Barcelona Consensus on Biomarker-Based Immunosuppressive Drugs Management in Solid Organ Transplantation

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Abstract: With current treatment regimens, a relatively high proportion of transplant recipients experience underimmunosuppression or overimmunosuppression. Recently, several promising biomarkers have been identified for determining patient alloreactivity, which help in assessing the risk of rejection and personal response to the drug; others correlate with graft dysfunction and clinical outcome, offering a realistic opportunity for personalized immunosuppression. This consensus document aims to help tailor immunosuppression to the needs of the individual patient. It examines current knowledge on biomarkers associated with patient risk stratification and immunosuppression requirements that have been generally accepted as promising. It is based on a comprehensive review of the literature and the expert opinion of the Biomarker Working Group of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology. The quality of evidence was systematically weighted, and the strength of recommendations was rated according to the GRADE system. Three types of biomarkers are discussed: (1) those associated with the risk of rejection (alloreactivity/tolerance), (2) those reflecting individual response to immunosuppressants, and (3) those associated with graft dysfunction. Analytical aspects of biomarker measurement and novel

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pharmacokinetic–pharmacodynamic models accessible to the transplant community are also addressed. Conventional pharmacokinetic biomarkers may be used in combination with those discussed in this article to achieve better outcomes and improve long-term graft survival. Our group of experts has made recommendations for the most appropriate analysis of a proposed panel of preliminary biomarkers, most of which are currently under clinical evaluation in ongoing multicentre clinical trials. A section of Next Steps was also included, in which the Expert Committee is committed to sharing this knowledge with the Transplant Community in the form of triennial updates.

Key Words: biomarkers of immunosuppression, immunologic biomarkers, consensus, assessment of acute rejection, graft outcome, graft injury, pharmacogenetics, pharmacokinetics, pharmacodynamics, personalized immunosuppression, solid organ transplantation

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INTRODUCTION

Most transplanted patients currently receive an immunosuppressive regimen consisting of induction therapy, combined with calcineurin inhibitors (CNIs) (mostly tacrolimus [Tac]), glucocorticoids, and mycophenolic acid (MPA). At present, treatment is not selected on the basis of individual immune alloreactivity, and immunosuppressive drug doses are guided mainly by the development of side effects and achievement of target drug concentrations in peripheral blood. The current strategy may lead, in a considerable number of cases, to either underimmunosuppression (resulting in rejections) or overimmunosuppression (resulting in opportunistic infections, malignancies, and toxicity). Thus, the treatment regimens currently used remain unsatisfactory, and new approaches are needed to address the issue of tailored immunosuppression.

In the last 10 years or so, several promising biomarkers have been identified for detecting the degree of alloreactivity of individuals, for determining personal response to treatment and individual drug doses, and for diagnosing graft dysfunction and injury. It is unlikely that one single biomarker will suffice to reflect all the complexities associated with organ transplantation, so consideration will have to be given to a comprehensive panel of distinct biomarkers to guide therapy in clinical practice.

Furthermore, recent scientific advances in the field of pharmacokinetics (PK), pharmacogenetics (PG), and pharmacodynamics (PD) have yielded a number of candidate biomarkers associated with outcomes and/or side effects. Other immunological biomarkers correlate well with rejection, graft dysfunction, and predicted tolerance, and offer a realistic opportunity for personalized immunosuppression. Use in the clinic of selected biomarkers that can predict the alloreactive susceptibility (assessment of risk of rejection) and response of the individual to treatment (risk of inefficacy and toxicity) will pave the way toward personalized immunosuppressive therapy in the care of transplanted patients. In this consensus document, a predictive biomarker can be considered as a tool for assessing the risk of rejection or drug-related adverse events. It may predict the risk of rejection, opportunistic infection, and malignancy, and may identify subpopulations that are likely to benefit from a certain immunosuppressive treatment.

The *Barcelona Consensus on Biomarker-Based Therapeutic Drug Monitoring in Solid Organ Transplantation* examines current knowledge on biomarkers associated with patient risk stratification and immunosuppression requirements that have been generally accepted in the literature as promising.

This consensus document was based on a comprehensive review of promising biomarkers and the expert opinion of the Biomarker Working Group (BWG) of the International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDMCT) (BWG acts as the Expert Committee of this consensus document). To achieve this goal, 3 types of biomarkers were identified: (1) those associated with the risk of rejection (alloreactivity/tolerance), (2) those reflecting individual response to immunosuppressants, and (3) those associated with graft dysfunction and injury. The analytical aspects of biomarker measurement and the requirement for standardized testing methods and new PK-PD models that are more accessible to the transplant community were also addressed.

This Expert Committee, consisting of 19 experts in the field of therapeutic drug monitoring (TDM) of immunosuppressive drugs and biomarkers in transplantation, reviewed articles published since 2000 and other data analyzed before 1999, as required. The Committee looked specifically for published clinical trials (retrospective or randomized) and existing meta-analyses. However, only a limited number of these types of studies were available, so many recommendations were developed from observational studies or small case studies. To evaluate evidence on promising biomarkers (biomarker panels), the Expert Committee followed the recommendations summarized in Table 1, which includes systematic weighting of the quality of evidence and a graded recommendation according to the Grading of Recommendations Assessment, Development and Evaluation (GRADE) Working Group,[1,2] and Table 2, which summarizes specific criteria for biomarker evaluation.

The Expert Committee communicated frequently by e-mail, and met in person on 2 occasions, the second time to arrive at a consensus. All members of the Barcelona Consensus Document Committee complied with the policy on conflicts of interest, which requires disclosure of any financial or other interest that might be construed as constituting an actual, potential, or apparent conflict. Potential conflicts of interest are listed in the disclosures of the article. The committee will determine the need for revisions to the consensus document at 3 yearly intervals.

This consensus document will incorporate, for the first time, the opinion of several groups of experts in the field. It has been designed to discuss the utility of measuring selected currently available biomarkers shown to be associated with the risk of rejection, immunosuppression requirements, drug-related efficacy and toxicity, and graft function. Biomarkers should help to tailor immunosuppressive therapy to the needs of the individual patient. The aim is to identify biomarkers with documented clinical utility that have been evaluated

TABLE 1. Grading System for Recommendations and Evidence Level Used in the Consensus Document

Category, Grade	Definition
Strength of recommendation	
A	Good evidence to support a recommendation for biomarker monitoring
B	Moderate evidence to support a recommendation for biomarker monitoring
C1	Recommendation for biomarker monitoring regardless of poor evidence
C2	Poor evidence to support a recommendation for marker monitoring
Quality of evidence	
I	Evidence from ≥1 properly randomized, controlled multicenter clinical trial using validated methodology
II	Evidence from ≥1 well-designed cohort or case–controlled nonrandomized clinical trial, multiple time series, standardized methodologies
III	Evidence from opinions of respected authorities, based on clinical experience, descriptive studies, or reports from expert committees

using standardized and validated methodologies in independent populations. The Expert Committee has decided that donor-specific antibodies (DSA)/anti-HLA antibodies will not be covered here, because these biomarkers are discussed in depth in the literature. Likewise, other interesting biomarkers such as gene microarrays[3,4] and miRNAs[5] will not be considered in this document; the authors' general position is that further studies are required to assess the combination of these biomarkers with TDM of immunosuppressive drugs.

BIOMARKERS ASSOCIATED WITH THE ASSESSMENT OF THE RISK OF REJECTION

T-Cell IFN-γ and IL-2 Cytokines as Predictive Markers of the Risk of Allograft Rejection

Background

Multiple cytokines can mediate effector and regulatory effects on the immune response,[6,7] and their production and secretion can be modified by immunosuppressive drugs after ex vivo stimulation. The impact of these drugs on the synthesis of the cytokines interleukin (IL)-2 and interferon (IFN)-γ has shown wide interindividual variability, which suggests that monitoring cytokines may be useful both for predicting the risk of rejection, by identifying biomarkers of alloreactivity, and for reflecting personal susceptibility to immunosuppressive drugs.[8,9]

TABLE 2. Criteria for Biomarker Evaluation

- Status of the clinical validation
  - Role of the biomarker in the improvement of conventional TDM
  - Utility of the biomarker for predicting individual drug response
    - Assessment of minimal necessary exposure
    - Prevention of overimmunosuppression
  - Efficacy of the biomarker for predicting the risk of rejection
  - Usefulness of the biomarker for predicting graft and patient outcome
- Analysis of agreement or controversy in the results obtained from different studies (pros and cons for each biomarker), taking into consideration:
  - Single-center experience
  - Multicenter experience
  - Multicenter randomized-controlled trials
  - Methods used in the study: standardized or certified (interlaboratory cross-validation)
- Define the clinical utility and limitations of each biomarker
  - Implementation
    - Pretransplant, to select the appropriate immunosuppressive regimen
    - Pretransplant, to recommend initial dosing
    - Posttransplant, repeatedly, to support better individualized dosing (than traditional TDM alone)
    - Complexity of the bioanalytical method
  - Application
    - Biomarker as a "robust," "standalone" predictor for drug selection or tailored dosing
    - Biomarker that should be included in a panel of selected biomarkers for predicting rejection risk, graft function, and individual response to immunosuppressive agents
    - Biomarker included in a formal (population-PK/PD) (outcome) model as one of several cofactors
    - Availability of algorithms to convert biomarker information into clinical recommendations

IFN-γ as a Predictive Biomarker of Individual Alloreactivity and Risk of Rejection

Executive Summary

- Monitoring intracellular or total IFN-γ before and early after transplantation can help to identify kidney and liver transplant recipients at high risk of acute rejection (B, II).
- Monitoring IFN-γ production with donor-specific stimulation can help identify patients who are candidates for immunosuppression minimization (B, II).
- Ongoing multicenter clinical trials using validated methods are now evaluating the clinical utility of IFN-γ production, both pretransplantation and posttransplantation as an early predictive biomarker of the risk of rejection and graft clinical outcome.

