Rapamycin for Treatment of Chronic Allograft Nephropathy in Renal Transplant Patients

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Chronic allograft nephropathy (CAN) represents the main cause of renal allograft loss after 1 yr of transplantation. Calcineurin inhibitor (CNI) use is associated with increased graft expression of profibrotic cytokines, whereas rapamycin inhibits fibroblast proliferation. The aim of this randomized, prospective, open-label, single-center study was to evaluate the histologic and clinical effect of rapamycin on biopsy-proven CAN. Eighty-four consecutive patients who had biopsy-proven CAN and received a transplant were randomized to receive either a 40% CNI reduction plus mycophenolate mofetil (group 1; 50 patients) or immediate CNI withdrawal and rapamycin introduction with a loading dose of 0.1 mg/kg per d and a maintaining dose aiming at through levels of 6 to 10 ng/ml (group 2; 34 patients). The follow-up period was 24 mo. At the end of follow-up, 25 patients (group 1, 10 patients; group 2, 15 patients) underwent a second biopsy. CAN lesions were graded according to Banff criteria. α-Smooth muscle actin (α-SMA) protein expression was evaluated in all biopsies as a marker of fibroblast activation. Graft function and Banff grading were superimposable at randomization. Graft survival was significantly better in group 2 ($P = 0.0376, \chi^2 = 4.323$). CAN grading worsened significantly in group 1, whereas it remained stable in group 2. After 24 mo, all group 1 biopsies showed an increase of α-SMA expression at the interstitial and vascular levels ($P < 0.001$); on the contrary, α-SMA expression was dramatically reduced in group 2 biopsies ($P = 0.005$). This study demonstrates that rapamycin introduction/CNI withdrawal improves graft survival and reduces interstitial and vascular α-SMA expression, slowing down the progression of allograft injury in patients with CAN.


The introduction of new medications in the 1990s offered several options for maintenance immunosuppression in kidney transplant recipients. New immunosuppressive drugs were introduced on the basis of their ability to reduce the incidence of acute rejection and to demonstrate short-term outcomes at least equivalent to those achieved with the use of established immunosuppressive therapy (1,2). With the success of kidney transplantation established in the short term, the focus of transplant clinicians has shifted toward improving long-term outcomes. Chronic allograft nephropathy (CAN), the main cause of renal allograft loss (3), can be identified in 40 to 60% of routine allograft biopsies 24 mo after transplantation and may cause a progressive deterioration in renal function ultimately resulting in allograft failure (3,4). Seron et al. (5) clearly demonstrated that renal allografts may develop chronic lesions despite a stable and even optimal graft function.

Although the pathogenesis of chronic vascular, interstitial, and glomerular damage featuring CAN is still largely unclear, both immune and nonimmune mechanisms may participate in the development of this pathologic condition. Among the latter, a key role was suggested for the potential nephrotoxic effects of calcineurin inhibitors (CNI), such as cyclosporine (CsA) and tacrolimus (6). CNI nephrotoxicity is characterized at the molecular level by a significant increase of profibrotic cytokines within the graft (6). These soluble mediators that are produced mainly by epithelial cells may prime their transdifferentiation into fibroblast and their subsequent activation, leading directly to interstitial fibrosis (7). α-Smooth muscle actin (α-SMA) is a specific marker of activated fibroblast also known as myofibroblast (7). Myofibroblasts are believed to play a central role in the pathogenesis of renal fibrosis regardless of the initial cause (8).

The patients with CAN present a clear need for new strategies to achieve adequate immunosuppression with simultaneous reduction of the possible detrimental effect of CNI influence on long-term graft function. CNI dose reduction with the addition of mycophenolate mofetil (MMF) resulted in the improvement of renal function in patients with established
chronic allograft damage (9,10). This observation led to a broad interest in the possibility to reduce CsA dose introducing newer agents in the immunosuppressive protocols. Rapamycin is a macrocyclic lactone with a novel mechanism of immunosuppression (11). Previous studies demonstrated a beneficial role of this new immunosuppressive drug on long-term graft function in association with CSA for the first 3 mo after transplantation and the subsequent withdrawal of the CNI (12,13). In addition to its effects on T cells, rapamycin has been shown to inhibit in vitro growth factor–driven proliferation of smooth muscle cells and fibroblasts (14) and to reduce intimal hyperplasia in immune and nonimmune models of vascular injury (15,16). On the basis of this observation, it is conceivable that rapamycin may be beneficial in the treatment of CAN.

