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ABOUT COVER

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AIM AND SCOPE

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FRONTIER

Is it time to give up with calcineurin inhibitors in kidney transplantation?

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BIOGRAPHY

Maurizio Salvadori, after his medical degree at the University of Florence (Italy) in 1968, became professor of Nephrology in 1975. He specialized in Clinical Nephrology, Endocrinology and Clinical Immunology. In 1991, with the beginning of renal transplant activity at the University of Florence, he was nominated chief of the Renal Transplant Department. Under his supervision to date approximately 500 renal transplants have been performed. He trained tens of post-graduated in the activity of renal transplantation both Italians and foreigners. Over the last 20 years he focused his scientific interest on the new immunosuppressant drugs receiving funds for his researches from Novartis, Roche and Astellas. Many new immunosuppressants were studied by professor Salvadori and his team. As a result he has been the top enroller in the world for fingolimod (FTY720), enteric-coated mycophenolate sodium, and everolimus. Repeatedly was called by foreign University, principally in USA to give lectures on his studies. He his author of more than 300 papers published in peer revised international journals. He is the member of the European, American and International Society of Nephrology and of the European, American and International Society of Transplantation. In recent years he was called as Italian representative in the European working group to generate the new European Guidelines on Renal Transplantation.

Abstract

Calcineurin inhibitors (CNIs) represent today a cornerstone for the maintenance immunosuppressive treatment in solid organ transplantation. Nevertheless, several attempts have been made either to minimize their dosage or to avoid CNIs at all because these drugs have the severe side effect of chronic nephrotoxicity. This issue represents a frontier for renal transplantation. The principal problem is to understanding whether the poor outcome over the long-term may be ascribed to CNIs nephrotoxicity or to the inability of these drugs to control the acute and chronic rejection B cells mediated. The authors analyze extensively all the international trials attempting to withdraw, minimize or avoid the use of CNIs. Few trials undertaken in low risk patients with an early conversion from CNIs to proliferation signal inhibitors were successful, but the vast majority of trials failed to improve CNIs side effects. To date the use of a new drug, a co-stimulation blocker, seems promising in avoiding CNIs with similar efficacy, better glomerular filtration rate and an improved metabolic profile. Moreover the use of this drug is not associated with the development of donor-specific anti-human leukocyte antigen antibodies. This point has a particular relevance, because the failure of CNIs to realize good outcomes in renal transplantation has recently ascribed to their inability to control the acute and chronic rejections B-cell mediated. This paper analyzes all the recent studies that have been done on this issue that represents the real frontier that should be overcome to realize better results over the long-term after transplantation.

Key words: Calcineurin inhibitors nephrotoxicity; Calcineurin inhibitors withdrawal; Calcineurin inhibitors minimization; Calcineurin inhibitors avoidance; Donor specific antibodies; Antibody mediated rejection; New drugs in renal transplantation

Core tip: Calcineurin inhibitors (CNIs) based therapy is still a cornerstone in renal transplantation. Nevertheless, with the use of such drugs the long-term graft survival did not improve. Causes may be nephrotoxicity, underimmunesuppression or both. All the trials attempting to CNIs sparing have been examined, but nephrotoxicity doesn't seem to be responsible for the lack of long-term improvement. In recent years emerged the problem of anti-human leukocyte antigen antibodies not adequately suppressed by the CNIs based therapy. New drugs are necessary, but the pipeline seems to be almost empty now. To date the only promising drug strategy is the co-stimulation blockade, whose four-year results are reported.

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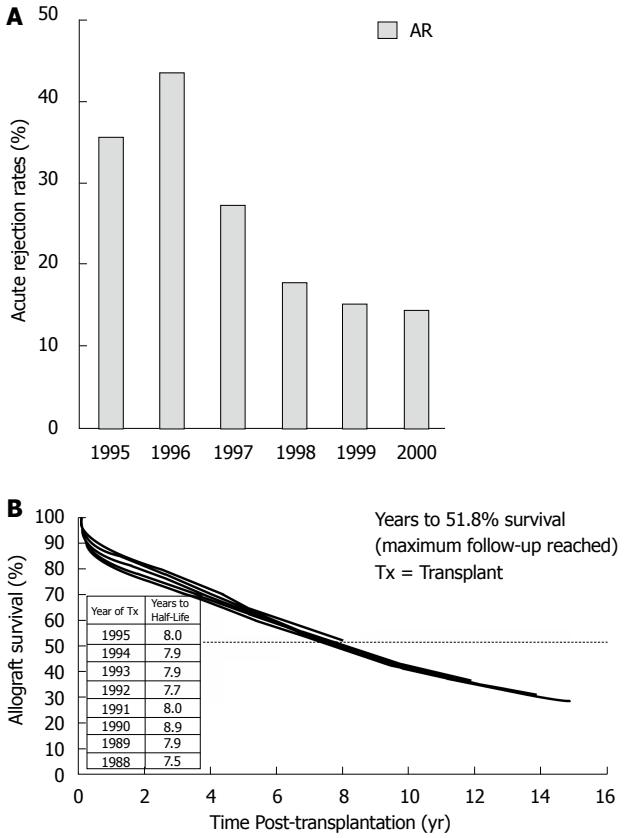

Figure 1 Kidney transplantation from deceased donors. Reduction in rejection rates (A) and half life graft survival according the era (B). AR: Acute rejection.

INTRODUCTION

The evolution of immunosuppressive therapies in renal transplantation beginning in the 1980s has led to lower rejection rates and improved recipient and short-term allograft survival rates primarily because of calcineurin inhibitor (CNI), which continues to be the cornerstone in the maintenance phase of immunosuppression. By the early part of the last decade, the one-year graft survival rates approached 90%, whereas the acute rejection rates were less than 20% (Figure 1). Nevertheless, long-term CNI-based immunosuppression [Cyclosporine A (CsA), tacrolimus (TAC)] is associated with nephrotoxicity and other adverse events, including hypertension (CsA), hyperlipidemia (CsA) and diabetes mellitus (TAC). As a consequence, long-term improvement in allograft-survival has been more elusive[1].

Studies conducted in the early- to mid-1990s suggested that decreasing early acute rejection rates would lead to an improvement in long-term allograft survival rates. However, since 1995, the reduction observed in acute rejection rates has not directly correlated with improvements in allograft survival. Indeed, between 1988 and 1995, the cumulative increase in the length of graft survival for primary renal transplants was less than 6 mo[2]. Moreover the spectacular success of CNI-based regimens has a dark side that continues to hinder better long-term patient and graft survival. The observed poor improvement in half-lives highlights the possibility that CNI-based immunosuppression is unable to improve long-term graft survival. Three important reasons are involved in the failure of CNI-based regimens to improve long-term outcomes (Figure 2): (1) late graft failure may be due to mechanisms unrelated to alloimmune injury, such as: nephrotoxicity, accelerated senescence and glomerular disease; (2) persistence of graft loss due to premature death from infections and cardiovascular disease; and (3) immunosuppression with CNIs may be inadequate in controlling the emergence of donor-specific antibodies (DSA) and chronic antibody-mediated rejection (AMR), a major cause of late graft failure because of minimization regimens and/or non-adherence.

CNI NEPHROTOXICITY

At the beginning of the 2000s, nephrotoxicity was thought to be an important player in hindering better long-term survival.

Chronic nephrotoxicity was first identified in cardiac transplant recipients. Moreover, the permanent histological hallmarks of striped interstitial fibrosis, tubular atrophy, medial arteriolar hyalinosis and tubular microcalcification were also observed in renal transplants and in patients treated for autoimmune disease[3-5]. Analysis of transplant recipients of organs other than kidney, reported a 16.5% risk of chronic kidney disease, with 28.9% of patients requiring dialysis or renal transplantation[6]. An elegant study of the Sidney group on kidney-pancreas

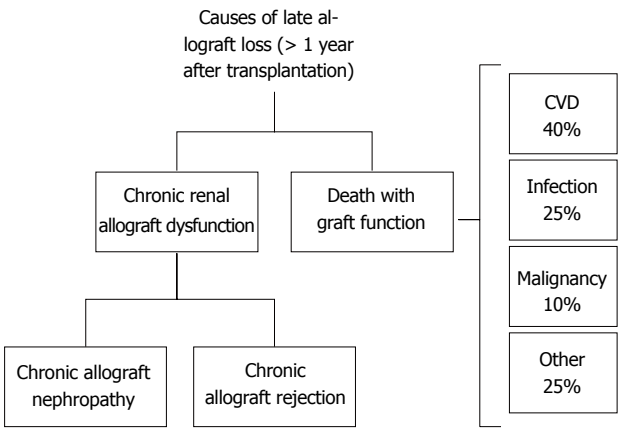

Figure 2 Main causes of graft losses beyond 1-year after renal transplantation.

transplant patients has demonstrated the progressive nature of pathological damage over a 10-year time frame. The 10-year graft survival of these kidneys was approximately 80%, and the mean measured glomerular filtration rate (GFR) was approximately 50 mL/min per square meter. Despite these promising outcomes, the histology was alarming due to progressive fibrosis and tubular atrophy, arteriolar hyalinosis and glomerulosclerosis[7-9].

As currently understood, the mechanisms causing CNI nephrotoxicity[10], are due both to a decrease in vasodilators such as prostaglandin E2 and nitric oxide as well as to an increase in vasoconstrictors, such as thromboxane, endothelin and the renin-angiotensin system. Direct toxicity to the tubular epithelium has been demonstrated both clinically and experimentally: isometric vacuolization resulted from the presence of giant mitochondria, most likely as a result of a CsA blockade of mitochondrial permeability transition pores (Figure 3).

In addition to direct nephrotoxicity, CNIs have other, primarily metabolic, side effects that can influence both kidney and patient survival. Hypertension, hyperlipidemia, hyperuricemia and gingival hyperplasia are associated with cyclosporine treatment; however, neurotoxicity and diabetes mellitus more commonly occur with tacrolimus treatment[11].

As a consequence of the aforementioned side effects, the short-term benefits of CNIs are unquestionable; however their effects on long-term outcomes are debatable[12]. Efforts to minimize the toxicities ascribed to CNIs, in particular nephrotoxicity, include various strategies aimed at eliminating (withdrawal), minimizing and avoiding these agents, such as: (1) CNIs withdrawal occurs either in CNI elimination, with the removal of the drug after a predetermined time, thereby diminishing CNI side effects, or in CNI substitution, with the use of alternative agents, keeping the total amount of immunosuppression comparable; (2) CNI minimization is a reduction in the dose of CNIs followed by therapeutic drug monitoring to target CNI levels lower than in the standard treatment; and (3) CNI avoidance is the intentional non-use of the drug from the beginning of transplantation[13].

