isoprostanes trimethylamine N-oxide 			

Table 4
Most Promising Candidate Molecular Markers of Gastrointestinal Toxicity

Molecular Markers	Description	Transplantation	References
Citrulline in serum	<ul style="list-style-type: none"> Citrulline is an amino acid formed almost exclusively in enterocytes and is not present in food proteins [165]. Clinically relevant are decreased citrulline levels as they reflect a lack of functional mass of enterocytes. 	Citrulline was studied as a molecular marker after bowel transplantation. Of 5195 citrulline samples, average serum citrulline levels decreased significantly when the patients presented a rejection episode [68]. The potential of citrulline to assess and monitor immunosuppressant-induced gastro-intestinal toxicity and inflammation has not yet been studied.	[165–170]
Calprotectin in feces	<ul style="list-style-type: none"> Calprotectin is measured in feces and is a member of the S-100 protein family. Calprotectin is present in squamous epithelial cells (not in normal cells), neutrophils and macrophages Inflammation stimulates its excretion into gut. 	Calprotectin levels were measured in 11 intestinal transplantation patients during 2 years' follow-up. Calprotectin determinations were correlated with histological and clinical findings. fecal calprotectin dosage showed a good sensitivity but low specificity for the diagnosis of intestinal rejection because high calprotectin levels can also be observed in other clinical conditions [171]. The potential of calprotectin to assess and monitor immunosuppressant-induced gastro-intestinal toxicity and inflammation has not yet been studied.	[171–174]
Lathosterol in feces	<ul style="list-style-type: none"> indicates bile mal absorption when mucosa is dysfunctional 	It has been used as a marker to study transplant ileum function in a pig model [175]. Since lathosterol also reflects cholesterol synthesis and hepatic parenchymal function, serum lathosterol has been used as a marker of liver transplant function [176]. Lathosterol as a marker of immunosuppressant-induced intestinal toxicity has not yet been explored.	[175,176]
¹³ C sucrose absorption	The ¹³ C-sucrose breath test measures enterocyte sucrase activity as a marker of small intestinal villus integrity and function.	The sucrose absorption test has not yet been tested to assess immunosuppressant-induced intestinal toxicity and inflammation.	[177]
⁵¹ Cr EDTA absorption	Is specific for small intestine and the integrity of tight junctions	Has been used to study intestinal integrity in bone marrow transplant patients after cytotoxic therapy. The potential of ⁵¹ Cr-EDTA absorption to assess and monitor immunosuppressant-induced gastro-intestinal toxicity and inflammation has not yet been studied.	[178]