Literature

IFN-γ has a pleiotropic effect[10]; in some physiological circumstances, it elicits inflammatory T helper (Th) 1–driven immune responses, whereas in others, it enables regulatory T cells (Treg) to control immune responses.[11]

Assessment of IFN-γ by the enzyme-linked immunospot (ELISPOT) assay has been used to evaluate the pretransplantation and early posttransplantation frequency of donor-specific IFN-γ-producing T cells and their impact on posttransplantation clinical outcome.[12] High frequencies of

donor-reactive memory effector T cells are associated with increased IFN-γ production, a high risk of acute rejection, and poorer first-year renal graft function.[13–15] Graft function was defined by the authors considering the simplified modification of diet in renal disease (MDRD) formula to calculate the glomerular filtration rate (GFR) and creatinine levels. Furthermore, delayed graft function was defined as the need for dialysis during the first week after transplantation, and acute rejection episode was defined as an increased creatinine level that was not attributable to other reasons, with a subsequent return to baseline after antirejection treatment. Moreover, the finding that circulating donor-specific alloreactive T cells were detectable long after transplantation suggests that T-cell-mediated chronic graft damage may persist in the long term and that those biomarkers of alloimmunity could be useful to identify patients with progressive immune-mediated graft injury.[16]

Because the type of cell subpopulation can determine whether the immune response will be effector or regulatory, there is a growing interest in determining which cell subpopulations synthesize specific cytokines. Several studies have used flow cytometry to analyze intralymphocytary IFN-γ changes, in alloreactive T cells, as a biomarker of risk of rejection.[17] In stable liver transplant recipients undergoing weaning from immunosuppressive therapy, % $CD3^+CD4^+$IFN-γ^+ and %$CD3^+CD8^+$IFN-γ^+ were identified as surrogate markers for the risk of rejection.[18] This promising finding was corroborated later in de novo adult liver transplant recipients.[19,20] Patients with acute rejection had an early significant increase in IFN-γ production by $CD4^+$ and $CD8^+$ cells during the first month after transplantation before the acute rejection was diagnosed (biopsy-proven acute rejection [BPAR]).

In line with these findings, the results from a multicenter prospective study indicate that pretransplantation and posttransplantation analysis of intracellular % $CD3^+CD4^+CD69^+$IFN-γ^+ and %$CD3^+CD8^+CD69^+$IFN-γ^+ T cells, measured by interlaboratory standardized methods, can help to identify liver and kidney transplant recipients at high risk of acute rejection.[19] All patients who rejected organs showed pretransplantation levels of % $CD3^+CD4^+CD69^+$IFN-γ^+ and %$CD3^+CD8^+CD69^+$IFN-γ^+ above the cutoff value established for the risk of acute rejection.[19]

IL-2 as Predictive Biomarker of Individual Alloreactivity and Risk of Rejection

Executive Summary

- Monitoring intracellular IL-2 before and early after transplantation can help to identify kidney and liver transplant recipients at high risk of acute rejection (B, II).
- IL-2 inhibition may reflect interindividual response to CNIs (B, II).
- Ongoing multicenter clinical trials using validated methods are now evaluating the clinical utility of IL-2 production, both before and after transplantation, as an early predictive biomarker of the risk of rejection and personal susceptibility to CNIs.

Literature

IL-2 drives T-cell growth, induces T regulatory (Treg) differentiation, and mediates activation-induced cell death.[21,22] Several studies have shown that IL-2 is necessary for the survival of activated cells and the successful generation of effector responses[23] and regulatory responses.[24]

The %$CD3^+CD8^+$IL-2^+ expression could be a surrogate marker to identify patients at high risk of rejection.[18,25] Pretransplantation IL-2 production in $CD8^+$ T cells was closely related to the onset of acute rejection and was also correlated with the Banff score in adult liver transplant recipients.[26] In stable liver recipients, an increase in the %$CD8^+$IL-2^+ during withdrawal was identified as a prelude to rejection.[18] More recently, in a cohort of de novo liver transplant recipients, % $CD3^+CD8^+$IL-2^+ was significantly higher in rejectors than in nonrejectors, both before and at 1 week after transplantation.[20] Along the same lines, in a multicenter prospective study,[19] both liver and kidney transplant patients with acute rejection showed significantly higher pretransplantation IL-2 production in $CD3^+CD8^+CD69^+$ T cells.

Preliminary studies have shown that intracellular IL-2 may reflect the individual response to CNI.[20,27] The incidence of BPAR was significantly related to inhibition of % $CD3^+CD8^+$IL-2^+ and %$CD3^+CD8^+$IFN-γ^+ during the first week after transplantation, and was unrelated to Tac exposure. BPAR occurred in patients with less than 40% inhibition of %$CD3^+CD8^+$IL-2^+ and %$CD3^+CD8^+$IFN-γ^+ during the first week after transplantation, compared with pretransplantation values.[20]

Limitations of the Methods and Clinical Use of IFN-γ and IL-2 Cytokine Assessment

Monitoring IFN-γ production with donor-specific stimulation can identify patients with an increased immune response to a defined donor antigen. Interestingly, cross-validation data of the IFN-γ ELISPOT assay performed in several European laboratories have shown that this method is effective for the assessment of circulating alloreactive memory effector T cells in renal transplant recipients.[28] However, there are 2 clear disadvantages to the ELISPOT assay: first, donor-specific cells are not usually available in routine clinical practice; and second, it is impossible to simultaneously analyze different lymphocyte subsets and/or effector/regulatory cytokines, which are donor nonspecific immune parameters that can also correlate with graft outcome.

Multiparameter flow cytometry has the advantage of allowing simultaneous analysis of multiple cell phenotypic markers and intracellular cytokine production. Results from multicenter studies[19,29] indicate that protocols for this type of peripheral blood cell phenotyping can be successfully transferred to multiple laboratories with experienced personnel and provide highly comparable results. One drawback of this method is its lack of specificity, given that the intracellular production of some cytokines may also be modulated by other inflammatory conditions (eg, infections). The validated IFN-γ ELISPOT assay[28] with donor-specific stimulation and intracellular cytokine measurement by flow cytometry[19] have shown similar median interlaboratory and intralaboratory coefficients of variation. For both methods, the results of the

analyses are interpreted on the basis of cutoff values determined in previous multicenter studies in kidney and liver transplant recipients.[16,25]

Further investigation in this field is warranted. The impact of confounding clinical factors in the application of these biomarkers for predicting the risk of rejection must be more appropriately evaluated. The optimal time point(s) and frequency for monitoring these cytokines as predictive biomarkers of risk assessment have yet to be established. Combined with other biomarkers and drug exposure, the %$CD3^+CD4^+IFN\text{-}\gamma^+$, %$CD3^+CD8^+IFN\text{-}\gamma^+$, and %$CD3^+CD8^+IL\text{-}2^+$ may complement pharmacokinetic TDM in transplant recipients receiving CNIs.

T-Cell Surface Antigens

Background

T lymphocytes play a central role in the cellular-mediated process of acute graft rejection after solid organ transplantation (SOT).[30] They are characterized by expression of the CD3 (CD = cluster of differentiation) receptor or T-cell receptor on their surface. T-cell activation is a hallmark of the early rejection process in SOT.[31] Upregulated surface antigens as markers of activated T cells (eg, CD25, CD26, CD28, CD38, CD44, CD69, CD71, CD95, CD134, CD152, CD154, CXCR3, CCR5, and HLA-DR) can be assessed either in nonstimulated whole blood or after ex vivo stimulation of whole blood, as well as in isolated peripheral blood mononuclear cells (PBMC) in cell function assays.[31] Some surface antigens are also cleaved off the cell surface and can be determined in the serum or plasma.