Since the mid and late 1980s, several trials have evaluated the weaning of patients off CNIs, months or years following transplantation[14]. However, kidney function in the early period post-transplantation emerged as a potent determinant of subsequent graft outcomes. However, an ever increasing array of powerful "non-nephrotoxic" agents may facilitate CNI reduction early in the post-transplantation time period. The 1990s witnessed the emergence of new anti-proliferative agents such as mycophenolate mofetil (MMF) and the mammalian target of rapamycin inhibitors (mTORi). Later, the immunosuppressive armamentarium expanded to include anti-CD52 leuco-depleting antibody, alemtuzumab; the proteinase C inhibitor, sotrastaurin (AEB071); the janus kinase (JAK) 3 inhibitor, tofacitinib (CP-690,550); and the CD28 co-stimulation blocker, belatacept[15].

CNI-SPARING STRATEGIES

Overall, the CNI-sparing strategies may be conducted under the protection of mycophenolic acid (MPA), proliferation signal inhibitors [sirolimus (SRL) or everolimus] or the newer aforementioned agents.

CNI withdrawal with mTORi

CNI withdrawal after kidney transplantation is usually performed early after transplantation or late more often because of grafts with deteriorating function.

Early CNI withdrawal with mammalian target of rapamycin inhibitors (mTORi) immunosuppression: In 2005, Mulay *et al*[16] published a systematic review of randomized trials on CNI withdrawal using SRL-based therapy. These trials were conducted in an attempt to improve renal allograft function. Six trials involving 1047 patients have been analyzed[17-19]. CNI withdrawal was associated with an increased risk of acute rejection (P = 0.002), but higher creatinine clearance at one year (P < 0.0001) and reduced blood pressure. The review concluded that longer follow-up was necessary to determine whether these changes will result in a better outcome in the long term.

The rapamune maintenance regimen (RMR) has data available over four years[20,21]. Overall, 510 patients treated after transplantation with triple therapy including CsA, SRL and steroids were randomized (1:1) at 3 mo to remain with the triple therapy or to stop CsA treatment. At four years patients with CsA withdrawal, experienced significantly better graft survival, also censoring for death rates. Calculated GFR and mean blood pressure also improved. Patients remaining on triple therapy had significantly higher rates of adverse events, such as hypertension, lower GFR and a higher incidence of cancers; nevertheless the RMR study has several drawbacks. For example several transplant physicians observed that the group that underwent triple therapy received an excess of immunosuppression and, as a consequence, these results should be observed with caution. Moreover at four

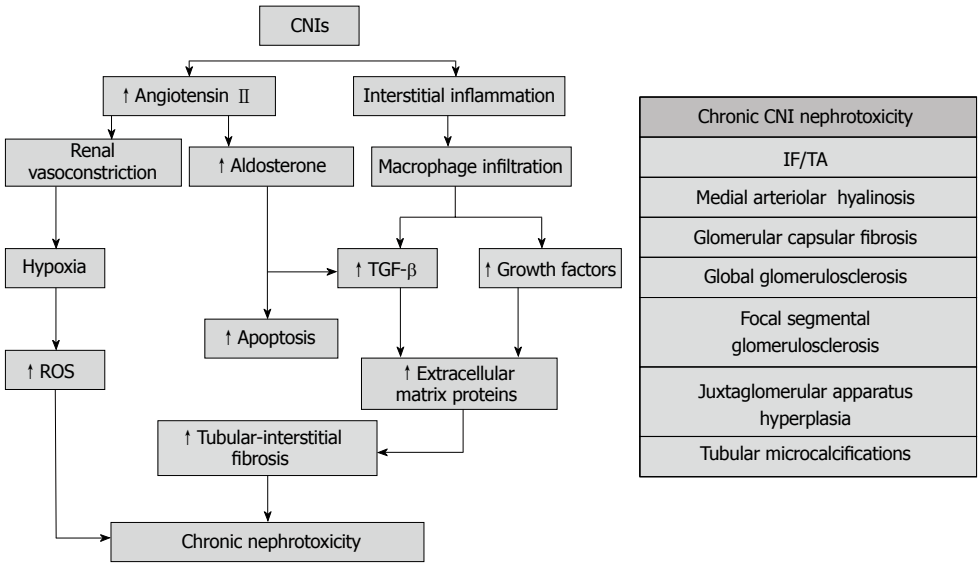

Figure 3 Pathogenesis of calcineurin inhibitors mediated nephrotoxicity (left) and morphological aspects. TGF-β: Transforming growth factor beta; ROS: Reactive oxygen species; IF/TA: Interstitial fibrosis/tubular atrophy; CNI: Calcineurin inhibitor.

years 113/215 recipients on triple therapy disappeared and could not be considered and the same happened for 118/215 patients in the withdrawal group. In the "Spare the Nephron" trial, 299 recipients of kidney transplantation after initial maintenance therapy with CNIs, (primarily TAC) and MMF were randomized (1:1) to remain in the same therapy group or were switched to a group who received maintenance therapy with MMF + Sirolimus. After a two-year follow-up period, renal function in the CNI withdrawal group was significantly better, with similar biopsy proven acute rejection (BPAR) and graft loss rates[22,23].

Lebranchu *et al*[24] in the CONCEPT study group, enrolled (1:1) 237 patients to remain in triple therapy with CsA, MMF and steroids or to switch CsA to SRL by the 3rd month. All patients underwent steroid discontinuations by the 8th month. The SRL group had higher BPAR incidence, most of them occurring after steroid discontinuation and GFR was significantly better in the SRL group. Guba *et al*[25] in the SMART study group, enrolled 141 recipients to receive induction therapy with anti-thymoglobulin (ATG) and maintenance therapy with CsA, MMF and steroids. Early post-transplantation (10-24 d) patients were randomized to switch from CsA to SRL or to remain on triple therapy with CsA. After one year the SRL group had higher GFR, while BPAR incidence rates were not different between groups. Drug discontinuation was higher in the SRL group due to higher incidence of side effects. Overall, 132 patients in this study were followed for 36 mo. At 36 mo renal function remained higher in the SRL group, however more patients discontinued therapy in the SRL group in the follow-up study. Interestingly, in a multivariate analysis, donor age > 60 years, serum creatinine at conversion > 2 mg/dL and immunosuppression with CsA were predictive of worse renal function. The authors concluded that patients selection is the key to understanding which patients will benefit from an mTOR inhibitor-based immunosuppressive regimen[26]. The ZEUS (CRAD001A2418) study utilized everolimus, a different mTOR inhibitor with an improved pharmacokinetics profile, to withdraw CsA[27]. Overall, 300 patients were enrolled in the study. After induction therapy with anti-interleukin 2 receptor inhibitors (anti-IL2Ri) and maintenance therapy with CsA, MPA and steroids, the patients were randomized 4.5 mo after transplantation, to remain in CsA-based immunosuppression or to switch from CsA to everolimus. By 36-mo data were available from 284 patients (94.7%), and GFR was higher at one year in the everolimus group and remained significantly higher at three years. The incidence of acute rejection was higher in the everolimus group. Most of the BPAR was verified early after randomization, but it did not exerted a deleterious effect on renal function by three years post-transplantation. The HERAKLES (CRAD001ADE13) study also utilized everolimus to withdraw CsA[28]. After initial therapy similar to the therapy utilized in the ZEUS study, 499 recipients, were randomized (1:1:1) by month three into three arms, to continue standard treatment, to convert CsA to everolimus, or to start everolimus treatment associated with low dose CsA. Data at one year showed that the withdrawal group had similar BPAR rates but higher GFR compared to the control group. Patients discontinuing therapy occurred more frequently in the conversion group, and adverse events were the most common cause for discontinuation. Moreover, out of 800 patients initially enrolled, only 499 were randomized.

In conclusion the early discontinuation of CNIs after transplantation with mTOR inhibitors in patients with good renal function appears to be safe and effective. Data related to the main studies are shown in Tables 1 and 2. Overall, acute rejections occur soon after CNI discontinuation, and physicians should be aware of this timetable. GFR is higher in CNI withdrawal recipients both at one

Table 1 Withdrawal/conversion study

Study (yr)	Intervention arm	Control arm	CNI sparing strategy	Study length (mo)
RMR (2004)	SRL, Steroids, CsA→Withdrawal by 3 mo (n = 215)	SRL, Steroids, CsA (n = 215)	Withdrawal by 3 mo	48
Spare the Nephron (2011)	MMF, S, CNI→SRL (30-180 d) (n = 148)	MMF, S, CNI (n = 151)	Conversion by 30-180 d	24
CONCEPT (2009)	MMF, S, CsA→SRL by 3 mo (n = 95)	MMF, S, CsA (n = 97)	Conversion by 3 mo	12
SMART (2010)	MMF, S, CsA→SRL 10-24 d (n = 70)	MMF, S, CsA (n = 71)	Conversion by 10-24 d	12
ZEUS (2012)	EC-MPS, S, CsA→EVR by 4.5 d (n = 154)	EC-MPS, S, CsA (n = 146)	Conversion by 4.5 mo	36
HERAKLES (2012)	EC-MPS, S, CsA→EVR by 3 mo (n = 171)	EC-MPS, S, CsA (n = 166)	Conversion by 3 mo	12

CNI: Calcineurin inhibitor; CsA: Cyclosporine; EVR: Everolimus; MMF: Mycophenolate mofetil; EC-MPS: Enteric coated mycophenolate sodium; SRL: Sirolimus; S: Steroids.

