Molecular markers of rejection and tolerance: lessons from clinical research

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In terms of finding specific molecular markers associated with graft outcome, attempts have been made to study whole genome transcripts using microarray assays or to study the effect of number of genes of interest using quantitative real-time polymerase chain reaction. Using these techniques, molecular phenotypes of rejection have been characterized, and the variability of the clinical outcome besides similar morphology explained in part. Recently, several specific transcripts including naïve B cell regulation have been identified in the peripheral blood of operationally tolerant kidney transplant recipients. The decrease in immature B cell-related transcripts in the peripheral blood in patients with immunosuppression was shown to be associated with acute rejection. Similarly, tolerance-associated antigen 1 transcripts were identified in biopsies and regulatory T cell transcripts in urine and biopsies in patients without rejection. Better understanding of molecular processes associated with allograft rejection or alloantigen hyporesponsiveness/tolerance may help to improve our knowledge about graft pathology and identify novel markers suitable for future monitoring and guided therapy and finally improve the outcome of kidney transplantation.

INTRODUCTION

Kidney transplantation represents the most efficient way to treat end-stage renal disease. However, long-term kidney allograft survival has not improved substantially, and both acute and chronic rejections have been suggested to be implicated in late kidney allograft loss [1, 2]. Deterioration of kidney
graft function reflects the already developed and frequently untreatable structural changes. Recently, our one diagnostics tool frequently fails to correctly predict the kidney graft outcome. Better understanding of mechanisms of how the kidney grafts are being rejected could yield novel therapeutic approaches. Similarly, new biomarkers identified in these studies may change our clinical praxis.

To detect molecular fingerprints of developing injury, the identification of molecular markers of rejection and the evaluation of intrarenal and systemic molecular events in various clinical situations are of great importance. Similarly, long-term immunosuppression is associated with several side effects and, for many patients, the triple drug regimen is not necessary because of the weak allospecificity. However, drug weaning strategies have not yet been supported by reliable tests to allow for the monitoring of changes in both alloreactivity and tolerance in order to pre-emptively modify immunosuppression according to the real biological need of the patient.

Since 2003, several studies have evaluated DNA microarray profiling in kidney graft biopsies and in the whole blood of transplant recipients who suffered from different graft pathology. Using this technique, it was possible to identify groups of patients with similar molecular phenotype (specific expression of hundreds or thousands of genes), despite the knowledge of histology or outcome. Moreover, single genes that best predicted diagnosis or the outcome were identified and further validated by simpler techniques such as quantitative real-time polymerase chain reaction (PCR) in other studies. In this review, we focus on findings obtained from descriptive studies that search for molecular fingerprints of rejection and/or tolerance in clinical kidney transplantation.

**Gene Expression Profiling**

Gene expression microarray assays have become a popular tool in discovery-based genomic and biomedical research [3]. However, the reliability of the gene microarray results is being challenged due to the existence of different technologies and non-standard methods of data analysis and interpretation. Studies evaluating and comparing the performance of various microarray platforms have often yielded conflicting conclusions. The reason for this fact seems to be very complex. There are several flaws that may occur during samples collection and storage. Similarly, statistical biases and incorrect data interpretation may cause discrepancies between studies. Since technology development is very fast, it is sometimes difficult to compare data from recent studies with those performed several years ago. Gene expression microarrays allow functional and biological data interpretations such as gene-annotation enrichment analysis, functional annotation clustering, pathway mapping and gene-disease association. However, robust bioinformatics and biostatistics approaches are needed to ensure correct data interpretation.

Real-time PCR is often referred to as the 'gold standard' for gene expression measurements [4], due to its advantages in detection sensitivity, sequence specificity, large dynamic range as well as its high precision and reproducibility.

Only some groups have used the gene expression microarray technique to detect kidney graft disease-specific transcripts, while the majority of researchers used a later technique to analyse the role of several genes with an already known function in innate and adaptive immune responses.