Executive Summary

- Donor-specific CD154 expression in T-cytotoxic memory cells with a United States Food and Drug Administration (FDA)-approved ex vivo cell function assay may be used to predict the risk of transplant rejection after liver and small bowel transplantation in patients <21 years (B, II).
- Soluble CD30 (sCD30) in the serum/plasma before and shortly after renal transplantation is associated with long-term kidney graft outcome, but its usefulness as a biomarker to predict acute rejection in SOT is not yet entirely clear (B, II).
- Assessing surface antigens on T cells stimulated in vitro and ex vivo with mitogens in cell function assays reflects the inhibitory effect of immunosuppressants on lymphocyte activation (C2, II).
- CD26 and CD28 surface antigens on T cells assessed directly in nonstimulated whole blood are associated with acute rejection and/or malignancy after kidney and liver transplantation (C2, III).

Literature

The effect of immunosuppressants on T-cell surface antigens has been shown in vitro by supplementing incubation media of cell function assays with various concentrations of immunosuppressive drugs, or by stimulating cells isolated from immunosuppressed patients ex vivo. As a proof of principle, dose–response curves with immunosuppressants have shown their inhibitory effect on surface antigen expression in activated T cells in vitro,[32] and stimulated PBMC isolated from immunosuppressed patients showed less surface marker upregulation ex vivo when compared with cells from healthy controls.[33,34] There are only a few reports on the direct assessment of T-cell subsets (CD4 or CD8) expressing the costimulatory molecules CD26 or CD28. An association with acute rejection and long-term outcome (malignancy) has been reported for CD28 in liver transplantation,[35,36] and with acute rejection in renal transplantation for CD26.[37] One group has extensively explored the surface marker CD30, in its soluble form in serum (sCD30), and found an association with acute rejection and long-term kidney function in renal transplantation.[38–40] This was true for plasma concentrations determined both before and after transplantation.[39,41] High serum sCD30 concentrations before transplantation combined with panel reactive antibodies were associated with remarkably poor graft outcome.[40] However, a more recent meta-analysis questioned the value of pretransplantation sCD30 in predicting acute rejection.[42] The significance of sCD30 for organs other than the kidney is less clear. The soluble IL-2 receptor is another T-cell activation marker that can be measured like sCD30 by immunoassay of the serum or plasma and has been shown to be associated with acute rejection in renal transplantation.[43]

T-Cell Surface Antigens: Methods and Association With Clinical Outcome

Stimulation in cell function assays can be achieved by donor alloantigens, third party antigens (cells or peptides), antibodies to T cells or T-cell surface proteins, or by mitogens. Antigen expression is usually followed by flow cytometry using fluorescent antibodies. Immunoassays are available to measure soluble surface antigens in the serum/plasma.

Most data on the association between T-cell surface antigens and clinical outcome have been reported in the early phase after kidney transplantation, concluding that surface marker expression was more useful to rule out, rather than to predict acute rejection (high negative predictive values). This is conceivable because T-cell activation is not restricted to immune activation due to tissue incompatibility but can also be triggered by other events such as infections. However, the costimulatory molecule CD154 can be used to predict acute rejection in young patients with liver and small bowel transplantation.[44,45] The Pleximmune cell function assay, using donor-specific stimulation, has obtained FDA clearance for this indication. The assay is performed in a central laboratory in the United States, which improves its reproducibility, but limits its worldwide dissemination and turn-around time.

Limitations of the Clinical Use of T-Cell Surface Antigen Assessment

A drawback of most cell function assays is the need for cell isolation and incubation times from 7 hours to 120 hours.[46] Another difficulty is the lack of assay standardization and limited cell stability, which are obstacles for multicenter trials. In contrast, soluble proteins in the circulation can be assessed by commercial assays, thereby improving

comparability between laboratories. However, this approach also has limitations; because surface antigens can also be released from cells other than T cells, such as activated endothelial and B cells, compromising their specificity as biomarkers for T-cell activation.[47,48] It is a matter of debate whether nonspecific stimulation (eg, by mitogens) or donor-specific stimulation by donor cells or donor antigens is more meaningful in cell function tests. In the first case, the general effect of immunosuppression on T-cell activation can be compared between individuals; in the second case, a donor-specific effect is observed, which may be more useful to personalize immunosuppression.

T-Cell Surface Antigens: Clinical Implementation

None of the surface antigens can be currently recommended to tailor immunosuppression in clinical transplantation, or to complement TDM. No data have been reported to justify the use of surface antigens to predict the individual response to a specific drug. CD154 is intended to predict acute rejection in patients aged <21 years with liver and small bowel transplantation, whereas sCD30 may be used to estimate kidney graft outcome. Although information from the Pleximmune assay and sCD30 is potentially considered by transplant physicians in their choice of immunosuppression, there are no controlled prospective clinical trials that have proven that adjusting immunosuppression based on surface antigen expression or the concentration of soluble surface antigens in the serum will improve the outcome of graft recipients.

T-Cell Regulatory Populations

Background

Tregs are basically defined by their capacity to suppress effector immune responses, and in the context of transplantation, to control alloreactive responses. This is why they have been considered as potential biomarkers for SOT, to monitor immunosuppression, and to predict clinical events.

Among Tregs, the most extensively studied ones are the $CD4^+$ Tregs. In humans, they are characterized by high expression of CD25 (the α-chain of the IL-2 receptor), in contrast to the effector $CD4^+$ T cells, which express lower, transient levels of CD25, and expression of the transcription factor Foxp3.[49] It has recently been demonstrated that the expression of Foxp3 is not as specific, and the promoter must be demethylated to be specific to Tregs.[50] The phenotype that best characterizes Tregs at present is defined as $CD4^+CD25^{high}FoxP3^+CD27^+CD127^{low/-}$.[51,52] Other Treg subsets have also been identified, such as Tr1 or Th3, although mostly in experimental models, not yet extensively studied in humans as $CD25^+$ Tregs. In addition, several Treg subsets have been described, and the CD45RO expression on $CD4^+CD25^{high}$ Treg cells has been shown to identify activated Tregs with highly suppressive capacity.[53]

Executive Summary

- Low numbers of circulating activated Tregs before transplantation may help to identify renal transplant recipients at high risk of acute rejection (B, II).
- Increased levels of circulating Tregs may help to identify renal transplant recipients at high risk of developing squamous cell cancer (B, II).

Literature

Tregs and Clinical Outcome

The first evidence of the possible role of Tregs in organ transplantation was found in biopsies from renal transplant patients undergoing acute rejection.[54] However, it was later found that this was not specific to rejection. Because Tregs have the potential to control immune responses, many subsequent studies were conducted, in an attempt to demonstrate their role as biomarkers in SOT. Many were performed on whole blood instead of on biopsies, to have a minimally invasive biomarker. A serious limitation was that most of these were not multicenter studies, were limited to special clinical situations, and had very few time points in follow-up. Moreover, most did not measure Tregs in both biopsies and peripheral blood. A few studies monitored the numbers of Tregs in peripheral blood during the first 2 years after transplantation, reporting a decrease of these cells in patients with acute rejection.[55–57] Furthermore, increased levels of circulating Tregs during the first year after renal transplantation were associated with better graft survival at 4 years after transplantation.[58] None of the studies were able to demonstrate any predictive value for rejection from circulating Treg levels. It has also been proposed that the decreased levels of these cells in patients with acute rejection could be more related to a high load of immunosuppression.[56] However, measuring the whole Treg population in peripheral blood might not reflect the cell subset involved in alloreactivity control. Thus, the presence of increased pretransplantation levels of activated Tregs with the phenotype $CD4^+CD25^{high}CD62L^+CD45RO^+$ was associated with increased risk of acute rejection within the first year after transplantation.[57,59]

There is even less evidence of Tregs being associated with chronic rejection, and results in many cases are discordant.[60–62] In fact, international consortia, such as the European RISET or the North American ITN, did not consider monitoring circulating Tregs to be useful in renal transplantation,[61,62] but they did find an exacerbated humoral immune profile. In this regard, another regulatory cell subset, B regulatory cells (Bregs), has been proposed very recently. Data on Bregs in human transplantation are still limited. Moreover, it is unclear whether they represent a cell subset, and to date, no cell lineage transcription factor has been identified. Most of the evidence is limited to mouse models.[63]

The utility of Tregs as biomarkers of rejection or graft outcome in other SOT is even less well studied, and far from being demonstrated.