Table 2 Withdrawal/conversion trials

Study (yr)	Drugs	F-up (mo)	Patient survival		Graft survival		Biopsy proven acute rejection		Glomerular filtration rate (mL/min)	
			SRL/EVR	CNI	SRL/ EVR	CNI	SRL/EVR	CNI	SRL/EVR	CNI
RMR (2004)	SRL (SRL+CsA)	48	95.3%	92.1%	91.5%	84.2%	10.2	6.5	58.3	43.8
Spare the Nephron (2011)	SRL/CNI	24	100%	97.5%	100%	98.7%	9.5	11.3	59.5	58.8
CONCEPT (2009)	SRL/CsA	12	100%	100%	100%	99.0%	16.8	8.2	69.6	64.8
SMART (2010)	SRL/CsA	12	99.0%	99%	99.0%	97.0%	17.4	15.5	64.5	53.4
ZEUS (2012)	EVR/CsA	36	98.1%	97.9%	98.7%	98.6%	13.0	4.8	67.9	60.6
HERAKLES (2012)	EVR/CsA	12	99.4%	98.8%	99.4%	99.4%	10.0	8.4	68.6	63.0

CNI: Calcineurin inhibitor; CsA: Cyclosporine; EVR: Everolimus; SRL: Sirolimus.

and three years; however, the discontinuation rate in patients on mTOR inhibitors is high and is caused by drug-related adverse events. Overall, these data encourage longer studies that enroll a higher number of patients and highlight that only some patients have a greater beneficial effect from switching to mTOR inhibitors from CNIs. However, identifying which patients will have a beneficial effect before withdrawal remains a major problem.

Late CNI withdrawal with mTORi immunosuppression: Several studies have evaluated the outcomes of conversion from CNI-based to mTORi -based immunosuppression in kidney transplant recipients with CNI nephrotoxicity and chronic allograft nephropathy (CAN)[29-32]. A review of Mulay *et al*[33] found only five randomized controlled studies, but only one, the CONVERT (Sirolimus Renal Conversion Trial), had enrolled a sufficient amount of patients to draw conclusions based on the findings. The CONVERT trial randomized kidney transplant recipients at 6-20 mo after transplantation, to continue CsA-based immunosuppression or to convert from CsA to SRL. Conversion of immunosuppressive therapy from CNIs to SRL did not improve renal function. Moreover, the conversion was detrimental among recipients with impaired kidney function and/or proteinuria. The median urinary protein-to creatinine ratio was higher in SRL-converted recipients. The benefits shown by the early conversion from a CNI- based to SRL-based immunosuppressive regimen have not been documented for late conversion. These studies highlighted that proteinuria and accelerated loss of renal function were the major problems after conversion and primarily occurred, in patients with proteinuria and/or low renal function before conversion.

CNI withdrawal with MMF

Trials on CNI elimination in patients treated with MMF have been reviewed by Moore *et al*[34]. Similar to CNI withdrawal with mTORi, the studies can be divided in elective CNI elimination and CNI elimination for transplant dysfunction.

Elective CNI elimination: Abramowicz *et al*[35,36] withdrew CsA from recipients with stable renal function treated with MMF and steroids. Data at one and at 5 years were reported in two different papers. Recipients in the CsA-withdrawal group had better renal function and lipid profile at one and five years, and the incidence of BPAR was higher in the CsA-withdrawal group. The long-term results of this study[36] showed that although

Table 3 Selected randomized trials on calcineurin inhibitors minimization

Study (yr)	Intervention arm	Control arm	CNI minimization	Study lenght (mo)
Andres (2009)	IL2Ri + lCsA + MMF + S	IL2Ri + lowCsA + MMF + S	Minimization	6
US09 (2008)	IL2Ri + EVR + lowTAC + S	IL2Ri + EVR + TAC + S	Minimization	6
Ciancio (2005)	Alem + lowTAC + lowMMF	rATG + TAC + MMF + S	Minimization	12
De Sevaux (2001)	lowCsA + MMF + S	CsA + MMF + S	Minimization	6
CAESAR (2007)	IL2Ri +lowCsA + MMF + S	CsA + MMF + S	Minimization	12
ELITE SYMPHONY (2007)	DAC+lTAC/lCsA/SRL+MMF+S	DAC+sCsA+MMF+S	Minimization	36
OptiCept (2009	Induction + lowCNI +MMF + S	Induction + CNI + MMF + S	Minimization	24
Hernandez (2007)	IL2Ri + lowCsA + MMF + S	IL2Ri + CsA + MMF + S	Mimimization	24
Kandaswamy (2005	rATG + lowTAC + S	rATG + TAC + S	Minimization	24
RADB156 (2004)	IL2Ri + lowCsA + EVR + S	IL2Ri + CsA+ EVR + S	Minimization	36
EVEREST (2009	standEVR + lowCsA +S	HighEVR + vlowCsa +S	Minimization	36
Vathsala (2005)	Alem + lowCsA	CsA + AZA + S	Minimization	6
ASSET (2012)	EVR + lowTAC	EVR + vlowTAC	Minimization	12

rATG: Rabbit Antithymocyte globuline; Alem: Alemtuzumab; CNI: Calcineurin inhibitor; CsA: Cyclosporine; EVR: Everolimus; TAC: Tacrolimus; DAC: Daclizumab; IL2Ri: Interleukin 2 receptor inhibitors; MMF : Mycophenolate mofetil; S: Steroids.

improvement in GFR was maintained at five-year follow-up, increased graft loss in patients experiencing acute rejection was observed. The Cyclosporine Avoidance Eliminates Serious Adverse Renal-toxicity (CAESAR) trial evaluated the outcomes of reduced-dose CsA (50% lower) either with or without early withdrawal at 6 mo, in primary kidney allograft recipients receiving daclizumab induction, MMF and steroids[37]. The outcomes were compared with patients on standard dose treatment. By 12 mo, the incidence of BPAR was significantly higher in the CsA-withdrawal group compared with the other two groups; therefore this arm was stopped. The results of these two largest studies mirror two other studies enrolling a small number of patients with elective CsA elimination[38,39]. Overall, all of these studies have an improvement of GFR (5.5 ± 2.3 mL/min) with an OR for acute rejection of 2.23 (95%CI: 1.57-3.17) and an OR for graft loss of 1.34 (95%CI: 0.63-2.86) at one year. In this regard, complete elective elimination of CNI is a "double-edged sword": the improvement in GFR was balanced by an increase in acute rejection rates that in turn led to reduced graft survival over the long term. Reducing acute rejection episodes in patients undergoing CNI elimination is the key to graft survival. As in the mTORi studies, these results were achieved by strategies such as refining the criteria for patient selection to identify those patients at low immunological risk, the timing of CNI elimination and immunosuppression monitoring.

CNI elimination for transplant dysfunction: Three primary studies have focused on the issue of CNI elimination with the use of MMF in patients with transplant dysfunction[40-42]. All of these studies were conducted in the early 2000s, and the patients enrolled had maintenance therapy without MMF at enrollment. Patients with deteriorating renal function, documented by renal biopsy, were randomized to continue undergoing their original therapy or to withdraw from CsA treatment but begin MMF treatment. Among these studies, the "Creeping creatinine" study is the most important trial. Patients enrolled in MMF treatment did not experience acute rejection after CsA withdrawal, and they had a significant increase in GFR (6.7 ± 3.2 mL/min). Interestingly and in contrast to elective CNI elimination, no rejection occurred in these patients, which may be ascribed to screening withdrawal patients with preexisting subclinical rejection, to initiating CNI elimination later and to increasing MPA exposure after withdrawal.

CNI withdrawal with other immunosuppressant

To our knowledge the only attempt at conversion has been tried with sotrastaurin. Sotrastaurin is a new, low molecular weight immunosuppressant, that selectively blocks protein kinase C isoforms and inhibits early T-cell activation via a calcineurin- independent pathway. In a phase Ⅱ study[43], sotrastaurin was evaluated in de novo renal recipients. In the first 3 mo sotrastaurin was combined with tacrolimus (either standard or reduced exposure) with subsequent conversion to a CNI-free regimen of sotrastaurin + MPA. Initially, the acute rejection rate was very low and comparable with patients in the control arm; however when tacrolimus was withdrawn after 3 mo, the acute rejection rate increased to unacceptable levels, and the study was halted. To date no new immunosuppressant has been proven to be sufficiently efficient to allow systematic CNI withdrawal in renal transplant recipients.

CNI minimization

A different approach to reduce CNI toxicity and nephrotoxicity is CNI minimization, which consists of a reduction in CNI dose followed by therapeutic drug monitoring to target CNI levels lower than in the control arm. Additionally, this approach is possible due to proliferation signal inhibitors, MPA, heavy induction therapy or new drugs. The issue of CNI minimization has been extensively reviewed by several meta-analyses[13,15,44-46]. The

study of Sharif *et al*[15] is one of the most recent and extensive studies concerning CNI minimization. The most important trials regarding CNI minimization are shown in Table 3.

CNI minimization with mTOR inhibitors immunosuppression: Several studies have evaluated CNI minimization using everolimus. Nashan *et al*[47] study RADB156, a phase Ⅱ, randomized, open label three-year study, was performed with 111 patients to compare the efficacy and safety of everolimus (3 mg/d) in combination with basiliximab, steroids and either full-dose CsA or reduced-dose CsA. Efficacy failure was significantly higher in the full-dose group and mean serum creatinine was higher in the reduced-dose group both at 6 and 12 mo. GFR improvement was not significantly greater at 36 mo. The RADB156 study was able to document the efficacy of low dose CsA in association with everolimus; however, the primary weakness of the study was the low number of patients enrolled. Moreover, the available data at three years only included 49% of the enrolled patients.

The upper target EVErolimus RandomisEd (EVEREST) study enrolled 285 kidney transplant recipients who were randomized to everolimus 3-8 ng/mL plus standard levels of CsA or to everolimus at very high levels (8-12 ng/mL) plus very low levels of CsA[48,49]. At 12 and 24 mo, no differences in renal function and acute rejection rates were observed in the two groups. The primary weakness of this study involved the inability of the investigators to maintain low CsA levels as dictated by the study design. As a consequence, no differences in GFR between the groups were observed. Nevertheless, CsA blood levels were kept as never has been done before, with no increase in acute rejection rates. In the study A2309 833[50], *de novo* renal transplant patients were randomized to receive either everolimus 1.5 or 3.0 mg/d with reduced exposure CsA, or MPA plus standard exposure CsA. Overall, 12-mo efficacy failure rates were similar in the everolimus and in the MPA groups. Mean eGFR at month 12 was also similar in the everolimus group and in the MPA group. This study also proved that the association of reduced dose Csa with everolimus had a similar efficacy and a similar renal function compared with standard exposure to CsA plus MPA. In the CRADUS09 Study Group[51] 92 *de novo* renal transplant patients received everolimus, steroids and basiliximab with low or standard tacrolimus exposure. Mean eGFR and BPAR at 6 mo were similar, but the study had several biases such as a low number of enrolled patients and the shortness of the study. Moreover, the study results were affected by the relatively small differences in tacrolimus exposure between the two arms. More recently in the ASSET (A2426) study[52], 228 renal transplant recipients in a 12-mo study were randomized to receive everolimus (through levels 3-8 ng/mL) and a lower dose (through levels 1.5-3.0 ng/mL) or a higher dose (through levels 4-7 ng/mL) of tacrolimus. BPAR were comparable between groups and mean GFR was also similar between the groups, which was most likely due to a probable overlapping of achieved tacrolimus exposure levels.