**Prediction of Graft Outcome from Protocol Biopsies**

Protocol biopsies performed at defined post-transplant follow-up (commonly at 6 weeks or at 3 months) despite the stable graft function may represent one of the invasive tools for monitoring alloreactivity. Many centres do not recommend that they be used in routine praxis and, thus, the clinical usefulness of protocol biopsies remains unclear. However, protocol biopsies may represent the perfect tool for studying the significance of molecular signatures since they are performed either as 0-hour biopsies or during the stable post-transplant phase. The evaluation of ‘0-hour’ biopsies may detect transcripts associated with the risk of late graft dysfunction. It has been shown that the gene transcripts reflect kidney quality and susceptibility to delayed graft function better than available clinical and histopathological scoring systems [5]. The expression of *TNFA*, *TGFB*, *CD25* and *ICAM1* in ‘0-hour’ biopsy predicted increased risk of delayed graft function and acute rejection. Combination of molecular markers along with clinical indicators was shown to further enhance prognosis consistency [6]. Using gene expression microarray techniques, the up-regulation of genes related to immunity and defence, cell communication and apoptosis were described. Combination of histology with three from the set of differentially expressed genes (*NLRP2*, *IGJ* and *RGS5*) increased the predictability of 1-year serum creatinine *NLRP2* was shown to be involved in protein complexes that activate proinflammatory caspases. RGS5 has been known as signal transduction molecule with GTPase activity and has a critical role in blood pressure regulation. Immunoglobulin J polypeptide was detected in pre-B cell lines that may suggest its role in immune regulation and rejection [7].

Mengel et al. [8] used 6-week protocol biopsies to study the effects of specific transcripts determined by gene expression microarray technology on kidney graft outcome and found no association of gene expressions with future rejection episodes, functional deterioration nor allograft loss. Based on these observations, the authors concluded that early protocol biopsies reflect more injury-repair response to implantation stresses and have little relationship to graft outcome. Analysing the outcome of 163 protocol biopsies performed in our centre at 3 months, the intrarenal expression of nine genes with well-known function showed the high expression of chemokines *RANTES* and *IP-10* in rejection detected only in protocol biopsy despite the stable graft function to be associated with the higher risk of premature graft loss [9]. Another group used the gene expression microarray technique to evaluate transcripts in 6-month protocol biopsies associated with chronic rejection at 12 months after transplantation [10] and found several genes differentially expressed in patients with chronic rejection and stable ones.
Moreover, kidney grafts with interstitial fibrosis/tubular atrophy (IF/TA) without inflammation in 12-month protocol biopsies had better survival than patients with IF/TA and subclinical inflammation. The combination of fibrosis and inflammation was associated with increased activity of innate immune pathways including interferon-γ and TLR responses as well as increased T cell immunity ([IFNG, UBD, CXCL9, CXCL10, CD14 and FOXP3]) and down-regulation of protective gene products ([TOLLIP, VEGF and BCL2]) [11]. Clearly, the absence of donor-specific alloantibodies or C4d-staining evaluations does not allow the correct interpretation of these data.

Such data support the hypothesis that distinct intrarenal gene expression phenotypes may be detected early prior to apparent clinical and histological manifestations of kidney injury. Prospective validation of such observations in independent cohorts seems to be, however, mandatory before molecular methods enter the clinical routine.

## REJECTION PHENOTYPES

More information comes from studies dealing with biopsies performed for cause, i.e. indicated due to graft function deterioration (Table 1). The first evidence that the early acute rejection after transplantation is not a homogeneous molecular entity was given by Sarwal et al. [12]. In this study, the authors analysed 67 biopsy samples obtained from 50 paediatric patients treated with anti-IL-2R induction therapy, a calcineurin inhibitor (CNI)-based immunosuppressant, who experienced deterioration of graft function. On the basis of hierarchical clustering (without the knowledge of morphological diagnosis), acute rejection formed specific cluster that differed from non-rejection pathology. Moreover, three subtypes of acute rejection were defined according to the rejection outcome. The subtypes of rejection differed in the expression of genes reflecting apoptosis, infiltration and activation of lymphocytes, driven by NF-κB and interferon-γ, transcripts from T cells, natural killer cells and macrophages. The enhanced expression of cytotoxic T-lymphocyte-effector genes ([GZMA and RANTES]), adhesion molecules, cytokines, cytokine receptors and growth factors, and surprisingly also markers of B cells ([CD20, CD74, immunoglobulin heavy and light chains and other molecules associated with B-cell receptors]) was found in the subtype of acute rejection with the most unfavourable outcome. Clearly, antibody-mediated rejection was not diagnosed that time and the Banff classification of acute rejection has substantial changes that limits the current interpretation of the Marwal study.