Other possible clinical applications of Tregs should be explained in the context of patients receiving chronic immunosuppression. The aim is to define drugs able to suppress the effector T-cell responses while maintaining or inducing the activity of Tregs. In line with the previous argument, mammalian target of rapamycin (mTOR) inhibitors (mTORi) may favor the action of Tregs.[64] In addition, long-term treatment with CNIs in stable human renal

transplantation produces a decrease in the number of circulating Treg cells, whereas mTORi maintains the number of circulating Tregs.[65] These findings suggest that mTORi treatment could help to recover the blood levels of Tregs in patients previously treated with CNIs. Furthermore, the presence of high numbers of circulating Tregs before conversion from CNI therapy to mTORi treatment could predict renal recipients who develop squamous cell cancer.[66] This could be one of the most promising clinical applications of monitoring circulating Tregs. However, there are very few studies on the effect of induction therapies on Tregs in renal transplantation. Most are not comparable because of the use of different immunosuppression maintenance regimens. Finally, Tregs have been proposed as a tool to achieve donor tolerance in transplantation, although their implementation in clinical transplantation is limited by the relative success of immunosuppression to avoid acute rejection.[67]

Tregs: Methods and Clinical Implementation

There is clearly a need for clinical trials that investigate the determination of Tregs as biomarkers in peripheral blood. The main difficulties entailed in such trials are the number of phenotypic markers required to define Tregs, and the lack of standardized methods to measure them in peripheral blood. To our knowledge, there is only 1 multicenter study in which standard operating procedures were followed to quantify the numbers of circulating Tregs, even using the same reagent lots to minimize interlaboratory variability.[57] The lack of large, randomized, prospective multicenter cohorts has meant that monitoring Tregs as clinical biomarkers in organ transplantation is far from being implemented in routine clinical practice.

BIOMARKERS THAT REFLECT THE INDIVIDUAL RESPONSE TO IMMUNOSUPPRESSANTS

Background

The combination of synergistic drugs is the main strategy to prevent early acute rejection and to provide long-term effective rejection prophylaxis after organ transplantation. The necessary TDM of immunosuppressive drugs in clinical practice is currently based on measuring drug concentration levels in blood (PK). However, such PK monitoring of immunosuppressants may not predict the individual pharmacological effects on immune cells.[68] Thus, the direct determination of drug targets (eg, enzyme activity or T-cell subsets) as a PD surrogate of the immunosuppressive drug effects may help to better assess the individual response to the immunosuppressant. This review does not discuss the effect of different biologicals on lymphocyte populations but focuses on the clinical relevance and published methods for monitoring PD targets of commonly used classical chemical immunosuppressive drugs.

Target Enzyme Activity as Specific Biomarkers in Transplantation

It is notable that current combination maintenance immunosuppression is mainly based on the inhibition of different enzymes in immune cells, eg, inhibition of calcineurin activity by cyclosporine (CsA) or Tac, inhibition of inosine-monophosphate-dehydrogenase (IMPDH) by MPA, and inhibition of the mTOR complex by everolimus (EVR) or sirolimus (SRL). It is obvious that direct determination of target enzyme activity would provide a straightforward PD approach to directly determine the effect of the immunosuppressant in the individual. Despite 2 decades of research, clinical applicability of this approach is often limited by the complexity of the test systems. Development of a rapid, reliable, and robust assay system, which can be used in clinical practice, is a prerequisite for any PK-PD investigation in larger patient populations.[32] In addition to methodological issues, the validation of and transfer of such PD biomarkers to clinical practice is a long, step-by-step process, largely depending on international collaboration networks.[69]

IMPDH Measurement Methods and Clinical Outcome

Executive Summary

- Determination of IMPDH activity before transplantation might be useful to identify renal transplant recipients at higher risk of acute rejection or MPA-associated side effects (B, II).
- Monitoring IMPDH activity may complement the determination of MPA PK to better guide MPA therapy (B, II).
- Ongoing multicenter clinical trials are using cross-validated methods to evaluate the clinical utility of IMPDH activity to predict the risk of rejection or MPA-associated side effects.

Literature

Development of a rapid, reliable, and robust IMPDH assay system, which can be used in clinical practice, was an important step for thorough PK-PD investigations in larger numbers of MPA-treated patients.[70,71] New insights into the mechanism of action of MPA were obtained by this direct PD assay.[72,73] It was used in several clinical studies, including pediatric cohorts, by different research groups, and is based on the chromatographic determination of newly generated xanthosine 5′monophosphate (XMP) in mononuclear cell lysates. The assay requires only reasonable amounts of blood and can reliably be used in multicenter trials. Pretransplant IMPDH activity may be linked to the genetic background and may provide some valuable indications for the further clinical course (eg, risk of rejection or MPA-associated side effects), which could result in better tailored MPA dosing strategies. Although pretransplant IMPDH activity is not affected by MPA, all subsequent IMPDH determinations are directly influenced by the ongoing MPA treatment. Given the complexities of MPA PK, the best time point (eg, predose) and/or IMPDH sampling strategy (eg, maximum inhibition, area under the effect curve) has yet to be determined as a PD surrogate marker of MPA-associated immunosuppressive effects. In addition, more clinical data from larger cohorts are needed to determine the clinical utility of IMPDH monitoring.

mTOR Activity: Methods and Clinical Outcome

Executive Summary

Monitoring P-p70S6 kinase (phospho-70-kDa ribosomal protein S6 kinase)/pS6RP (phospho ribosomal S6 protein) may complement the determination of mTOR inhibitor trough concentrations to better guide mTOR inhibitor therapy (C1, III).

Literature

With respect to monitoring mTORi, early results using the Western blot or enzyme-linked immunosorbent assay providing data on measurement of mTOR pathway compounds (p70S6 kinase or pS6RP) seem to be promising for enhanced TDM of SRL and EVR after organ transplantation.[74–76] Compared with the Western blot and enzyme-linked immunosorbent assay, the technique of phospho-flow cytometry offers the ability to detect phosphorylated proteins, and to differentiate between activation-induced changes of signaling molecules inside the cell relative to unstimulated populations of identical cells in the same sample.[77]

Additionally, only microliters of whole blood are needed for multiparametric flow cytometric analysis to measure drug potencies and efficacies in vivo[78] and are therefore the ideal tool for PD cell monitoring.[33] At present, only the phospho-flow pS6RP assay has been validated in vitro for the analysis of SRL effects on phosphorylated S6 ribosomal protein (pS6RP) in vitro.[79] Phospho-flow analysis revealed that SRL suppressed pS6RP in human T cells in a dose-dependent manner. In the experience of some groups, storage of whole blood for 24 hours at room temperature or 4°C before analysis seems to display adequate robustness for its clinical use, although data on stability are not consistent across laboratories. Further evaluation of this pS6RP whole-blood assay in 87 EVR-treated heart transplant recipients showed that CsA blood concentration, the duration of EVR treatment, the comedication with thiazide diuretics, and different metabolic parameters could have an influence on the expression of pS6RP in T cells. Additionally, 4 different patterns of EVR responses on pS6RP expression were observed.[80]

Another phospho-flow assay measured p70S6K phosphorylation in PBMCs in renal transplant recipients.[81] Phosphorylation was significantly reduced in isolated PBMC from patients treated with CNIs and mTORi compared with patients on CNIs and mycophenolate. However, the effect did not correlate with the whole-blood trough concentrations of the mTORi. Additionally, it was observed that in the $CD4^+CD25^{low/-}$ subset of T cells, the p70S6K phosphorylation was significantly reduced for patients on EVR, whereas in the circulating $CD4^+CD25^{high}$ Treg cells, the phosphorylation was not affected by the mTORi. This assay has not been validated, so far.

IMPDH and mTOR Activity: Clinical Implementation

To demonstrate clinical relevance, both specific biomarkers of target enzyme activity, IMPDH and mTOR, must be validated in clinical settings and multicenter studies. Based on current findings, any future multicenter prospective study should be carefully designed to (1) formulate the study population, (2) identify inclusion and exclusion criteria, (3) establish a time frame for optimal enzyme activity measurement, and (4) assess baseline values for IMPDH and p70S6 kinase/pS6RP to investigate the outcome of MPA- and mTORi-treated patients after SOT.