Overall, all of these minimization studies documented no differences in renal function, rejection rates or survival among recipients receiving a lower dose of CsA in combination with everolimus. A lower dose of TAC with everolimus was also attempted but, no difference in renal function or rejection rates was observed between these drug associations. A hindering factor in these studies was the overlap of the achieved CNI exposure levels.

CNI minimization with MPA immunosuppression: Several studies have attempted to minimize CNIs with MMF association. In the de Sévaux *et al*[53] study, 313 renal allograft recipients were randomized for treatment with MMF, steroids and either conventional- or low dose-CsA. Data after 6 mo showed similar efficacy for the two groups, but no improvement in renal function was observed in the low CsA group.

Hernández *et al*[54] compared low dose CsA with low dose TAC and standard dose CsA + ATG. This 24-mo study enrolled 240 renal transplant recipients (1:1:1). The incidence of BPAR was similar across the groups, as well as graft and patient survival. Significantly better renal function was observed in patients enrolled in the low CNI groups. As mentioned above, the CAESAR study[37] enrolled 536 renal transplant patients in three arms: CsA withdrawal, CsA standard dose and CsA low dose. All patients received daclizumab, MMF and steroids. The arm with CsA withdrawal failed because an excess of acute rejection after withdrawal occurred, and the incidence of BPAR was similar in the low and standard CsA dose groups. At one year the GFR difference between these groups was small (2 mL/min). Moreover, the study had some weaknesses because out of 357 patients enrolled for minimization, only 257 had data available at one year, and an overlap of achieved CsA exposure levels occurred.

The Optimal CellCept Dosing (OptiCept) trial[55] in addition to CNI minimization, analyzed the relevance of MMF dosing. OptiCept was a two-year randomized trial comparing the efficacy and safety of concentration-controlled MMF (MMFcc) dosing with a fixed-dose regimen in 720 kidney transplant recipients. Patients received either MMFcc and reduced levels of CNIs or MMFcc and standard levels of CNIs or a fixed dose MMF and a standard dose of CNIs. At two-years, no major differences were observed between the fixed and the controlled dose groups. However, comparing patients with low vs. patients with high MMF trough levels, a relevant threshold was considered to be 1.6 μg/mL. Patients with a higher level had a lower frequency of acute rejections. In contrast, graft survival was the same, and patients with less CNIs had a slightly higher GFR, which was the only difference observed in the study. Thus, a trough level of MMF of less than 1.6 μg/mL was correlated with acute rejection episodes but did not predict worse long-term graft survival. Furthermore, a reduced CNI dose corre-

Table 4 Efficacy and effects on biopsy proven and glomerular filtration rate of the main minimization trials

	Calcineurin inhibitors	F-up (mo)	Acute rejection	Graft survival	Biopsy proven	Glomerular filtration rate
OptiCept	CsA/TAC	24	Similar	Similar	Similar	Similar
CAESAR	CsA	12	Similar	Similar	Similar	Similar
ASSET	TAC	12	Similar	Similar	Similar	Similar
Andres	CsA	6	Similar	Similar	Similar	Similar
2309	CsA	12	Similar	Similar	Similar	Similar
EVEREST	CsA	36	Similar	Similar	Similar	Similar
REFERENCE Study	CsA	24	Similar	Similar	Similar	Similar
De Sevaux	CsA	6	Similar	Similar	Similar	Similar

CsA: Cyclosporine; TAC: Tacrolimus.

Table 5 Glomerular filtration rate gain in different minimization trials

Study	Calcineurin inhibitors	Length	Glomerular filtration rate gain (mL/min)
US09	TAC	6 mo	+2.8
De Sevaux	CsA	6 mo	+4.0
CAESAR Study	CsA	12 mo	+2.3
SYMPHONY Study	TAC *vs* CsA	3 yr	+4.6
A2309	CsA	12 mo	+1.9
ASSET Study	TAC	12 mo	+5.3
B156	CsA	3 yr	+4.9
EVEREST	CsA	6 mo	+2.1

CsA: Cyclosporine; TAC: Tacrolimus.

lated with better GFR.

In the Efficacy Limiting Toxicity Elimination (ELITE-SYMPHONY) study[56,57], 1645 renal transplant recipients were randomized to four treatment groups: standard dose CsA, low-dose CsA, low-dose TAC, or low-dose SRL. All the recipients of the low-dose regimen groups received induction therapy with daclizumab, and all recipients of all the groups received MMF and steroids. At one year, the acute rejection rate as well as allograft survival were the lowest in the low-dose TAC group. The TAC group had significantly better renal function with respect to all other groups. The study was extended to a three-year follow-up, and the TAC low-dose group still had better renal function but was no more significant. Moreover, out of the 401 patients enrolled in the low-TAC arm only 249 were evaluated at three years.

Based on the aforementioned CNI toxicity theory, the aim of all trials studying CNI minimization was either to prove CNI low-dose efficacy by BPAR and graft survival rates or to document GFR improvement and reduction of cardiovascular events which are side effects of CNIs. Tables 4 and 5 summarize the results of the aforementioned trials. Both tables show that CNI minimization is effective, and BPAR and graft survival rates are similar to the arms receiving standard CNI doses. In contrast, these strategies failed to improve GFR. GFR was slightly better in patients undergoing minimization, but the difference was small in all the studies and was never significant. Additionally, in the studies with higher GFR improvement, the difference was not significant because of the small number of enrolled patients. Similar minimization strategies failed to realize a decrease in blood pressure. Another effect aimed by the minimization strategies was the reduction of cardiovascular mortality, but also this effect was not documented.

Moreover, all of these studies have several drawbacks. For example, all of the studies had a short-term follow-up. Under this condition, it was not possible to document the minimization effects on hard end-points as cardiovascular mortality or major cardiovascular events (MACE). The short-term follow-up had the consequence of using surrogate end-points instead of hard end-points. In addition, all minimization studies lacked specific searches for antibody-mediated rejections (AMR), circulating DSA and C4d in renal biopsy specimens. These finding may be an important bias because circulating DSA and AMR have been recently shown to be major causes of long term graft loss.

CNI minimization with alemtuzumab induction: Alemtuzumab is a CD52-specific monoclonal antibody that causes profound and sustained lymphocyte depletion. Its use in induction therapy in organ transplantation is increasing and allows a reduction in CNI maintenance therapy. Morgan *et al*[58] systematically reviewed and performed a meta-analysis of the most important randomized trials comparing alemtuzumab with other induction therapies as ATG and IL-2Ri. Data of the selected trials are shown in Table 6[59-64].

Alemtuzumab induction has a lower risk of BPAR compared with induction using IL-2R inhibitors. No significant difference in BPAR incidence was observed

Table 6 Immunosuppression regimens in alemtuzumab minimization trials

Ref.	Study group			Control group		
	Induction	Maintenance		Induction	Maintenance	
Chan *et al*[59]	Alem	TAC, Ster	82	Dac	TAC, MMF, Ster	41
Hanaway *et al*[60]	Alem	TAC, MMF,	164	Bas	TAC, MMF, Ster	171
		Ster	70	rATG	TAC, MMF, Ster	69
Ciancio *et al*[61]	Alem	TAC, MMF	13	Dac rATG	TAC, MMF, Ster	12
					TAC, MMF, Ster	13
Ciancio *et al*[62]	Alem	TAC, MMF, Ster	30	Dac rATG	TAC, MMF, Ster	30
					TAC, MMF, Ster	30
Farney *et al*[63]	Alem	TAC/CsA, MMF, Ster	85	rATG	TAC/CsA, MMF, Ster	95
Vathsala *et al*[64]	Alem	CsA, Ster	20	None	CsA, AZA, Ster	10

Alem: Alemtuzumab; TAC: Tacrolimus; Ster: Steroids; MMF: Mycophenolate mofetil; CsA: Cyclosporine; Dac: Daclizumab; Bas: Basiliximab; rATG: Rabbit antithymocyte globulin.

when alemtuzumab induction was compared with ATG. Almost all randomized controlled trials reported no significant difference in renal function in all time points after transplantation. In the largest trial conducted by Ciancio *et al*[62] reported a significantly lower mean calculated creatinine clearance at 1, 3, 6, 12 and 24 mo after transplantation when alemtuzumab was compared with the average values of both the daclizumab and ATG groups combined. In this trial, TAC through levels were significantly lower in the alemtuzumab group. Overall, in minimization trials with alemtuzumab induction minimization efficacy was confirmed, but no GFR improvement with respect to standard treatment was observed.

CNI avoidance

CNI avoidance with MMF and/or mTOR inhibitors: CNI avoidance is the complete omission of CNIs from the immunosuppressive regimen beginning after transplantation. Early attempts at de novo CNI avoidance were soon abandoned because of a very evident lack of efficacy. Vincenti *et al*[65] investigated the avoidance of CNIs with the use of MMF, daclizumab and steroids in a multicenter study. Although 6-mo recipient and graft survival rates were excellent, the 6-mo acute rejection rate was 48%. The authors concluded that such a high BPAR rate is not acceptable and that other immunosuppressive agents should be added to attempt CNI avoidance. Grinyó *et al*[66], in recipients of suboptimal donors, tried a calcineurin-free regimen with ATG, high dose MMF and steroids. The high dose of MMF was not well-tolerated and in many patients, reintroduction of CNIs was necessary. When SRL became available, the combination of two non-nephrotoxic agents (SRL and MMF) appeared promising as a possible CNI-avoidance strategy.