### Table 1. Molecular evaluation of kidney graft biopsies with rejection

<table>
<thead>
<tr>
<th>Study</th>
<th>Method used to analyse expression</th>
<th>Main findings</th>
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<tbody>
<tr>
<td>Perco et al. [7]</td>
<td>Microarray</td>
<td>NLRP2, IGJ and RGS5 combined with histology predicted 1-year serum creatinine</td>
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<tr>
<td>Seiler et al. [15]</td>
<td>qRT-PCR</td>
<td>NKG2D and granulysin up-regulation was associated with the restricted 6-month graft outcome</td>
</tr>
<tr>
<td>Sarwal et al. [12]</td>
<td>Microarray</td>
<td>Immune activation and cellular proliferation-related gene expression pattern distinguished subtypes of acute rejection with different outcomes</td>
</tr>
<tr>
<td>Viklicky et al. [26]</td>
<td>qRT-PCR</td>
<td>Low expression of CD20 was associated with poor graft prognosis after early acute rejection</td>
</tr>
<tr>
<td>Bunnag et al. [47]</td>
<td>Microarray, qRT-PCR</td>
<td>FOXP3 was evaluated in rejection (both TCMR and AMR); no relationship between FOXP3 or FOXP3/GZMB expression with future function</td>
</tr>
<tr>
<td>Einecke et al. [17]</td>
<td>Microarray</td>
<td>Transcripts reflecting tissue injury (e.g. ITGB6, HAVCRI, LTF, ADAMTS1, SERPINA3, MMP7, C3 and COL1A2), dedifferentiation and epithelial-to-mesenchymal transition (NNMT and VCAN) and tissue remodelling, but not those reflecting inflammation ([IFNG effects and T cell or macrophage infiltration]) predicted graft failure</td>
</tr>
<tr>
<td>Famulski et al. [18]</td>
<td>Microarray, qRT-PCR</td>
<td>The canonical form of TCMR in late biopsies was defined on the basis of molecular phenotype and independently on histopathology with γ-interferon suppressed transcripts-alternative macrophage activation set and γ-interferon and rejection induced transcript set as the main feature and with the best prognosis despite severe inflammation</td>
</tr>
<tr>
<td>Sis et al. [23]</td>
<td>Microarray</td>
<td>High endothelial-associated transcript expression with antibody predicts graft loss in AMR patients better than C4d</td>
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</table>
Earlier studies showed up-regulation of Fas ligand mRNA in biopsy samples with steroid-resistant acute rejections versus samples with therapy-sensitive acute rejection [13, 14]. Beside the up-regulation of granulysin, higher intrarenal mRNA expression of NKG2D, which is activating cytotoxicity receptor expressed by NK cells, was reported to be associated with the deteriorated renal function at 6 and 12 months after transplantation, mainly in grafts with acute rejection [15]. Recently in a retrospective study, molecular predictors of steroid resistance were evaluated by the Dutch group. With respect to therapy effect, molecular markers offered a superior prognostic value compared with conventional parameters and several T cell-related transcripts including CD25, CD3ε and lymphocyte activation gene 3 expression were predictive of steroid resistance; however, the low specificity and sensitivity and retrospective design should be noted [16].

Evaluating transcripts in late kidney graft biopsies, using gene expression microarray analysis, Einecke et al. [17] identified genes associated with graft loss. They developed the molecular risk scores strongly associated with graft loss and found that the transcripts predicting graft failure were those reflecting tissue injury, dedifferentiation and epithelial-tomesenchymal transition and tissue remodelling, but not those reflecting inflammation. Risk predictions were accurate when applied to the late biopsy population. It indicates that the active ongoing tissue response to injury is the final common pathway linking mechanisms of inflammation and non-inflammatory disease states to parenchymal loss, dysfunction and kidney failure. These data have not been validated by others so far.

Famulski et al. [18] defined the specific form of T-cell-mediated rejection in late biopsies on the basis of molecular phenotype. The pattern of this rejection did not occur in C4d-positive antibody-mediated rejection and was rare in other biopsies. The molecular features of this type of rejection were the expression of alternative macrophage activation transcripts and high levels of IFNG-induced transcripts. The biopsies with this type of rejection were the most severely inflamed, but surprisingly had the best prognosis. Interferon gamma was shown to be secreted by various cell types, including NK cells and CD4 helper and CD8 cytotoxic T lymphocytes as well as macrophages and dendritic cells and is involved in both innate and adaptive immune responses [19]. The data from the above-mentioned studies have not been confirmed yet since similar studies were not performed by other groups.