Nuclear Factor of Activated T-Cell-Regulated Gene Expression

Background

Several approaches have been undertaken to measure the biologic effects of CNI-based immunosuppression (CsA; Tac) including calcineurin phosphatase activity, cytokine release, and gene expression.[82–90]

Measuring calcineurin phosphatase activity has been proposed as a PD approach to optimize CNI dosing at the molecular target.[91,92] Only small cohorts have been monitored to date, and a consistent correlation between CNI concentrations and calcineurin activity in transplant patients has not been found. A new assay based on liquid chromatography–tandem mass spectrometry (LC–MS/MS) in multiple reaction monitoring (MRM) mode has been described recently, but data in large clinical cohorts are lacking.[93]

Quantitative analysis of gene expression has been established to calculate the functional effects of calcineurin inhibition, specifically inhibition of the transcription of nuclear factor of activated T-cell (NFAT)-regulated genes in peripheral blood.[94,95] This assay is based on the quantitative analysis of IL-2, IFN-γ, and granulocyte macrophage colony-stimulating factor (GM-CSF) gene expression in whole-blood samples collected at CsA/Tac troughs (C_0), and peak levels (2 hours for CsA and 1.5 hours for Tac) after an oral dose.

Executive Summary

- Determination of residual NFAT-regulated gene expression helps to identify renal transplant recipients at higher risk of opportunistic infections, malignancy, acute rejection, and cardiovascular risk (B, II).
- Monitoring residual NFAT-regulated gene expression complements CNI PK to better guide CNI therapy (B, II).
- Ongoing multicenter clinical trials are using cross-validated methods to predict the risk of opportunistic infection, malignancy, and acute rejection.

NFAT Gene Expression: Method

The real-time polymerase chain reaction (RT-PCR) technique provides a rapid, highly reproducible, and sensitive tool for the quantitative analysis of gene expression.[96] The test can be semiautomated, standardized, and performed in a specialized laboratory. Whole-blood samples are stable for 24 hours at 20°C. Although the overall gene expression is reduced on storage, the relative degree of NFAT inhibition remains stable in this period. Therefore, this monitoring technique can be used in larger patient cohorts and in multicenter clinical studies.

NFAT-regulated gene expression has shown low analytical variability in repeated measurements. Although interpatient variability is high, intraindividual variability is low in patients on stable CNI doses.[97] Establishment of this PD

monitoring assay in other specialized laboratories, and external validation of the method, is currently ongoing.

NFAT-Regulated Gene Expression and Clinical Outcome

Beneficial effects have been confirmed in long-term follow-up after transplantation, because most evaluations included maintenance allograft recipients.[97–101] These results summarize mostly data on opportunistic infections, malignancy (eg, nonmelanoma skin cancer), acute rejection, and cardiovascular risk. Monitoring of residual NFAT-regulated gene expression has been proven in observational cross-sectional and prospective clinical trials, including 1 prospective case–control study, as a beneficial and safe tool to reduce CsA therapy in stable renal allograft recipients.[88] An ongoing randomized controlled clinical study is evaluating the improvement in cardiovascular risk in stable renal allograft recipients on a CsA regimen by monitoring standard CsA trough levels, compared with the novel approach by monitoring residual NFAT-regulated gene expression.[102]

In Tac-treated patients, inhibition of NFAT-regulated gene expression is lower compared with CsA treatment, possibly because of a low relative increase of Tac levels from C_0 to C_{max}.[101] However, several studies on Tac treatment show that monitoring residual NFAT-regulated gene expression may help to identify allograft recipients at higher risk of infections or acute rejection.[101,103,104]

NFAT-regulated gene expression is a promising biomarker in CNI therapy as regards infectious complications, malignancies, acute rejection, and cardiovascular risk. A residual NFAT-regulated gene expression below <10% on CsA treatment and <30% on Tac treatment might be a risk factor for infectious complications and malignoma, whereas a residual NFAT-regulated gene expression above 40% in CsA-treated patients and 60%–80% in Tac-treated patients is a risk for rejection. Prospective interventional studies and randomized controlled studies are ongoing to confirm these encouraging results.

NFAT-Regulated Gene Expression and Clinical Implementation

The assessment of residual expression of NFAT-regulated genes is a minimally invasive, rapid, robust, and reliable assay system, which has proven its validity and practicality in clinical and research settings. In CsA-treated patients, NFAT-regulated gene expression has the potential to develop into a monitoring tool complementing PK, especially in long-term renal allograft recipients. However, the benefit of monitoring in de novo allograft recipients and in patients on Tac therapy has yet to be evaluated in additional long-term studies, to confirm the preliminary data in Tac-treated patients.

PHARMACOGENETIC MARKERS PREDICTIVE OF PK AND PD

Background

PG is based on the identification of constitutive genetic markers located in the genes influencing drug response. The majority of genes explored in the context of SOT are those coding for metabolizing enzymes or membrane drug transporters. Pharmacogenetic biomarkers useful to refine dose selection or, more interestingly, to select a priori the initial dose have been identified in rare cases but are not homogeneously used across transplantation centers. In addition, pharmacogenetic markers related to the fate of immunosuppressants in particular tissues (eg, lymphocytes, kidney graft) or to drug PD may be identified and implemented in the clinical decision process.

CNIs, Cyclosporine and Tac

Executive Summary

- CYP3A5 genotype-based dose adjustment of immediate-release Tac clearly improves initial dosing in renal transplantation (A, I). This is not the case for cyclosporine.
- No benefit on clinical outcomes has been demonstrated so far.
- Other candidate biomarkers requiring prospective validation include *CYP3A4*22*, especially for CsA (C2, III), and donor *ABCB1* variants (C1, III), for CsA.

Literature

CYP3A Enzymes

The *CYP3A5*3* allele (associated with decreased enzyme expression) is the main genetic biomarker of immediate-release Tac dosing requirements. In renal transplantation, genotype-based adjustment of initial dosing improves drug exposure[105,106] and, although not proven prospectively, might also improve clinical outcomes. The recently described *CYP3A4*22* allele, associated with decreased enzyme activity, might help to refine dose proposals, but its clinical utility has still to be proven in prospective studies.

Some studies and meta-analyses have also suggested a slight, and less significant, influence of *CYP3A5*3* single-nucleotide polymorphism (SNP) on CsA PK.[107–109] *CYP3A4*22* resulted in lower CsA clearance (−15%)[110] and higher CsA C_2/dose (+53%).[111] However, no genotype-based dose adjustment has been proposed so far for CsA in organ transplantation, as there is no evidence that this would improve clinical outcomes.[108,112,113]

ABCB1

The influence of *ABCB1* polymorphisms on the whole-blood PK of CNI is more controversial, with at best, weak associations between the c.3435C>T (rs1045642; Ile1145Ile) genotype and concentration-to-dose ratios and dose requirements. However, different *ABCB1* variants have been shown to influence intracellular CNI concentrations,[114,115] particularly in PBMC, an effect that, in turn, may theoretically influence PD parameters, because low intracellular CNI concentrations have been associated with a higher risk of acute rejection in renal and liver transplantation.[116,117] The recipient *ABCB1* genotypes have apparently no effect on Tac nephrotoxicity,[118] whereas the situation is less clear for CsA, with a few reports of positive associations with

decreased GFR and/or higher risk of delayed graft function in 3435T carriers.[119,120]

Donor *ABCB1* genotypes (at position 3435 or studied as haplotypes) can be considered as very promising biomarkers in renal transplantation, as they have been associated with nephrotoxicity and graft loss after CsA administration,[121,122] as well as with interstitial fibrosis (IF)/tubular atrophy severity over the first 3 years after transplantation, and with the degradation of renal graft function in Tac-treated patients.[123,124]

Clinical Implementation

In summary, data supports the use of pretransplant *CYP3A5*3* genotyping to adjust the initial Tac dose, which may be further individualized using *CYP3A4*22*. Initial CsA dosing may be improved by pretransplant *CYP3A4*22* determination. However, these genotypes may not add much to the precision of dose recommendations based on whole-blood concentrations in the maintenance phase. Donor *ABCB1* variant haplotype or genotype may be promising as a predictive biomarker of CNI-related nephrotoxicity.

MPA

Executive Summary

- *UGT1A9* genotype may serve as a biomarker to predict initial dosing of MPA in patients cotreated with Tac (C2, III).
- *IMPDH1* and *IMDPH2* genotype may explain, at least in part, some of the variability in the response to and toxicity of MPA when added as covariates to PK/PD population models (C2, III).