Flechner *et al*[67] randomly assigned 61 renal transplant recipients to receive maintenance therapy with MMF and either SRL or CsA. At two years, recipient and graft survival and BPAR rates did not significantly differ between the groups. At two-years, the SRL-treated recipients had better renal function and a reduced prevalence of chronic allograft nephropathy. These results were maintained five years after transplantation[68], and the authors concluded that excellent five-year kidney transplantation outcomes can be achieved without CNIs in patients at low to moderate risk with drug monitoring. In a more recent study, Larson *et al*[69] assigned 165 renal transplant recipients to receive SRL plus MMF and steroids or TAC plus MMF and steroids. Although adequate efficacy was achieved, no benefit to one-year GFR was observed. Additionally, the ELITE-Symphony study[56] included a CNI-free arm that used a combination regimen of SRL and MMF. This CNI-free strategy failed to demonstrate a benefit to renal function. Moreover, the acute rejection rate was significantly higher, and the graft survival rate was significantly lower with the SRL-MMF regimen. This fact should also be ascribed to the SRL blood levels that were much lower in the Symphony study with respect to the Larson and Flechner studies[68,69].

Hamdy *et al*[70] studied CNI avoidance in 132 living donor renal transplants. All patients received induction therapy with basiliximab and steroids. Patients were randomized to receive a maintenance immunosuppressive regimen with steroids, SRL and either low-dose tacrolimus or MMF. Over a mean follow-up period of approximately five years, patient and graft survival rates did not differ between groups; however, the SRL-MMF group had significantly better renal function. A relevant drawback of this trial is the association of SRL and TAC which may be nephrotoxic because of interaction between the two drugs. Moreover, in this study, SRL levels were not controlled.

In two small monocentric studies, Lo *et al*[71] and Ruggenenti *et al*[72] compared SRL- based therapy with CsA based therapy. Both studies documented similar patient and graft survival rates, similar incidence of BPAR and similar GFR between the treatments. Surprisingly at two years Ruggenenti observed higher chronic allograft damage index score in the SRL-treated patients.

In the Spiesser Group trial[73], 145 renal transplant recipients were randomized to receive either SRL or CsA in association with ATG induction, MMF and steroids. At one year, patient and graft survival and incidence of BPAR were not different. GFR was significantly higher in the SRL group, and adverse events (wound complications,

Table 7 Selected randomized trials on calcineurin inhibitors avoidance

Study (yr)	Intervention arm	Control arm	Study length (mo)
Flechner (2004)	IL2Ri+ SRLcc + MMF + S (n = 31)	IL2Ri + CsA + MMF + S (n = 30)	24
Flechner (2007)	IL2Ri +SRLcc + MMF+S (n = 31)	IL2Ri + CsA + MMF + S (n = 30)	60
Larson (2006)	rATG + SRL + MMF + S (n = 81)	rATG + TAC + MMF + S (n = 84)	12
SYMPHONY (2007)	IL2Ri + low SRL + MMF + S (n = 399)	IL2Ri+sCsA+MMF+S (n = 390)	36
Hamdy (2008)	IL2Ri + SRL+ MMF+S (n = 67)	IL2Ri + lowTAC+ SRL+S (n = 65)	60
Lo (2004)	rATG+SRL+MMF+S (n = 41)	rATG+TAC+MMF+S (n = 29)	12
Ruggenenti (2007)	Alem+SRL+MMF+ S (n = 11)	Alem+ CsA+MMF+S (n = 10)	24
Spiesser group (2007)	rATG +SRL + MMF + S (n = 71)	rATG + CsA +MMF+S (n = 74)	12
Glotz (2010)	rATG + SRL+ MMF + S (n = 71)	rATG+ TAC+ MMF+ S (n = 70)	12
ORION (2011)	IL-2Ri + SRL + MMF+S (n = 155)	IL-2Ri + TAC + MMF + S (n = 140)	24

IL2Ri: Interleukin 2 receptor inhibitor; SRLcc: Sirolimus concentration controlled; MMF: Mycophenolate mofetil; S: steroids; CsA: Cyclosporine; rATG: rabbit antithymocyte globulin; TAC: Tacrolimus; Alem: Alemtuzumab.

Table 8 Calcineurin inhibitors avoidance trials

Study (yr)	Drugs	F-Up (mo)	Patient survival		Graft survival		Biopsy proven acute rejection		Glomerular filtration rate (mL/min)	
			SRL	CNI	SRL	CNI	SRL	CNI	SRL	CNI
Flechner (2004)	SRL/CsA	24	93.50%	100%	93.50%	93.60%	6.50%	16.60%	60.6	49.2
Flechner (2007)	SRL/CsA	60	87.10%	90%	83.90%	76.70%	12.90%	23.30%	66.7	50.7
Larson (2006)	SRL/TAC	12	98.00%	96%	94%	92%	13%	10%	63	61
SYMPHONY (2007)	SRL/CsA	36	95%	94%	85%	87%	39%	27%	71.1	67.1
Hamdy (2008)	SRL/TAC	60	98.50%	93.80%	88%	83%	NA	NA	89	93
Lo (2004)	SRL/TAC	12	100%	98%	89%	80%	7%	10%	72.4	50.5
Ruggenenti (2007)	SRL/CsA	24	NA	NA	NA	NA	NA	NA	52	49.8
Spiesser Group (2007)	SRL/TAC	12	97%	97%	90%	93%	14.30%	8.60%	69	60
Glotz (2010)	SRL/TAC	12	95.80%	97.10%	85.90%	95.70%	16.90%	12.90%	56.1	58.4
ORION (2011)	SRL/TAC	24	94.5%	97%	89.9%	95.4%	32.8%	12.3%	63.4	66.7

CNI : Calcineurin inhibitor; SRL: Sirolimus; CsA: Cyclosporine; TAC: Tacrolimus; NA: Not available.

mouth ulcers, bronchopneumonia) leading to discontinuation were also higher in the SRL group. Similar results were recently obtained by Glotz *et al*[74]. These authors enrolled renal allograft recipients to receive either SRL or tacrolimus in association with ATG, MMF and steroids. At one year, patient survival and BPAR incidence were not different between groups. Graft loss and premature withdrawal from the study were higher in the SRL group. GFR was higher in the SRL group, but only in functioning grafts and in patients still undergoing the therapy.

Recently in the Optimizing Renal transplant Immunosuppression to Overcome Nephrotoxicity (ORION) study, Flechner *et al*[75] enrolled 469 patients in a multicenter study to receive SRL plus TAC or SRL plus MMF or TAC + MMF. The SRL plus MMF group was prematurely sponsor-terminated due to an excess of BPAR. Overall, one and two year GFRs were similar across the groups. CNI avoidance with MMF alone (at dose of 3 g/d) was abandoned early because of an excess of acute rejection associated with MMF side effects when given at high dose. CNI avoidance with antibody induction therapy, MMF and mTOR inhibitors gave discordant results as shown in Tables 7 and 8.

Overall, in the aforementioned studies, 2688 patients were reported. Even if several studies had good efficacy results, the overall combination of mTORi and mycophenolate was associated with increased graft failure (OR = 1.43, 95%CI: 1.08-1.90, P = 0.01) compared to CNI-based regimens. Although the attempts at CNI avoidance with SRL and MMF have not been successful, at least in the de novo setting; surprisingly, even in studies in certain subpopulations where efficacy was maintained, better renal function was difficult to document. Improved GFR in the SRL-based strategy was documented only in patients able to remain on treatment throughout the study; however, most of the patients enrolled in the SRL arms withdrew treatment due to either lack of efficacy or SRL related side effects.

CNI avoidance with newer drugs (the good promise): New agents that inhibit novel and critical pathways of immune activation are needed for the acceptance of CNI-free regimens in clinical practice. Two agents in clinical trials may help fulfill the promise of non-nephrotoxic, safe and effective immunosuppression. These agents are: Belatacept, a costimulation blocker and Tofacitinib, an inhibitor of the JAK3 signaling pathway[76].

Belatacept is a fusion receptor protein biological agent, that is administered intravenously for chronic immunosuppression and developed as a replacement for

CNIs[77,78]. Belatacept, a second-generation CTLA4Ig, binds with high affinity to CD86/CD80 and inhibits the delivery of costimulatory signals through the CD28 receptor, leading to T-cell anergy. In a phase Ⅱ trial and two pivotal phase Ⅲ trials [Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial (BENEFIT) for recipients of kidneys from standard deceased and living donors and Belatacept Evaluation of Nephroprotection and Efficacy as First-line Immunosuppression Trial-EXTended criteria donors (BENEFIT-EXT) for recipients of kidneys from extended criteria donors], two regimens (a more intense and less intense dosing schedule) of belatacept were used in combinations with MMF and steroids and compared with a regimen of CsA standard dose + MMF+ Steroids[79,80]. The less intensive regimen was recently approved by the United States Food and Drug Administration.

A careful analysis of these results at three years has been made. According such analysis the patient survival rate was similar to the CsA group, as well as the graft loss rate both in BENEFIT and BENEFIT-EXT[80,81]. In the belatacept group, most of the graft loss occurred in the first year. In the BENEFIT trial, a higher BPAR rate was observed in patients treated with belatacept compared with CsA-treated patients. The rejections occurred mostly in the first year. In the BENEFIT-EXT study, the BPAR rates were comparable across treatment groups. Concerning GFR in the BENEFIT trial, the patients on belatacept had higher GFR with respect to CsA patients (Δ = 15.1 mL/min by 1st year). In the BENEFIT-EXT trial, a similar phenomenon was observed with a smaller Δ due to the quality of donor kidneys. In both studies, a trend of GFR improvement in belatacept patients and a negative slope of -2 mL/min per year in the CsA treated patients were observed, therefore the Δ GFR increased over time, ultimately reaching a Δ of 20.8 mL/min by the 3rd year.

This trial is the first CNI avoidance study where we have a disparity between higher rejection rates and better outcomes of GFR. Less severe acute rejections with belatacept may be the cause of such disparity. More importantly, it has recently been observed that belatacept patients have lower levels of DSAs. DSAs are emerging as important biomarkers associated with subsequent graft loss[82]. Lower DSAs and lower AMR rates with belatacept have also been previously observed in experimental models of transplantation[83].

These data over three years show a better profile for belatacept with respect to CsA, for metabolic and cardiovascular parameters. The belatacept patients have a lower incidence of new onset diabetes after transplantation and a better metabolic profile with lower cholesterol and triglycerides. Lower diastolic and systolic blood pressure was also observed in belatacept patients. Nevertheless extrapolation of improvements in these surrogate markers to become therapeutic benefits requires the reporting of hard cardiovascular outcomes. To date the 4 year data both for BENEFIT and BENEFIT-EXT are available[84]. These data confirm what previously observed and described. In particular renal function in patients remaining on belatacept is stable over 4 years, differences in GFR between belatacept and cyclosporine treatment arms are sustained over time, safety profile of the belatacept regimen is consistent over time, few drop out from the studies happened and few deaths and graft losses in long term extension have been observed. Moreover, late acute rejections are rare.