When evaluating late biopsies, many groups including ours used TGF-β1 protein and mRNA expression as markers of ongoing fibrogenesis. TGF-β1 mRNA up-regulation in late for-cause biopsies predicted poor renal graft function within the 18-month follow-up. Similarly, the higher intrarenal expression of several other genes associated with inflammation and fibrosis was shown to discriminate patients at a higher risk for the earlier kidney graft failure [20, 21].

**Antibody-mediated rejection**

Recently, antibody-mediated rejection represents the main obstacle in clinical kidney transplantation. It is obvious that current diagnostic procedures are insufficient since several phenotypes of this diagnosis have been discovered. The importance of molecular pathology is further documented by studies revealing the C4d-negative phenotype of antibody-mediated rejection was shown to be characterized with several histological parameters similar to the C4d-positive phenotype [22]. Endothelial-associated transcripts were reported to be up-regulated in both C4d-negative and -positive forms of antibody-mediated rejections. Moreover, higher endothelial-associated transcript expression predicted a poor kidney graft outcome and graft loss better than the presence of C4d-positive staining itself [23–25]. It is important to note, however, that the results of these interesting studies have not yet been confirmed by other groups, mainly due to the use of demanding microarray techniques that limit wide spread into clinical laboratories. In the future, however, molecular evaluation of biopsies might improve the, so far, imperfect diagnostics of this entity.

The Banff classification of histological findings in transplanted grafts has been developing continuously and thus it happened in earlier studies that T-cell and antibody-mediated rejections were mixed up into one entity. This fact precludes the correct interpretation of observed differences in earlier studies. To discriminate a role of several genes known to be associated with rejection and tolerance, we studied the effects of transcripts on the outcome of early acute rejection that occurred during first 14 days. At first we found several genes to be expressed differentially between early acute antibody-mediated rejection and early acute T cell-mediated rejection. Compared with patients with T cell-mediated rejection, those with antibody-mediated rejection had significantly higher intrarenal mRNA expression of the cytoprotective heme oxygenase-1, but had lower expression of the regulatory T cell marker forkhead box P3 (FoxP3), the B cell marker CD20 and the chemokine regulated upon activation, normal T cell expressed and secreted (RANTES). T cell infiltration was similar in both groups of patients. Compared with grafts that had a favourable 3-year outcome, those that failed as a result of antibody-mediated rejection had expression profiles suggesting a lack of regulation (less FoxP3, TGF-beta1, RANTES and CD20). Of note, grafts that failed as a result of T cell-mediated rejection only revealed lower expression of CD20 mRNA [26]. These data suggest that severe antibody-mediated rejection and T cell-mediated rejection result in graft loss by distinct mechanisms involving lower B cell-related transcripts.

To summarize the important observations from molecular pathology studies, using both the whole genome approach and profiling of several gene expressions, distinct molecular phenotypes associated with acute rejections were identified. In antibody-mediated rejection, higher endothelial transcripts and lower extent of inflammation were repeatedly observed. The interferon-γ-related inflammation in both early and late biopsies does not necessarily indicate a mean disadvantage, while inflammation in late biopsies with already present fibrogenesis represent a risk for rapid progression. In both T cell-mediated and antibody-mediated early acute rejections,
lower expression of B cell markers might be associated with a lack of regulation associated with poor outcome.

**A dualistic role of B cells**

The role of intragraft B cells in renal transplantation is the subject of many studies with controversial results. The B cell infiltrate during acute rejection was shown to be associated with poor prognosis in some [12, 27] but not all reports [26, 28]. Recently, many immune cell subpopulations, including B cell subpopulations were shown to be involved in the induction and maintenance of transplantation tolerance. Previously, it was thought that B cells were primarily associated with the development of antibody-mediated immune response, and B cell clusters in kidney graft tissue were shown to be associated with poor prognosis [29]. More recently, and contrary to this observation, several groups including ours have found immature B cell signatures to be associated with better early rejection outcomes [30, 31].