Literature

Metabolizing Enzymes

Of the many variants in the various UGT genes, 3 SNPs in *UGT1A9* seem to be the most promising as biomarkers. *UGT1A9* c.-2152C>T and c.-275T>A, which are in linkage disequilibrium, have been associated with reduced exposure to MPA, and patients carrying these SNPs may have an increased risk of acute renal graft rejection when treated with concomitant Tac therapy[125,126]; *UGT1A9* c.-98T>C ([or *UGT1A9*3*]) has been associated with higher MPA exposure, but data demonstrating a reduced rejection risk or increased toxicity are lacking.[125–128]

IMPDH

In some studies, selected *IMPDH1* gene variants have been correlated with rejection episodes,[129–131] leukopenia, and other adverse events, whereas other major studies have not reproduced these findings.[132,133] Although variants of *IMPDH2* were expected to influence the effect and outcome on the basis of their upregulation in activated lymphocytes, the influence of genetic variants has not been conclusive for this isoform.[132,134] The conflicting results may in some cases relate to relevant, but low-frequency, gene variants,[131] whereas for others, the relevance for IMPDH activity of some variants has not been identified.[129–131]

Clinical Implementation

The potential of *UGT1A9*, *IMPDH1*, and *IMPDH2* genotyping as biomarkers for MPA dose individualization and to predict outcome has not yet been clarified and is a definite role for these as biomarkers will require further evidence.

mTOR Inhibitors (mTORi), Sirolimus and EVR

Executive Summary

- There are no validated pharmacogenetic biomarkers for mTORi.
- *CYP3A5* genotyping might be useful for the initial dose adjustment of SRL provided that CNI are not coadministered (the PG of mTORi being presumably influenced by drug interactions with CNI) (C1).

Literature

CYP3A Enzymes

Although still controversial, the *CYP3A5*3* allele may influence SRL PK, but without any proven impact on the risk of acute rejection, graft clinical outcomes, or adverse effects. This effect would only concern renal transplant patients not receiving concomitant CNI treatment, perhaps because they compete with SRL for CYP3A5.[135–137] In contrast, there is no evidence so far to recommend the prospective genotyping of CYP3A5 for EVR dose adjustment.[110,138–142] The defective *CYP3A4*22* allele might have a moderate influence on EVR and SRL hepatic metabolism, but probably is not strong enough to justify dose adjustments.[110,143]

ABCB1

No clinically significant *ABCB1* pharmacogenetic effect has been reported on SRL or EVR PK, or on SRL effects in vivo in SOT.[135,137,139,140,142,144] Only few data are available regarding the impact of these polymorphisms on intracellular mTORi concentration. A recent study suggests that ABCB1-mediated efflux of EVR would have a minor role in its distribution in PBMC; ABCB1 SNPs showed no effect on this distribution.[145]

Clinical Implementation

In summary, there is no clinical evidence as yet to support the usefulness of mTORi pharmacogenetic biomarkers.

BIOMARKERS ASSOCIATED WITH GRAFT DYSFUNCTION OR INJURY

Chemokines as Biomarkers of Graft Clinical Outcome

Chemoattractant cytokines or chemokines (CXCs) are small-molecular-weight proteins (8–14 kDa) that are secreted by several types of cells.[146] The chemokine protein family consists of at least 45 ligands and 20 receptors.[146,147] They direct leukocyte navigation and are associated with inflammation and immune response after transplantation,[148,149] among other conditions. An increasing number of studies have suggested that the IFN-γ-inducible CXC-receptor 3 (CXCR-3) ligands CXCL-9 and CXCL-10 are rapidly increased after reperfusion and are abundant in rejecting allografts. They

are assessed by either protein or mRNA levels in urine, serum, and the transplant organ, and are associated with Banff scores of T-cell and antibody-mediated rejection after kidney transplantation.[150] Graft parenchymal cells can secrete CXCL-9 and CXCL-10, thus recruiting $CXCR3^+$ T cells into the transplanted organ, which enhances the alloimmune response.[151]

Executive Summary

- CXCL-9 and CXCL-10 proteins in urine as markers for kidney graft inflammation and alloimmune response have been validated in multicenter clinical trials, providing sufficient evidence to support the next steps toward clinical implementation (A, II).
- Urinary CCL-2 has been found to be a promising marker for inflammation and IF in renal allografts. Further validation in multicenter trials is justified (B, II).

Literature

Chemokines and Clinical Outcome

Earlier studies in renal transplant patients indicated that urinary CXCL-9 and CXCL-10 concentrations could differentiate patients with acute graft rejection and BK virus infections from stable patients[152] and could identify patients with subclinical tubulitis.[153] CXCL-9 and CXCL-10 had better diagnostic sensitivity and specificity than serum creatinine concentrations.[152] Evidence that CXCL-10 may also be a predictor for short- and long-term kidney graft function has been reported.[154]

In a multicenter study, the serially collected protein and mRNA levels in urine from 280 adult and pediatric de novo kidney transplant patients were analyzed. CXCL-9 mRNA and protein indicated the presence or absence of active inflammation in the graft, and were associated with BPAR within the first 6 months after transplantation.[155] Moreover, low urinary CXCL-9 protein levels 6 months after transplantation indicated a low risk of acute rejection and decreased GFR 6–24 months after transplantation, which suggests that CXCL-9 may be used for risk stratification of renal transplant patients.[155]

The benefit of urinary CXCL-9 and CXCL-10 levels in the diagnosis and prognosis of antibody-mediated rejection was studied in a highly sensitized cohort of 244 renal allograft recipients, 67 of whom had preformed donor–specific antibodies.[156] Urinary CXCL-9 and CXCL-10 levels, with or without normalization to urine creatinine concentrations, were correlated with tubule interstitial and microvascular inflammation. CXCL-10 normalized to urine creatinine concentrations were also associated with T-cell-mediated and antibody-mediated rejection, even in the absence of tubule interstitial inflammation. Moreover, the results suggested that the combination of urinary CXCL-10 levels normalized to urine creatinine with donor-specific antibody monitoring, significantly improved the noninvasive diagnosis of antibody-mediated rejection, and may allow for the stratification of patients at high risk for graft loss.[156]

In addition, it was found that urinary CXCL-10 levels normalized to urine creatinine levels are related to microvascular inflammation, and are a potential sensitive and specific biomarker for subclinical and clinical T-cell-mediated rejection in children.[157]

In a prospective study, nonsensitized stable living donor renal transplant patients were randomized to remain on or to be withdrawn from Tac.[158] CXCL-9 was measured in serially collected urine samples, and it was found that high urinary CXCL-9 levels predated clinical detection of acute rejection by a median of 15 days.[158]

Other chemokines also seem to be of potential interest as markers after transplantation. In renal transplant patients, 6-month urinary CCL-2 concentrations normalized to urine creatinine were found to be associated with IF and tubular atrophy in 24-month biopsies[159] and were a predictor of death-censored graft loss.[160] In a follow-up study, urinary CCL-2 levels normalized to urine creatinine concentrations in samples collected 6 months after transplantation were independently correlated with IF and inflammation scores in biopsies after 6 months and 24 months.[161] Moreover, 6-month urinary CCL-2 normalized to urine creatinine was also able to differentiate between the absence or presence of inflammation in renal tissue.[161]

The potential value of chemokines as biomarkers after liver and lung transplantation has also been explored. There is evidence that chemokines are involved in organ damage such as ischemia/reperfusion injury, rejection, inflammation, viral infection, biliary injury, fibrosis, and cirrhosis after liver transplantation.[147] In a study in 94 liver transplant patients, serum CXCL-9 concentrations were significantly higher before transplantation and on day 1 after liver transplantation in patients with acute cellular rejection within the first 6 months.[162] This is consistent with the results of an earlier study in liver transplant patients showing that, among other markers, high serum CCL-2, CXCL-9, and CXCL-10 concentrations were associated with early allograft dysfunction.[163] Plasma CXCL-10 levels at 6 months after liver transplantation in recipients with recurrent hepatitis C (n = 130) were lower in patients with slow, compared with rapid, fibrosis progression.[164] In this study, 6-month plasma CXCL-10 concentrations correlated with fibrosis stages and necroinflammatory scores in liver biopsies, as well as serum transaminases 12 months after liver transplantation.

Chronic lung allograft dysfunction, which limits long-term survival after lung transplantation, is heterogeneous, and different clinical phenotypes have been identified. In a biomarker discovery study in lung transplant patients, CXCL-8, CXCL-10, CCL-2, CCL-3, CCL-4, and CCL-7 in bronchial lavage fluid could differentiate between neutrophilic bronchiolitis obliterans syndrome (n = 17 patients) and restrictive allograft syndrome (n = 20), as well as discriminate between those from patients with stable (n = 20) and nonneutrophilic bronchiolitis obliterans syndrome (n = 20).[165]

Chemokines and Clinical Implementation

Clinical validation studies have provided sufficient information and agreement, specifically in terms of CXCL-9 and CXCL-10 protein in urine as markers for kidney graft inflammation and alloimmune response, to justify further

steps toward implementation of these markers in clinical practice.[150] Based on the published evidence, as briefly summarized above, it is reasonable to expect that these chemokine markers will help to guide and individualize immunosuppressive regimens, predict acute and chronic T-cell and antibody-mediated rejection, and may be a useful tool for risk stratification of patients. It has also been shown that measurement using standard immunoassay platforms is adequate,[155] which should facilitate clinical implementation and acceptance.