To our knowledge belatacept is the first approved immunosuppressant that allows safe and effective CNI avoidance. Nevertheless, as we have observed for all the other examined trials, also the data concerning belatacept should be taken with caution. Indeed also from these studies we do not have yet the answer to the outcomes over the long-term.

Tofacitinib is an oral JAK inhibitor that suppresses intracellular signal transduction of multiple cytokines that are essential for homeostasis and function of T-cells, B-cells and natural killer cells. A small pilot study with tofacitinib showed promise in preventing acute rejection in kidney allografts[85]. In a phase Ⅱb study, tofacitinib was used at two different dosages in association with basiliximab, MMF and steroids and compared with CsA[86]. At 12 mo, patient and graft survival rates, as well as BPAR rates, were similar in all groups. GFR was significantly higher in patients who were on tofacitinib. Overall, 331 patients were enrolled in the three arms, but more patients discontinued the treatment in the tofacitinib groups. Discontinuations were mostly due to infections or hematological abnormalities, and as a consequence, only 50% of patients enrolled in the tofacitinib trial were able to complete the one-year study. Tofacitinib appears to be effective and attractive because it can be taken in pill form. Lower doses that are similar to the doses used for rheumatoid arthritis treatment should be tested. Longer phase Ⅲ studies will further our understanding of whether tofacitinib is truly a safe CNI-sparing agent.

DARK SIDE OF CNI SPARING

Overall, the aforementioned CNI-sparing strategies did not support the theory that suggests that CNI nephrotoxicity is the most important factor determining the lack of improvement in long-term graft survival. Only early CNI withdrawal with conversion to mTOR inhibitors demonstrated good results concerning efficacy, although a large number of patients who converted to mTORi did not continue using the therapy because of its side effects. The belatacept study is the only trial with positive results concerning progressive GFR improvement; however, we need a longer follow-up period to evaluate the long-term results.

CNIs can be nephrotoxic and induce progressive renal failure. The progressive and inexorable nature of CNI nephrotoxicity was documented in an elegant study by Nankivell *et al*[7,8] using renal allografts that were biopsied yearly over a 10-year follow-up period. However, at the time of the Nankivell study, DSAs and C4d staining

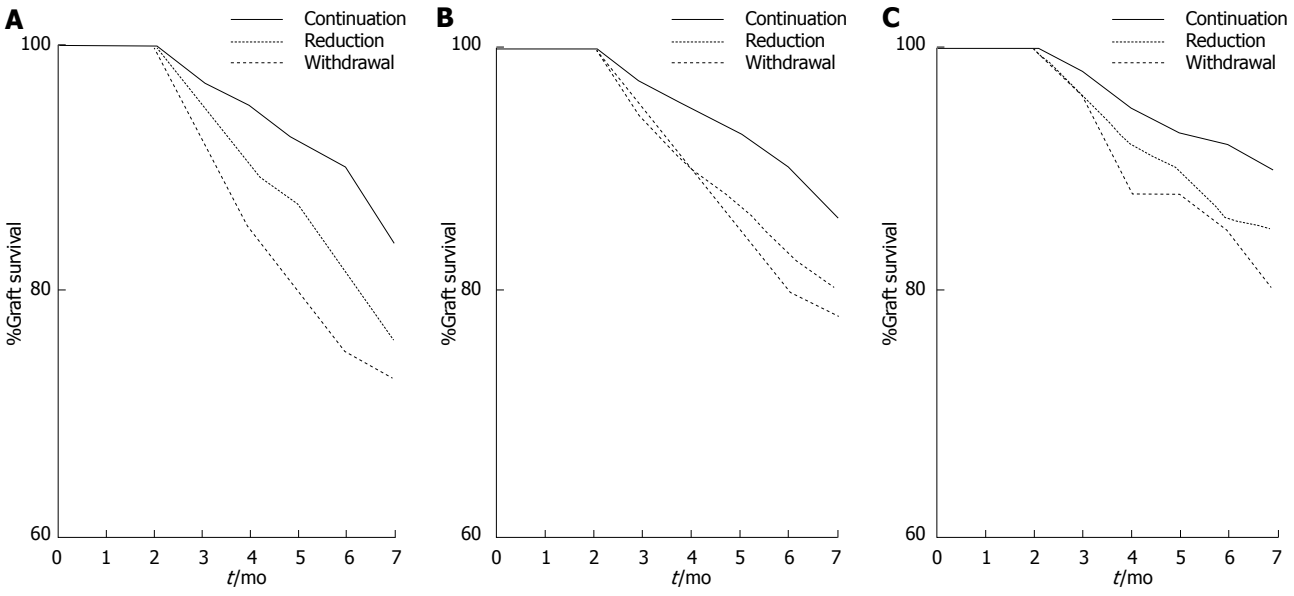

Figure 4 Collaborative Transplant Study Data. A, B: Renal graft survival between 3 and 7 years after transplantation of graft functioning at 2-year after transplantation. Survival according continuation of calcineurin inhibitor (CNI) dosage, reduction or withdrawal. Cyclosporine (A), Tacrolimus (B); C: Collaborative transplant study (CTS) data. Renal graft survival for patients on CNI immunosuppression from 3 to 7 years after transplantation. Data related to kidneys having an optimal graft function al 2-year after transplantation. Survival according continuation of CNI dosage, reduction or withdrawal.

techniques were not available, which may represent an important bias in understanding the relevance of CNI neprotoxicity in long-term outcomes and the failure of CNI minimization strategies. Three recent histological analyses dispute the conclusion by Nankivell *et al*[7,8] and Cosio *et al*[87] in a study of protocol kidney biopsies performed at one year in low immunological risk recipients reported that the presence of histological abnormalities usually ascribed to CNIs were not associated with progressive renal dysfunction. In a study by Snanaudj *et al*[88], histological lesions associated with CNI toxicity were present in the kidneys of patients who were never exposed to CNIs. In the multicenter Long Term Deterioration of Kidney Allograft Function (DeKAF) trial[89], transplant recipients who had a histological diagnosis of CNI nephrotoxicity had better outcomes than patients without this diagnosis. Furthermore, in the DeKAF trial, chronic antibody-mediated rejection (diagnosed by DSA or C4d or both) and not nephrotoxicity was the predominant cause of late graft function. The DeKAF trial and the study by Cosio *et al*[87] concluded that alloimmune injury (from underimmunosuppression) rather than nephrotoxicity (from elevated exposure to CNIs) may be the primary cause of late graft failure, which implies that strategies that were advocated to minimize CNI exposure to decrease nephrotoxicity and to improve renal function in the short term may have the unintended consequence of increasing the risk of chronic rejection and of accelerating the loss of renal allografts[90]. Registry data from the CTS study[91] by 2008 documented that CNIs reduction and/or withdrawal were associated with worse graft survival by three to seven years post-transplantation compared with the graft survival of patients continuing to receive an unchanged dose (Figure 4), which is similar to the case involving kidneys with a two-year excellent function (Figure 4C). Registry data have the limitation of not having the scientific accuracy of a randomized controlled trial, but they have the strength of reporting data of a large number of patients.

The relevance of anti-human leukocyte antigen (HLA) antibodies on long-term graft survival has been recently reviewed by Loupy *et al*[92]. Loupy highlighted that antibody-mediated rejection is now recognized as an underestimated culprit in organ failure, superseding the historical dogma that CNIs toxicity is the leading cause of graft failure. The development of DSAs after transplantation is a process that occurs at different time points, and as a consequence, AMR represents a continuum between acute and chronic damage, which can be indolent, but ultimately progresses to graft loss. Sellarés *et al*[93] followed 315 allograft recipients who underwent indication biopsies from 6 d to 32 years post-transplantation. The author aimed to relate morphological data with graft loss, and they found that the major cause of graft failure was rejection (64%), and every rejection loss could have evidence of antibody-mediated rejection according to the time of failure. In biopsies performed beyond one-year, AMR was the leading cause of graft failure, confirming that both acute and chronic AMR can occur late after transplantation and is influenced by immunosuppression reduction. In a ZEUS sub-study, Liefeldt *et al*[94] found an increase of circulating DSAs and AMR beyond the three-year point after transplantation in the cohort of patients with conversion from CsA to everolimus. As aforementioned, the ZEUS three-year data documented both good efficacy and GFR improvement after conversion. The finding of DSA after three years, shadows this important conversion study and has been a debated issue among the authors[95,96]. Similarly, an increase of acute rejection was recently described after early reduction of the TAC dose

post-transplantation[97].

A relationship between maintenance immunosuppressive treatment, development of DSA, AMR and graft failure was found by Hourmant *et al*[98] in 2005. The study investigated 1229 recipients of a kidney graft who were screened annually over a five-year period for HLA antibodies. Correlations were established between the presence and the specificity of the antibodies and clinical and therapeutic parameters. Non-donor specific antibodies appeared earlier (1-5 years post-transplantation) than DSAs (5-10 years). The presence of either DSAs or non-DSAs significantly correlated with lower graft survival and poorer transplant function. As DSAs were more frequently observed in patients between five and 10 years post-transplantation, the authors hypothesized that this finding may have resulted from a decrease of immunosuppression over time. Indeed, an international cooperative study has shown that patients who received CsA-MMF had significantly fewer antibodies than patients who received CsA-azathioprine[99].

In another smaller study conducted by Hoshino *et al*[100] on 72 kidney transplant patients from a living donor, an association between the appearance of DSAs and the immunosuppression level was observed. The risk of DSA development was greater and occurred earlier at a low immunosuppressive dosage. Thus, the risk of DSA development is inversely proportional to drug dosage, implying that greater care must be exercised with low doses of drugs. In conclusion, it is reasonable to assume that DSA development may be higher after immunosuppression weaning compared to standard triple-drug therapy. Wiebe *et al*[101] in an elegant study analyzed 315 consecutive renal transplants with a mean follow-up of 6 years. All patients underwent biopsies either per cause or per protocol, and clinical and histological correlations with serological data were then made. At the multivariate analysis independent predictors of DSA development were HLA-DRβ 1 MM and non-adherence. A strong trend toward clinical rejection episodes preceding DSA was also present. The median 10 years graft survival for patients with DSA was lower compared with patients without DSA.