Interestingly, operationally tolerant patients who had stable graft function despite the absence of immunosuppressive therapy for >1 year presented with distinct molecular signature. Most prominent and reproducible in different studies and cross-over validations performed in USA and EU consortia (IOT, RISET and ITN; for details, see Table 2) was the relation between operational tolerance and enhanced expression of B cell-related transcripts as well as enhanced flow cytometric B cell counts in the peripheral blood compared with patients with chronic rejection. Drug-free tolerant patients displayed increased numbers of B cells in the peripheral blood, with decreased memory pool and increase in transitional and naïve B cell subsets. Enhanced expression of B cell differentiation and activation genes in tolerant patients was also reported [32–36]. Little is, however, known about the expression of B-cell signatures in stable transplant patients who are still on immunosuppressants, and, particularly, about the kinetics of this particular expression pattern post-transplantation. Therefore, we performed a 12-month prospective observational study to monitor known markers associated with kidney transplant operational tolerance. We demonstrated that immature and naïve B cells-related and operational tolerance-associated transcripts were up-regulated in the peripheral blood in rejection-free kidney transplant recipients within the first year post-transplantation. In this study, patients with combined T cell—and antibody-mediated rejections had lowest immature B cell-related transcripts in the peripheral blood. Moreover, expression levels of tolerance-associated gene-1 (TOAG-1), a mitochondrial protein involved in the suppression of T’ cell activation [37], were observed in kidney graft tissue in rejection-free patients [38].

Recently, the composition of the B-cell compartment was shown to be important to determining graft outcome. Naïve and transitory B cells have been suggested to be associated with long-term graft function and operational tolerance, in contrast, memory B cells have been associated with limited graft survival and rejection [14, 15, 34]. Clearly, larger prospective biomarker-driven studies might verify the clinical usefulness of monitoring or therapeutic activation of naïve or immature B cells.

**Regulatory T cells and rejection**

A role of regulatory T cells in acute rejection has been widely discussed as well, although frequently with conflicting results. It was shown that induction immunosuppressive

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**Table 2. International research consortia in rejection/tolerance**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
<th>Year</th>
<th>Web page</th>
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</thead>
<tbody>
<tr>
<td>ITN</td>
<td>Immune tolerance network</td>
<td>Since 2002</td>
<td><a href="http://www.immunetolerance.org">www.immunetolerance.org</a></td>
</tr>
<tr>
<td>RISET</td>
<td>Reprogramming the immune system for establishment of tolerance</td>
<td>2005–2010</td>
<td><a href="http://www.nds.ox.ac.uk/riset">www.nds.ox.ac.uk/riset</a></td>
</tr>
<tr>
<td>GAMBIT study</td>
<td>Genetic analysis and monitoring of biomarkers of immunological tolerance</td>
<td>2010</td>
<td><a href="http://www.kcl.ac.uk/medicine/research/divisions/timb/research/tolerance/gambit/index.aspx">www.kcl.ac.uk/medicine/research/divisions/timb/research/tolerance/gambit/index.aspx</a></td>
</tr>
<tr>
<td>The One Study</td>
<td>A unified approach to evaluating cellular immunotherapy in solid organ transplantation</td>
<td>2011</td>
<td><a href="http://www.onestudy.org">www.onestudy.org</a></td>
</tr>
<tr>
<td>Bio-DRIM</td>
<td>Personalized minimization of immunosuppression after solid organ transplantation by biomarker-driven stratification of patients to improve the long-term outcome and health-economic data of transplantation</td>
<td>2012</td>
<td><a href="http://www.charite.de">www.charite.de</a></td>
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</tbody>
</table>
agents that deplete T cells from the periphery allow early repopulation of regulatory T cells \textit{ex vivo} [39]. This observation was confirmed \textit{in vivo} in clinical cohorts by several groups including ours. These studies showed enhanced repopulation of peripheral regulatory T cells including the preservation of its suppressive activity after induction with rabbit antithymocyte globuline [40, 41].