Graft-Derived Cell-Free DNA as a Marker of Transplant Injury

Graft-derived circulating cell-free DNA (GcfDNA) is a promising new approach in the detection of graft injury.[166–168] Plasma donor DNA is a cell death marker, released from necrotic or apoptotic cells in the transplanted organ. GcfDNA accounts for a small fraction of total cfDNA in the recipient's blood. Because organ transplants are also genome transplants, GcfDNA could be specifically determined in plasma and used as a marker of allograft injury, like a "liquid biopsy."[169] During acute rejection, high amounts of GcfDNA are shed into the blood stream.[170] Monitoring GcfDNA could potentially detect rejection episodes at early stages when other diagnostic methods are still ineffective.

Executive Summary

- Graft-derived circulating cell-free DNA (GcfDNA) as a "liquid biopsy" may be useful for early detection of graft injury due to subclinical or full-blown rejection, specific infections, or ischemia (A, II).
- Serial GcfDNA determinations can help to guide changes in immunosuppression, and to monitor minimization in combination with TDM, to achieve personalized immunosuppression.
- Ongoing multicenter clinical trials are currently evaluating the clinical utility of this biomarker as a potential universal marker of graft injury.

Literature

Graft-Derived Cell-Free DNA Measurement: Methods and Association With Clinical Outcome

Current methods do not provide rapid and cost-effective direct assessment of graft integrity after SOT,[171–175] and there is a lack of reliable conventional, noninvasive markers for cardiac rejection. A newly developed droplet digital PCR (ddPCR) method[166] has advantages over expensive high-throughput sequencing methods[176] in the rapid quantification of GcfDNA percentages and absolute amounts. This procedure does not require donor DNA and can therefore be applied to any organ donor–recipient pair. GcfDNA rises sharply after engraftment, because of ischemia reperfusion damage. It then decays to the baseline level within about 1 week. This can be used as a threshold for the diagnosis of acute rejection. Episodes of acute rejection are accompanied by a significant increase of GcfDNA (>5-fold) compared with values in patients without complications.[170] Elevated GcfDNA values were already observed 6–10 days before early acute graft rejection after liver transplantation[177] and 2–3 months before late acute rejection in heart transplantation.[176] The direct measurement of graft integrity using GcfDNA can be used to establish the minimally effective concentrations of immunosuppressive drugs in the individual patient.[178] The test could therefore be helpful for guiding the minimization of immunosuppression. The ddPCR method permits early, sensitive, specific, and cost-effective direct assessment of graft integrity.

Graft-Derived Cell-Free DNA Measurement: Clinical Implementation

Although prospective, multicenter clinical trials in liver ($n \approx 120$), heart ($n = 80$), and kidney ($n = 300$) transplant patients have not been completed,[170] interim results suggest that GcfDNA can be combined with TDM to guide changes in immunosuppression and to monitor immunosuppression minimization to provide more effective, less toxic treatment. Gielis et al[167] have recently reviewed currently published studies on this promising biomarker in transplantation. GcfDNA monitoring will provide actionable health care information, with the aim of achieving the right therapy for the right patient. Effective, truly personalized immunosuppression has the potential to shift emphasis from reaction to prevention and to reduce the cost of health care.

ANALYTICAL ASPECTS OF BIOMARKER MEASUREMENT

Because there is a broad consensus that not a single biomarker but rather a panel of complementary components is needed to cover most clinically relevant issues, such analytical strategies may have to deal with a wide variety of molecules with very different properties and behaviors.[179] To meet this challenge, a large body of techniques combined with a plethora of assay protocols is available. Some of these strategies, particularly those allowing for multiplexing, require complex software-based data evaluation and reporting. Although some analytical procedures are of great value for research purposes, they may be too complex for implementation in a clinical setting. From a clinical point of view, potential biomarkers should be noninvasive or minimally invasive, available within a reasonable time frame to allow timely adjustment of the immunosuppressive therapy, not too laborious, accurate, precise, and cost effective. Importantly, they should be robust and suitable for standardization to ensure reproducibility of results across laboratories.[179,180] Assay performance should guarantee that the observed tendency in a biomarker is related to clinical evolution and not an analytical artifact.

New biomarkers have to compete with current biochemical markers (eg, creatinine, troponins, bilirubin), which often have limited diagnostic performance if used for monitoring immunosuppressive therapy, but for which well-established analytical methods with highly optimized performance are available around the clock. This is a challenging goal for new, more comprehensive, yet more complex biomarkers. Assays to measure such biomarkers are often "in house" developments, and publications of

clinical studies commonly do not report details of the analytical protocol or their analytical performance, which limits their implementation in other laboratories. Commercial kits are available for only a small number of biomarkers; they are rarely approved for clinical use, often do not have established cutoff values to guide clinical decisions, and are also seldom cross-validated among laboratories. Consolidation of a panel of biomarkers available so far is limited to single technical platforms (eg, Luminex, MesoScale Discovery), and measuring different biomarkers often requires multiple instruments and expensive consumables and reagents. This is further accompanied by the need for in-depth expertise of the operators, and training has to be continuously provided. Many procedures are laborious, time-consuming and difficult to automate (eg, functional cell-based assays of cell isolation, culture, and stimulation are needed).

Appropriate method validation and standardization of the analytical process, 2 issues of critical importance to allow clinical implementation of biomarkers, are still insufficiently addressed. Both are often aggravated by many factors: the fact that biomarkers are mostly endogenous molecules; many of them, such as proteins, represent complex biopolymers; their biological origin and heterogeneity complicates development of appropriate reference standards; and their stability is a complex issue including chemical and physical properties and biological integrity. Potential predictive biomarkers clearly need to be analytically validated, using different patient cohorts before being integrated into routine clinical practice (Table 3). Although an analytical validation plan should be adopted to cover the specifics of the diverse techniques, the availability of general uniform guidance (currently often absent) is a prerequisite for method harmonization and standardization. Although the proof of "fitness for purpose" is appropriate for validation of biomarker assays used in exploratory drug development studies, a higher level of analytical validation must be achieved before diagnostic application in a clinical setting. Guidelines for method validation published by national and international authorities, eg, FDA, European Medicines Agency,[181,182] the College of American Pathologists (CAP, www.cap.org), the Clinical and Laboratory Standards Institute (CLSI, www.clsi.org), and the International Organization for Standardization (ISO, www.iso.org), together with some proposals for the validation of specific methodologies,[183–187] offer an advanced basis for a consensus on method validation.

It is important that method validation and efforts for method harmonization or standardization should cover all steps of the analytical process. This starts with the choice of appropriate sample matrices, collection and handling, including storage, sample preparation for analysis, and bioanalytical procedures, and ends with postanalytical issues such as the appropriateness of proposed cutoff levels and translation of results into valid clinical recommendations. New biostatistics models should be developed to establish the most appropriate correlation among biomarkers, drug effect and clinical outcome, which allows personalized treatment.

Important considerations for clinical implementation of promising new biomarkers are:

- To select analytical techniques and protocols appropriate for use beyond a research setting and capable of providing the analytical performance needed to ensure that data generated with the assay are reliable for the intended diagnostic application.
- To define the most appropriate sample matrices and clear protocols for sample collection, handling, storage, and shipment.
- To have consensus on method validation plans and acceptance criteria.
- To evaluate the feasibility and develop strategies for standardization of the analytical process.
- To establish training programs.
- To endeavor to make reference materials, stable calibrators, and quality control materials available and to develop external quality assurance tools.

A major initiative to foster the establishment of standardized protocols for monitoring of transplant recipients, suitable for sharing within the global transplant community and offering the capability for providing appropriate training (The Global Virtual Laboratory for Transplantation), was recently launched.[188]

NEW MODELS TO DESCRIBE AND PREDICT THE PK/PD RELATIONSHIP

In medicine, physicians face increasing amounts of complex information. In the past, decisions for patient care were based on medical history, physical examination, some basic laboratory tests, and an x-ray; but now, information from advanced biomedical techniques needs to be integrated into patient management. Typically, such data are too complex to be handled by individual MDs, and clinical decision support is required for implementation in patient care, both to reduce variability in decision making and to reach personalized medicine.

Biomarkers can provide guidance in clinical decision-making, by adding information on disease severity, treatment effects, or adverse events. By integrating biomarkers in mathematical models, the relationship between drug exposure (PK variables) and drug response (PD variables) can be characterized. With these models, both desired and undesired clinical outcomes can be studied and hopefully predicted. The models describe the time course of disease and the effects of interventions. Furthermore, the relationship between drug treatment, changes in the biomarker, and various clinical outcomes can be studied. A better understanding of PK and PD is therefore required to optimize drug therapy in transplant patients, corresponding to integrating pharmacometrics—the science of quantitative pharmacology—in clinical practice to develop evidence-based personalized pharmacotherapy.