The relevant finding of this study was that non-adherence was the major cause of DSA development and graft loss over time. Non-adherence is clearly defined as patient admission of medication non-adherence documented by clinic staff and/or drug levels below the detectable limit. Repeated failure to attend clinic visits or perform laboratory evaluation constituted a pattern of behaviour defined as non-adherence in a minority of patients. This finding is extremely important, and non-adherence is common in all transplant patients, but it is easy to understand that this behaviour is more common and dangerous in all the trials aimed at CNI sparing. Indeed, in these strategies, the therapeutic window of immunosuppressant drug level is extremely narrow and non-adherence can have dramatic consequences under these conditions.

All the aforementioned studies should be considered with caution because the finding of anti-HLA-Ab is recent and lacks in the older studies on CNI sparing strategies. Moreover, several studies on the relevance of anti-HLA antibodies on graft loss lack of enough long-term follow-up. Nevertheless the study of Sellarés *et al*[93] has almost three-year follow-up and the study of Wiebe *et al*[101] has a mean follow-up of 6.2 years. In a recent paper by Everly *et al*[102], 20% of patients immunosuppressed with CNIs and in triple therapy developed DSA after 4 years and 24% of them lost the graft within three years. Additionally, in the review of Loupy *et al*[92] the natural history of antibody-mediated allograft deterioration is quite well described. DSAs are complement activating and this fact leads to endothelial injury with glomerulitis and peritubular capillaritis. Chronic ABMR lesions are not reversible and will worse with time. Graft function decreases at different rates depending on the severity of the initial presentation, type of treatment and response to treatment itself. The DeKAF study[89] analyzed recipients with new onset late kidney graft dysfunction to determine the relevance of C4d staining and circulating DSAs on subsequent late graft failure. Evidence of antibody mediated injury (DSAs or C4d) is common (57%) in patients with new onset late kidney allograft dysfunction. Importantly, 96% of these patients were treated by CNIs. The question that arises from these and other studies is why CNIs are not enough effective to control B cell mediated acute and chronic rejection. While T cell mediated alloimmunity has been largely controlled using CNIs based immunosuppression, the role of B cells is just beginning to be understood. Recent studies have highlighted several important clinical issues involving the antibodies, including early acute humoral rejection and late post-transplant glomerulopathy. These studies have identified the relevant role of bone marrow derived long lived plasma cells that appear to be a major source of donor-specific alloantibodies[103].

CNIs have an important and valuable inhibitory action on T cells, and affect the humoral immune response by interfering with T helper signals, but not targeting B cells directly[104]. Moreover, in transplantation as well as in autoimmune diseases, B cells in addition to their role in the humoral response to alloantigens, act as efficient antigen presenting cell, so participating in the activation of T cells[105]. Evidence that CNI immunosuppression suppresses $CD4^+$ T cell function would suggest that antibody production may be severely limited in CNIs treated patients and related to an indirect pattern. In a study on heart transplantation CNI therapy does not prevent the production of alloantibody with the capacity to mediate allograft vasculopathy[106]. The reduced efficacy of CNIs on bone marrow derived long lived plasma cells, on memory B cells coupled with the observation that T cell tolerance does not always convey tolerance in naive B cells, seem to represent the basis of the reduced action on CNIs on antibody production.

One of the issue raised by this work is to find out

why CNIs based strategies did not improve long term renal allograft outcome as waited from the first results of CNIs in transplantation. The hope that CNIs sparing strategies, lowering nephrotoxicity could improve graft long-term outcomes is not documented by almost all the trials analyzed. In contrast, sparing strategies carry the risk of increasing acute or chronic AMR. The impact of CNIs sparing strategies on this issue is documented by a small number, but significant, studies. Indeed, the vast majority of CNI sparing studies have a short term follow-up and do not include in the protocol the research for DSAs. Moreover, we have documented that DSAs often appear after a long term from transplantation. The Liefeldt *et al*[94] study studying patients enrolled in the ZEUS study beyond three years, found that 10.8% of patients on cyclosporine developed DSAs, while 23% of patients randomized to everolimus developed DSAs (P = 0.048). Similarly, out of 10 patients who developed biopsy proven AMR, eight had been randomized to everolimus based immunosuppression and three with continued use of CsA (P = 0.036). This study demonstrates for the first time that an everolimus based immunosuppression and a CsA sparing strategy may be associated with an increased risk of developing DSAs and AMR.

In a further analysis of the DeKAF study[89], patients reducing TAC dose early post transplantation (2-3 mo) are at higher risk for acute rejection. Indeed higher TAC levels lowered the risk of rejection by 78% (HR = 0.22, P < 0.001). The study of Sellarés *et al*[93] documents in the multivariate analysis that major causes of AMR is the non-adherence to therapy, that consists in the reduction of the prescribed doses spontaneous and documented by physicians. Non-adherence is a common behaviour in transplant patients and may be particularly dangerous in the setting of CNIs reduction.

CONCLUSION

The major causes of late graft loss include chronic allograft nephropathy (CAN: a useful but limited term, because it lacks specificity) and death with a functioning graft. The perception that most grafts are lost due to the inexorable progression of CNIs nephrotoxicity has led to several trials based on CNIs sparing which has been extensively described. Except for several studies with early CNI withdrawal converting CNIs to mTORi, the other studies failed at their attempts. Moreover, CNIs early conversion of CNIs to mTORi has proven to be effective only in some patients; however, understanding which patients would benefit from this conversion before the start of the therapy would increase the success of the method. Until now, only the belatacept trial appears to have been effective, as it led to a stable improvement of GFR in CNI-free patients without emerging post-transplant DSAs. Also this study has a follow up of 4 years and the data should be considered with caution and probably do not allow to foresee the effects of this drug in the long-term. The CNI nephrotoxicity as a cause of late graft failure is also being challenged by the findings of the DeKAF[89,107,108] and others studies[109,110]. According to these studies, chronic immune injury mediated by anti-donor antibodies may account for the majority of late graft losses. Thus a new paradigm for immunosuppression regarding long-term maintenance of renal allografts is emerging. This paradigm includes the use of immunosuppressants able to control chronic humoral anti-donor injury. Indeed, the reason for CNI failure in improving long-term outcomes may be the relative ineffectiveness of CNIs in combating acute and chronic humoral-mediated injury. Furthermore, the minimization and withdrawal protocols of CNIs, implemented to combat late allograft loss by minimizing nephrotoxicity and metabolic derangements from CNIs, may contribute to late allograft loss from chronic and sub-acute immune-mediated injuries[111].

The two main frontiers we have ahead are the following: (1) In the setting of CNIs sparing, we need safe tools for immune-monitoring of every patients[112]. These tools include the monitoring of protocol biopsies that are the gold standard for analyzing graft damage, checking anti-HLA-antibodies that are known to have a negative impact on the graft outcome, by pharmacogenomics to better understand CNIs disposition and drug-related nephrotoxicity, checking soluble CD30 and other molecules known to have a negative impact on graft outcomes and microarray analysis of graft biopsies to detect signatures of chronic allograft nephropathy; and (2) With respect to finding new agents with novel mechanisms of action, devoid of the toxicities associated with CNIs, non-protein drugs targeting intracellular pathways and biological agents targeting B-and T- cell surface receptors and ligands are already in phase Ⅱ and Ⅲ clinical trials. These new drugs represent hope in the field of solid organ transplantation.

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CASE REPORT

Residual renal function in peritoneal dialysis with failed allograft and minimum immunosuppression

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Abstract

Immunosuppression (IS) is often withdrawn in patients with end stage renal disease secondary to a failed renal allograft, and this can lead to an accelerated loss of residual renal function (RRF). As maintenance of RRF appears to provide a survival benefit to peritoneal dialysis (PD) patients, it is not clear whether this benefit of maintaining RRF in failed allograft patients returning to PD outweigh the risks of maintaining IS. A 49 year-old Caucasian male developed progressive allograft failure nine years after living-donor renal transplantation. Hemodialysis was initiated via tunneled dialysis catheter (TDC) and IS was gradually withdrawn. Two weeks after IS withdrawal he developed a febrile illness, which necessitate removal of the TDC and conversion to PD. He was maintained on small dose of tacrolimus (1 mg/d) and prednisone (5 mg/d). Currently (1 year later) he is doing exceedingly well on cycler-assisted PD. Residual urine output ranges between 600-1200 mL/d. Total weekly Kt/V achieved 1.82. RRF remained well preserved in this patient with failed renal allograft with minimal immunosuppressive therapy. This strategy will need further study in well-defined cohorts of PD patients with failed allografts and residual RRF to determine efficacy and safety.

Key words: Immunosuppression; Kidney transplantation; Nephrectomy; Peritoneal dialysis; Renal function reserve

Core tip: Making decision regarding the optimal management of immunosuppression is one the most challenging decisions following allograft failure. The use of low dose immunosuppressive medications is the most reasonable approach. Many patients with failed allograft require renal replacement therapy. Peritoneal dialysis (PD) remains underused modality in failed renal allograft, especially in patients with residual renal function (RRF). Our patient failed renal transplant and was initiated on PD and maintained on minimal immunosuppression. Interestingly, his RRF remained well preserved. We recommend further study in well-defined cohorts of PD patients with failed allografts and RRF to determine efficacy and safety.

Elmahi N, Csongrádi É, Kokko K, Lewin JR, Davison J, Fülöp T. Residual renal function in peritoneal dialysis with failed allograft and minimum immunosuppression. *World J Transplant* 2013; 3(2): 26-29 Available from: URL: http://www.wjgnet.com/2220-3230/full/v3/i2/26.htm DOI: http://dx.doi.org/10.5500/wjt.v3.i2.26

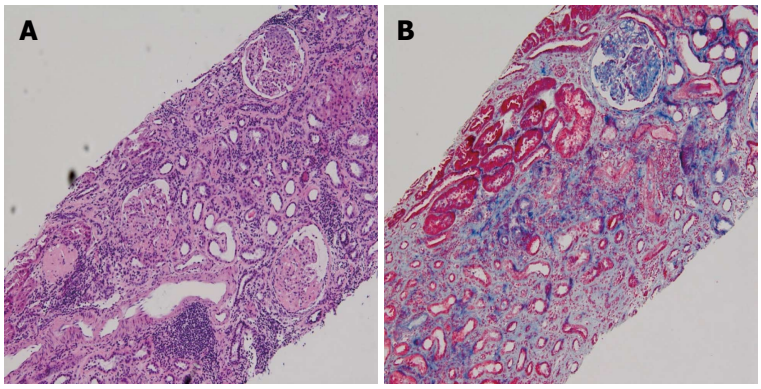

Figure 1 Patient was admitted to the hospital and underwent a diagnostic percutaneous ultrasound guided renal biopsy (HE stain, ×100). Hematoxylin and eosin (A) and periodic acid-Schiff stains of the kidney biopsy specimens (light microscopy) (B) showing the histopathology examination of the kidney, which tissue confirm the presence of focal segmental glomerulosclerosis as evidenced by involvement of approximately 50% of the glomeruli with segmental lesions and some of the glomeruli had total global glomerulosclerosis. There was also associated interstitial fibrosis and tubular atrophy.