To test this hypothesis, we performed a prospective, open-label, single-center study that was designed to provide preliminary information on a previously untested therapy. In this study, we evaluated the impact of CsA withdrawal and introduction of rapamycin versus CsA dose reduction plus MMF on graft outcome in patients with histologic diagnosis of CAN. As a secondary aim, we evaluated the expression of α-SMA in renal allograft biopsy at the CAN diagnosis and after 24 mo of treatment as a surrogate marker of progressive renal damage.

Materials and Methods

Study Design

This is a prospective, open-label, single-center study that was designed to provide preliminary information on a previously untested therapy. Starting from September 2000 to December 2002, all patients who had received a kidney transplant; were 12 to 36 mo after transplantation; had stable graft function and serum creatinine levels ranging from 1 to 3 mg/dl; and were in treatment with corticosteroids and CsA/tacrolimus. Patients with biopsy-proven CAN were enrolled. Fifty patients (group 1) were randomly assigned to receive a 40% CNI dose reduction plus MMF and 34 patients (group 2) to immediately withdraw CNI with the introduction of rapamycin. Eighty-four consecutive patients with biopsy-proven CAN were enrolled. Fifty patients (group 1) were randomly assigned to receive a 40% CNI dose reduction plus MMF and 34 patients (group 2) to immediately withdraw CNI with the introduction of rapamycin. The first 15 patients were enrolled consecutively in group 1; subsequently, all patients were randomly assigned 1:1 in the two arms. The reason for this enrollment was an initial failure in the mass ultraviolet spectrometric system to determine rapamycin blood-through concentrations for the first 6 mo of enrollment. A second protocol biopsy was offered to all patients 24 mo after randomization, but only patients with a grade 1 (G1) CAN at the first biopsy were considered for the histologic study in an attempt to define better the influence of both immunosuppressive regimens on CAN progression. The study was approved by the local Ethical Committee, and all patients gave their written informed consent.

Renal Allograft Biopsy

Renal specimens, obtained by needle-core biopsies (16 gauge) that were performed under ultrasonographic guidance, were fixed in 4% formaldehyde, paraffin-embedded, and then processed for routine histologic staining (hematoxylin-eosin, periodic acid-Schiff, silver methenamine, and Masson’s trichrome). The histologic lesions of the four renal compartments (glomeruli, tubules, interstitium, and vessels) were scored independently by two pathologists (G.G. and L.G.) who were unaware of immunosuppressive therapy of the patients when analyzing the second biopsy. Semi-quantitative scoring for acute and chronic tubular, interstitial, vascular, and glomerular changes was performed according to the Banff 1997 criteria (17). For limiting imprecision in score estimation, only biopsy specimens with at least 10 glomeruli and at least two arterial sections were considered representative. All examined biopsies satisfied listed criteria.

Confocal Microscopy

The expression of α-SMA in graft biopsies at the diagnosis of CAN and 24 mo after treatment assignment was evaluated by indirect immunofluorescence and confocal microscopy analysis using a specific mAb (Dako, Milan, Italy). To this purpose, 4-μm-thick frozen tissue sections were incubated for 1 h with a blocking solution of BSA 3% in PBS. Subsequently, the tissue slices were incubated with the primary antibody (1:500 dilution in BSA 3%/PBS) for 2 h at room temperature in a humidified container. After washing, the sections were incubated with a secondary antibody (Alexa Fluor 488 goat anti-mouse IgG-FITC conjugate, 1:200 dilution; Molecular Probes, Eugene, OR) for 1 h at room temperature and Alexa Fluor 543 goat anti-rabbit IgG-TRITC conjugate (1:600 dilution; Molecular Probes). The slides then were mounted in Gel/Mount (Biomedia, Milan, Italy) and sealed.

Immunosuppressive Regimens before Randomization

Before randomization, 60 patients were receiving 2.9 to 4.1 mg/kg per d CsA in two equally divided doses, and 24 patients were receiving 0.1 to 0.2 mg/kg per d tacrolimus in two equally divided doses; all patients received 1000 mg/d MMF and 5 mg/d prednisone.

Immunosuppressive Regimens after Randomization

In group 1, 40 patients received a dose reduction of CsA, adjusted to maintain C2 levels of 400 to 500 ng/ml, and 10 received a dose reduction of tacrolimus adjusted to maintain trough levels at 4 to 6 ng/ml. After randomization, the actual dose of CsA was 1.5 to 2.5 mg/kg per d, and the dose of tacrolimus was 0.05 to 0.14 mg/kg per d. In all patients, the dose of MMF and prednisone remained the same after randomization. In group 2, CNI and MMF were withdrawn and rapamycin was introduced with an initial loading dose of 0.10 mg/kg for the first day, then 0.04 to 0.06 mg/kg per d, with dosage adjusted to maintain trough levels at 6 to 10 ng/ml; in all patients, the dose of MMF and prednisone remained the same after randomization.