Although limited to the clinical practice of large centers, modeling is increasingly performed for drug therapy. For optimal dosing strategies, it is important to be aware of the concentration–effect relationship and of the factors that influence the variability in drug exposure in individual patients. Population PK modeling is used to select the best dose for complex patients. Data from a patient population are first fitted into a model, which is tested to see whether the model

TABLE 3. Panel of Biomarkers in SOT

Biomarkers	Clinical Utility	Interlaboratory Validated Methods	Single-Center Observational Studies
IFN-γ	Predictive of the risk of acute rejection, can be used for risk stratification and immunosuppression selection	Yes	Yes
Interleukin-2	Predictive of the risk of acute rejection, can be used for risk stratification and immunosuppression selection	No	Yes
CD154 in T-cytotoxic memory lymphocytes	Predictive of the risk of acute rejection after liver or intestine transplantation in young patients <21 years. May assist in immunosuppression minimization	No	Yes
sCD30	Serum concentration before and after renal transplantation predictive of long-term kidney graft outcome	No	Yes
CD26 and CD28 T-cell surface antigens	Associated with acute rejection and/or malignancy after kidney and liver transplantation	No	Yes
Regulatory T cells	Predictive of the risk of acute rejection, can be used to decide immunosuppression conversion	No	Yes
Chemokines	Predictors for kidney graft inflammation and alloimmune response, can be used for risk stratification	No	Yes
Target enzymes: IMPDH	May assist in determining patients at risk for rejection or MPA-associated toxicity	No	Yes
Target enzymes: mTOR	mTORi	No	Yes
NFAT	May assist to identify transplant recipients at higher risk of opportunistic infections, malignancy, acute rejection, and cardiovascular risk. May complement CNI pharmacokinetics to better guide CNI therapy	Ongoing (manuscript written)	Yes
CYP3A5 genotype	May assist in determining the optimal Tac starting dose	Yes	Yes
GcfDNA	Early detection of transplant injury ("liquid biopsy"). Guide changes in immunosuppression and minimization	No	Yes

Biomarkers	Multicenter Observational Studies	Randomized Controlled Multicenter Studies (Clinical Qualification)	References
IFN-γ	Yes	Ongoing	12–20,28,29
Interleukin-2	Yes	Ongoing	18–20,25–27
CD154 in T-cytotoxic memory lymphocytes	No	No	44,45
sCD30	Yes	No	38–40
CD26 and CD28 T-cell surface antigens	No	No	35–36
Regulatory T cells	Yes	No	55–59,64–66
Chemokines	Yes	No	161–164
Target enzymes: IMPDH	No	No	70–73
Target enzymes: mTOR	No	No	83–85
NFAT	Ongoing (clinical trials)	No (monocentric randomized controlled study ongoing)	82–104
CYP3A5 genotype	Yes	Yes	106–107
GcfDNA	Yes	No	175–179,180,181

adequately describes the data. New data from individual patients can then be entered, and using Bayesian estimation, the next dose for a particular patient is defined. These techniques have been applied in drug development for a long time, but have now reached the clinic as well, and are most used for critical dose drugs, in particular in patients treated with antibiotics. Especially in patients in intensive care, many factors will influence drug exposure, and for serious infections in these vulnerable patients, it is essential that target concentrations are reached as quickly as possible. Population PK can account for an increased clearance or for a changed volume of distribution in critically ill patients. After the assessment of patient-specific drug exposure data, adaptive feedback control algorithms can predict the best dose adjustment to reach the target concentrations.

Biomarker development, and subsequent implementation of biomarkers into transplant patient management, would benefit from following a similar approach. For the research

side, the ultimate methodology is systems biology. In systems biology, there is an integration of complex interactions within biological systems to describe and understand physiological and pathophysiological processes. The term "systems pharmacology" is also used to describe the effects of drugs on these processes. Such models however are very complex and are not suitable for clinical application.

Typically E-max models are used to describe the relationship between drug concentrations and biomarkers that reflect the PD effect of this drug. The inhibition of IMPDH by MPA has been promoted as a method to monitor the effects of MPA treatment.[189] If there is a better correlation between the PD parameter and outcome, than between the drug concentration and outcome, the PD marker should be studied in more detail. Several investigators have used multivariate logistic regression analyses to determine the influence of multiple variables on clinical outcome after transplantation.[190,191] A complicating factor in these analyses is the fact that risk of rejection depends on various covariates including the time after transplantation and that the target concentrations for most of the drugs used to prevent rejection change over time. To deal with this problem, new PK-PD models have recently been proposed.[192] These so-called time-to-event models are of special interest for the transplant field, as they consider the whole longitudinal history of the explanatory time-dependent variable.[193]

If a single biomarker had sufficient positive or negative predictive power to be used as a stand-alone variable on which to base drug treatment, then supportive models would not be required. However, in our view, it is unlikely that in the transplant setting, in which many factors influence the outcome, such a highly predictive biomarker will be found. The more likely scenario is that the information provided by the biomarker will need to be integrated with parameters such as time after transplantation, concentrations, or dosages of one or more immunosuppressive drugs, and previously observed rejections and infectious complications. Assistance from clinical pharmacologists or pharmacists will be necessary to develop the models and to generate treatment recommendations for individual patients. For the multidisciplinary field that SOT already is, this should not be a major hurdle. Improvement and increased use of PK-PD modeling are most likely to occur in the coming decade.

RECOMMENDATIONS

- Monitoring a panel of valid biomarkers in combination with TDM by applying appropriate PK-PD models may be a better approach to designing personal immunosuppressive therapy to improve outcomes and long-term graft survival.
- Preliminary proposal for a panel of biomarkers (discussed above), currently under clinical evaluation in ongoing multicentre clinical trials:
  1. Expression of IFN-γ and IL-2 for the assessment of the risk of rejection and graft outcome.
  2. Urine CXCL10 synthesis for short- and long-term kidney graft function.
  3. Residual NFAT-regulated gene expression for personal response to CsA and Tac as well as risk of rejection and infections.
  4. GcfDNA for early detection of graft injury.
  5. CYP3A5*1 genotype for Tac dose requirement.

When deciding to implement a new biomarker, laboratories should be aware that:

- A higher level of analytical method validation must be achieved before diagnostic application, compared with exploratory drug development studies or general research projects. Suitable validation plans should follow well-established guidelines (eg, CLSI, ISO) for the use of biomarkers in a clinical setting and should be adapted to reflect technique-specific characteristics. Data derived from validation experiments should be compared against the predefined performance goals that reflect clinical needs, rather than simply the capability of a technique, to guarantee the intended support of therapeutic decisions.
- Appropriate cutoff values that should prompt intervention need to be defined and validated in independent populations, and at the interlaboratory level in multicenter, randomized controlled clinical trials.
- A system for assay life cycle management should be established to ensure consistency of results over time, namely a comprehensive internal quality assurance program that includes quality controls, system suitability testing, and continuous revalidations of critical analytical parameters. The quality assurance program should address not only analytical but also preanalytical issues such as sample collection, storage, and transport. In addition, measures for a permanent education and training of both the personnel involved and the customers should be implemented. Laboratories should have established protocols for all of these procedures, covering all these aspects.
- To ensure that results for biomarker analysis are comparable between laboratories, long-term external quality assurance programs should be established. Before this is achieved, cross-validation between laboratories is recommended on a regular basis. In addition, efforts to harmonize and standardize analytical services should be obligatory.
- To develop and establish new PK-PD models, particularly time-to-event models. In the transplant setting, in which the outcome is influenced by many factors, information provided by some biomarkers will have to be combined with parameters such as time after transplantation, immunosuppressive drugs concentrations or doses, previously observed rejections, and infectious complications. Assistance from clinical pharmacologists or pharmacists will be necessary to develop the models and to issue treatment recommendations for individual patients. In addition, efforts to perform external/cross validation of standardized PK-PD model between laboratories should be mandatory.

NEXT STEPS

The Expert Committee of this consensus document, as members of the BWG of the Immunosuppressive Drugs Scientific Committee of the IATDMCT, has a commitment to optimize the analysis of the biomarkers discussed. It intends to:

- Develop and disseminate standard operating procedures for monitoring immune responses and immunosuppression adjustment in transplant recipients to the transplantation community.

- Develop and make available through the Educational Web site of IATDMCT measures for permanent education and training that facilitate the implementation and maintenance of these biomarker assays with the aim to ensure that procedures are being performed properly.
- Actively participate in the multidisciplinary design and conduct of multicenter, randomized controlled clinical trials for biomarker evaluation in SOT.
- Revise the consensus document and update the proposed panel of biomarkers on a regular basis. The need for updating will be determined at 3-year intervals.

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