It remains, however, unclear whether the intrarenal compartment reflects the situation in peripheral blood. Therefore, we analyse the effect of induction therapy with various depletion agents on intrarenal transcripts at 3-month protocol biopsy only in patients who remained rejection free. Surprisingly, there were no signs of different FoxP3 expression among patients with and without rATG. However, despite normal morphology in graft biopsy, the intrarenal transcriptome differed in patients treated with different rATGs. In the thymoglobulin, the transcriptome profile was identical to the low-risk group without induction therapy and down-regulation of the nuclear factor-κB pathway was noticed [42]. Similarly to our study where all patients had received CNIs-based immunosuppression, CNI-free regimen including belatacept had no effect on regulatory T cell expansion in 3-month protocol biopsy [43]. On the contrary, in rATG induction followed by sirolimus-based CNI-free regimen, the recruitment of Tregs in the allograft at 6-month protocol biopsy was suggested to play a role for graft acceptance [44]. In other studies, patients with subclinical rejection without Foxp3+ T(reg) cells within graft infiltrates showed significantly worse 3- and 5-year graft function evolution than patients with subclinical rejection and Foxp3+ T(reg) cells. The presence of Foxp3+ T(reg) cells in patients with subclinical rejection with IF/TA was associated with a favourable long-term allograft outcome [45, 46]. In their study, Bestard \textit{et al.} found subclinical rejections at 6-month protocol biopsy with Foxp3+ T(reg) cells infiltrate in patients who received rATG induction and sirolimus, while patients treated with basiliximab induction and CNI-based immunosuppression exhibited no infiltrates in biopsies. On the contrary, Bunnag \textit{et al.} [47] showed an elevated expression of FOXP3 in rejection and described no association of FOXP3 expression with kidney allograft outcome. Thus, inconsistencies in therapeutic regimes cannot offer conclusive results about the proper role of infiltrating regulatory T cells in kidney grafts so far. However, adoptive transfer of regulatory T cells represents the scope of several experimental therapies under recent investigation (i.e. The One Study).

Using microarray assays and qRT-PCR techniques, different regulations of several genes or biological processes associated with rejection or operational tolerance have been recently described. Expression profiles are likely to be more disease specific than other laboratory tests used for graft monitoring and allows one to discriminate between different mechanisms of injury and firmly predict poor outcome. The relevance of many molecular markers associated with rejection or tolerance remains to be further validated by the reanalysis of microarray gene expression data sets using improved bioinformatics and finally in larger prospective biomarker-driven trials.

\section*{Acknowledgements}


\section*{Conflict of Interest Statement}

None declared.

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Corticosteroid and calcineurin inhibitor sparing regimens in kidney transplantation

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corticosteroids and the nephrotoxicity of calcineurin inhibitors (CNIs) may increase recipients’ morbidity and mortality in the long run. The purpose of the current report is to review emerging data on corticosteroid and CNI sparing modalities and to identify deficiencies in the available evidence.

ABSTRACT

Chronic kidney disease is a major public health problem that is associated with increased risks of kidney disease progression, cardiovascular disease and death. Kidney transplantation remains the renal replacement therapy of choice for patients with end-stage kidney disease. Despite impressive strides in short-term allograft survival, there has been little improvement in long-term kidney graft survival, and rates of death with a functioning allograft remain high. Long-term safety profiles of existing immunosuppressive regimens point to a need for continued search for alternative agents. This overview discusses emerging evidence on a few promising therapeutic approaches, juxtaposes conflicting findings and highlights remaining knowledge gaps.

INTRODUCTION

In the past three decades, the field of kidney transplantation has seen significant advances in early graft and patient survival. However, the adverse metabolic profile from chronic use of corticosteroids and calcineurin inhibitors (CNIs) may increase recipients’ morbidity and mortality in the long run. The purpose of the current report is to review emerging data on corticosteroid and CNI sparing modalities and to identify deficiencies in the available evidence.

Corticosteroids

Corticosteroids are a cornerstone of immunosuppressive regimens. Chronic corticosteroid therapy, however, is complicated by hyperglycemia, osteoporosis, exacerbation of hypertension, impaired wound healing and cataracts. Improvements in short-term allograft survival have created an impetus to reduce steroid exposure. The approach used by most centers is to give high-dose corticosteroids intraoperatively followed by a taper in the ensuing months, with indefinite continuation of low-dose prednisone. An alternative is to withdraw corticosteroids at a specified time post-transplant or avoid their use altogether. The major randomized controlled trials (RCTs) in this area have predominantly focused on the timing of withdrawal.