CsA whole-blood C2 levels were determined locally using a RIA mAb technique (Behring Diagnostics, Milton Keynes, UK). Rapamycin blood-trough concentrations were determined locally using an HPLC assay coupled with ultraviolet spectrometric detection.

Outcome Measures

Actuarial graft survival at 24 mo after randomization in all groups of patients was considered as the primary outcome measure. As additional parameters, we considered graft function evaluated by serum creatinine (sCr), creatinine clearance (Nankivell formula) (18), and 24-h proteinuria at 24 mo and changes in the degree of histologic CAN lesions. Moreover, we evaluated in the two groups of patients the expression of α-SMA in renal allograft biopsy at time of CAN diagnosis and after 2 yr as a surrogate marker of damage progression.

Statistical Analyses

The results of the quantitative variables were expressed as mean ± SD, and those of the qualitative variables were expressed as proportions. Actuarial graft survival was calculated from the date of renal transplantation to graft failure or patient death. Survival curves were generated using the Kaplan-Meier method and compared using the log-rank (Mantel-Cox) test. All tests were two-tailed. P < 0.05 was
Results
The patients in the two groups had similar baseline features and did not differ significantly for donors’ clinical features, number of mismatches, cold ischemia time, and incidence of delayed graft function (Table 1). Both groups displayed the same low incidence of acute rejection (10%) before randomization; specifically, the grade of acute rejection was mild in all patients and presented within 3 mo posttransplantation. Patients in the two groups failed to show any significant difference in the whole incidence of posttransplantation diabetes, dyslipidemia, and arterial hypertension before randomization (Table 2). The mean time between transplantation and CAN diagnosis was similar in the two group of patients (group 1, 24 ± 7; group 2, 21 ± 9 mo).

After randomization, no acute rejection episode was observed. Group 2 patients showed increased cholesterol and triglyceride levels (total cholesterol: at randomization 165 ± 40 mg/dl; at 24 mo 225 ± 21 mg/dl; triglycerides at randomization 174 ± 31 mg/dl; at 24 mo 220 ± 51 mg/dl; NS), which dictated the extensive use of lipid-lowering agents: All patients were prescribed either fibrates or statins, as appropriate. In contrast, in group 1, serum lipid levels remained unchanged (total cholesterol at randomization 177 ± 34 mg/dl; at 24 mo 185 ± 25 mg/dl; triglycerides at randomization 190 ± 29 mg/dl; at 24 mo 187 ± 33 mg/dl; NS). The incidence of arterial hypertension was slightly, although not significantly, higher in group 1 (mean arterial pressure at randomization 104 ± 3 mmHg; after 24 mo 108 ± 4 mmHg), compared with group 2 patients (mean arterial pressure at randomization 102 ± 4 mmHg; after 24 mo 99 ± 3 mmHg), and group 1 patients required more antihypertensive drugs. A mild decline of hemoglobin concentration was observed in the whole incidence of posttransplantation diabetes, and all tissue samples were negative for C4d. No significant difference between the two treatment groups was observed in scoring graft biopsies; in fact, CAN lesions were scored as mild (G1) in 60% and moderate to severe (G2) in 40% of group 1 and mild in 50% and moderate to severe in the remaining 50% of group 2 patients.

After 2 yr of treatment, we performed a protocol graft biopsy in 25 patients: 10 of group 1 and 15 of group 2. All examined biopsies were considered as representative. Immunofluorescence excluded signs of recurrent or de novo glomerulonephritis and were C4d negative. All patients who underwent second biopsy had grade 1 lesions at first allograft biopsy (Figure 1). All 2-yr biopsies of group 1 patients showed CAN lesions scored as moderate to severe (Figure 1). On the contrary, the biopsies of group 2 patients showed CAN lesions scored as mild (G1) in 13 of 15, and only two biopsies were scored as G2 (P = 0.002; Figure 1).

α-SMA Expression
We observed a diffuse positivity of α-SMA expression in all biopsies performed at randomization (Figure 2, A and B). In particular, α-SMA staining was diffusely positive within the interstitial area, directly and significantly correlating with the extent of interstitial fibrosis. When we analyzed the second biopsies, at 2 yr after randomization, we observed in group 1 a slight increase of interstitial α-SMA expression compared with the first biopsy (Figure 2C). On the contrary, α-SMA protein expression was significantly reduced in group 2 biopsies (Figure 2D). Quantification of α-SMA expression revealed a significant reduction of interstitial expression only in group 1 (Figure 2E).