INTRODUCTION

Data from the United States Renal Data System revealed that the number of patients with a failed transplanted kidney in the United States has increased over the past few years[1]. The management of those patients with a failed transplant involves two major decisions: optimal management of immunosuppression (IS) and whether or not to perform graft nephrectomy. While there might be a survival advantage in maintaining dialysis patients on long-term immunosuppressive therapy after allograft failure[2], immunotherapy comes with its own risks, which include increased susceptibility to infections and cancers[3,4]. This case report and review of the literature illustrates the fact that not all dialysis patients with allograft dysfunction are created equally and that different cohorts deserve further study regarding the benefits of maintenance of low dose IS after declared allograft failure.

CASE REPORT

A 49-year-old Caucasian male with past medical history of hypertension was diagnosed with end stage renal disease (ESRD) and was started on peritoneal dialysis (PD) in 2001. One year later he had living-donor renal transplantation, after which he maintained fair allograft function with new baseline creatinine around 1.8-2.2 mg/dL. His initial immunosuppressive therapy included tacrolimus, mycophenolate mofetil (MMF) and prednisone. His medications were adjusted over the next few months and he was maintained on tacrolimus 3 mg twice daily, MMF 500 mg twice daily, and prednisone 5 mg/d. 3 mo after his transplant, he had a biopsy-proven type ⅡA acute rejection, which responded well to treatment with steroids.

He presented to the transplant clinic on September 13, 2010 for a routine visit with elevated serum creatinine of 3.2 mg/dL compared to creatinine of 1.8 mg/dL one year prior to that. Further testing revealed nephrotic range proteinuria of around 3 g by a spot urine analysis. The patient has been compliant with his immunosuppressive medications and has no major change in his medical, surgical, and social history. The patient was admitted to the hospital and underwent a diagnostic percutaneous ultrasound guided renal biopsy (Figure 1). Histopathologic examination of the tissue confirm the presence of focal segmental glomerulosclerosis as evidenced by involvement of approximately 50% of the glomeruli with segmental lesions and some of the glomeruli had total global glomerulosclerosis. There was also associated interstitial fibrosis and tubular atrophy. Immunofluorescence studies were consistent with a diagnosis of focal segmental glomerulosclerosis with 2+ staining for IgG and a segmental distribution, 2+ staining of IgM and a segmental distribution, negative staining for IgA and 2+ staining for kappa and lambda light chains. The patient had one area of questionable crescent formation on a single glomerulus but the biopsy was unrevealing otherwise for any other disease process. There was no evidence of transplant rejection or antibody mediated rejection as the patient had a negative C4d immunofluorescence. His medications were adjusted where his prednisone dose was increased to 60 mg/d and lisinopril was resumed to reduce proteinuria.

His creatinine worsened gradually over the next five months. He was readmitted to the hospital in February 2011 with herpes zoster involving his eye and was treated with ganciclovir and local erythromycin ointment. The serum creatinine was 5.02 mg/dL at the time of admission and 5.42 mg/dL at the time of discharge. It was clear that the patient was experiencing progressive renal allograft failure and the options of dialysis were explained to the patient.

Few days after his discharge, he was readmitted to the hospital for evaluation of pneumonia and was treated with antibiotics. During that hospitalization his renal function continues to worsen with associated oliguria and clinical uremia that required initiation of dialysis. Tunneled dialysis catheter (TDC) was placed and the patient was discharged in stable condition. He remained oliguric with minimal urine output and he continued hemodialysis *via* TDC. In the interim, he also had a PD catheter placed. MMF was discontinued but he was maintained on low dose of tacrolimus (1 mg twice daily). Two months later he was re-admitted to the hospital with suspected sepsis and associated TDC infection. He was treated with antibiotics, stress dose steroids and removal of the hemodialysis catheter. During the hospitalization he had increased urine output up to 1.0-1.5 L per 24 h. However, he continued to be dialysis dependent with elevated creatinine around 7-8 mg/dL. At that point of time, PD was

initiated and we opted to continue his tacrolimus at 1 mg daily (serum levels not measurable) and prednisone 5 mg daily. Currently (1 year later) he is doing exceedingly well on cycler-assisted PD regimens of 10 L exchanged over 8 h. Residual urine output ranges between 600-1200 mL/d. Total weekly Kt/V achieved 1.82 (dialysate: 1.30; endogenous: 0.51) and global creatinine clearance 64.8 L/wk per 1.73 m^2 (dialysate: 39.3; endogenous: 25.4). A renal scan confirmed that all endogenous renal function is originating from the partially functioning renal allograft. Furthermore, his albumin remained stable at 4 g/dL and hemoglobin well controlled (11.6 g/dL) on darbopoteinalfa 12.5 mg/wk. He is currently awaiting another renal transplant and has an arteriovenous fistula in place.

DISCUSSION

Management of immunosuppression after graft failure

Approximately 20% of all renal patients on the transplant waiting list in the United States have had a previously failed allograft[5]. Initiating dialysis on those patients with failed renal transplant usually prompts the clinician to withdraw immunotherapy to reduce the risk of infection. Gregoor *et al*[4] showed that patients with allograft failure who were maintained in low-dose IS suffered from high infectious complications, in addition to higher cardiovascular-related death. Those findings were supported by more recent study done by Johnson *et al*[3], who studied more than 5000 patients who initiated dialysis after failed renal transplant. Their study revealed overall sepsis rate of 12 per 100 patient years and the sepsis rates were higher in the first 76 mo after transplant failure. Along the same line, Smak Gregoor *et al*[6] argued against the value of using low dose immunosuppressive medications based on the perceived morbidity and mortality associated with immunosuppressive medications. His group analyzed data from patients' files, with renal failure after at least 3 mo graft function. The authors found that continuation of immunosuppressive medication did not lead to fewer rejections. They revealed an increase in morbidity and mortality in the group with low immunosuppressive medications[6]. Closer scrutiny of this study, however, revealed that many of the conclusions might not be applicable to the current era where the majority of the transplant occurred in the pre-cyclosporine era with a large variation of maintenance prednisone doses and about one-third of the patients were on significant doses of azathioprine[6]. It is also unclear, how many of them have been placed upfront on PD to reduce the risk of infection and sepsis typically caused by infection of TDC.

There has been no consensus on the optimal management of IS in patients with a failed transplant. Nonetheless, the decision to continue low-dose IS *vs* IS withdrawal must be individualized as both options have their inherent advantages and disadvantages. Immunosuppressive withdrawals' protocols vary among transplant centers with most centers discontinue anti-metabolites abruptly and taper calcineurin inhibitors over several weeks and prednisone over a 3-6 mo period. Certain adverse effects should be considered in the process of withdrawing IS that include precipitation of rejection, the potential need for transplant nephrectomy, secondary adrenal insufficiency, and loss of RRF[2,7].

The role of nephrectomy after graft failure

Nephrectomy of the failed allograft remains a controversial issue. Failed allograft with no symptoms may not require an immediate intervention. However, some centers routinely refer these patients for nephrectomy in the absence of symptomatic rejection to prevent potential future complications[8,9]. Recent retrospective study by Ayus *et al*[10] suggested that patients who undergo allograft nephrectomy after graft failure might experience superior outcomes to those who did not. The limitations of this study include its retrospective nature and the unclear reasons for nephrectomy. Madore *et al*[11] revealed that the need for late allograft nephrectomy was correlated with the number of previous episodes of acute rejection. The authors suggested more gradual tapering of IS or continuation of low-dose IS indefinitely to reduce the need for nephrectomy.

It is more acceptable practice to perform post allograft failure nephrectomy when patients develop symptoms attributed to the failed renal allograft[11]. The surgical risk, rising number of circulating antibodies, reduced erythropoietin, and preserved urine output are among the arguments for observing or supporting a failed allograft[12,13]. On the other hand, chronic inflammation, potential for malignancy and infections has been raised as arguments for surgical intervention[13,14].

Need for dialysis and the choice of dialysis modality

Among transplant-native, those treated with PD enjoy an early survival advantage compared with those treated with hemodialysis (HD) but this advantage is not sustained over time. However, it is not clear if this advantage persist in post allograft failure in patients treated with PD. On the other hand, survival of patients initiating PD after graft loss may be equivalent to that seen in transplant-naïve patients on PD[15-18]. The outcome of the dialysis modality (PD or HD) can be affected by the use of immunosuppressive medications and the need for transplant nephrectomy[19]. However, no survival benefit was found when using PD versus HD. Perl *et al*[20] studied 2110 adult patients who initiated dialysis after renal transplant failure and after adjustment, the authors found no difference in overall survival between HD-treated and PD treated patients with similar results seen for both early and late survival.

Nevertheless, PD remains underused modality in patients with failed renal allograft as suggested by many researchers[18,21]. Davies[21] revealed that PD would appear to be a good option for patients with failing allograft. His study also demonstrated that the earlier loss of residual Kt/V in those patients might be prevented by continuing IS after commencement of dialysis.

In summary, the management of patients with a failed transplant involves two major decisions: optimal management of IS and whether or not to perform graft nephrectomy. The use of low dose immunosuppressive medications in failed renal allograft is the most reasonable approach. Transplant nephrectomy is not routinely indicated but might be required in certain group of patients with morbidities related to transplant. Many patients with failed allograft require a period of renal dialysis while relisted for new renal transplant. There is no clear evidence to support the superiority of hemodialysis or PD in the treatment of patients with failed allograft. However, PD remains underused modality in failed renal allograft, especially in patients with RRF. Our patient failed renal transplant and was declared ESRD. PD was initiated and he was maintained on minimal immunosuppressive regimen with tacrolimus 1 mg/d. Interestingly, his residual renal function remained very well preserved. We recommend further study in well-defined cohorts of PD patients with failed allografts and residual renal function to determine efficacy and safety.

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Acknowledgments

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5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean ± SD or mean ± SE.

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