At the vascular level, we observed, as expected, a diffuse staining for α-SMA in the arterial wall (Figure 3, A and B). It is interesting that in the second biopsies of group 1 patients, we observed a significant increase in α-SMA staining intensity at the vascular level, suggesting an increase in vascular smooth muscle cells (Figure 3C), whereas in group 2 biopsies, the vascular α-SMA staining remained unchanged (Figure 3D). Quantification of α-SMA expression revealed a significant reduction of vascular expression only in group 1 (Figure 3E).

Graft Outcome
Both groups showed 100% actuarial patient survival at 2 yr after randomization. Graft survival was significantly better in group 2 patients (P = 0.0376, χ² = 4.323; Figure 4). Indeed, we observed eight events in group 1 patients versus only one event in group 2 patients.

In addition, sCr, calculated creatinine clearance (Nankivell formula), and daily proteinuria were evaluated at discharge, at randomization, and 2 yr after randomization. Graft function was similar in the two groups at discharge (sCr 1.8 ± 0.99 versus 1.90 ± 0.48 mg/dl; calculated creatinine clearance 53.6 ± 19.7 versus 50.7 ± 14.3 ml/min; proteinuria 0.69 ± 0.24 versus 0.58 ± 0.39 [group 1 versus group 2]), at randomization (sCr 2.01 ± 0.63 versus 2.00 ± 0.55 mg/dl; calculated creatinine clearance 50.8 ± 22.9 versus 50.1 ± 19.3 ml/min; proteinuria

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Table 1. Donor and recipient features at the time of transplantation

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<th>Group 2</th>
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<tr>
<td>Donors (n)</td>
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<td>32 (18 M, 14 F)</td>
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<tr>
<td>mean age (yr)</td>
<td>49 ± 19</td>
<td>42 ± 20</td>
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<td>creatinine clearance (ml/min)</td>
<td>70 ± 15</td>
<td>66 ± 21</td>
</tr>
<tr>
<td>Recipients (n)</td>
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<td>34</td>
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<tr>
<td>mean age (yr)</td>
<td>43 ± 9</td>
<td>49 ± 10</td>
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<tr>
<td>cold ischemia time (hr)</td>
<td>14 ± 3.1</td>
<td>16 ± 2.8</td>
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<td>HLA-MM</td>
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<td>delayed graft function (%)</td>
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<td>29.4</td>
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Table 2. Main clinical features of the patients included in the study, divided according to the immunosuppressive regimen adopted, at randomization and 2 yr after randomization

<table>
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<th>Group 1</th>
<th>Group 2</th>
<th>Group 1</th>
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<td>Patients (n)</td>
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<td>33</td>
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<td>Acute rejection (%)</td>
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<td>Hemoglobin (g/dl)</td>
<td>11 ± 2.1</td>
<td>12 ± 1.3</td>
<td>12.3 ± 3.2</td>
<td>10 ± 2.8</td>
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Discussion

To our knowledge, this is the first prospective, randomized study to provide information on the conversion from a CNI- to a rapamycin-based immunosuppressive regimen in a cohort of patients who received a renal transplant and had a histologic diagnosis of CAN. Several studies have previously shown that patients who receive a kidney transplant benefit from CNI withdrawal and treatment with rapamycin (12,13). In all multicenter trials based on de novo kidney transplant recipients, the immunosuppressive regimen was based initially on CsA, rapamycin, and steroids; after a short time, CsA was withdrawn, and this led to an improvement of renal function compared with patients who continued on CsA, rapamycin, and steroids (12,13). Recently, Ruiz et al. (19) demonstrated that early CsA withdrawal prevents progression of CAN lesions in patients who receives a kidney transplant. In this study, we simultaneously introduced rapamycin and stopped CNI administration, and for the first time we indicate both rapamycin loading and maintenance dose on a mg/kg basis. The safety of the “stop and go” approach to switch from CNI to rapamycin is confirmed by the absence of acute rejection after the procedure. This approach will avoid any potential side effect related to the concurrent administration of CNI and rapamycin. Indeed, simultaneous administration of CsA and rapamycin may amplify the nephrotoxic effects of the CNI, also in reduced doses (20,21).
The findings of our study confirm that rapamycin-based immunosuppressive regimens are as effective as those that comprise CNI, at least during short-term follow up (2 yr), in preventing acute rejection. In addition, it suggests that CNI withdrawal and rapamycin introduction is a safe option, which may significantly delay the progression of chronic histologic damage. This is demonstrated by a stabilization of CAN lesion grade in group 2 patients compared with the progression of CAN grading in group 1 patients in a protocol biopsy that was performed 2 yr after the randomization. In addition, rapamycin introduction dramatically reduced intragraft α-SMA expression, whereas the reduction in CNI doses resulted in a significant increase of this marker of fibroblast activation in the 2-yr biopsy. Surprising, these beneficial effects of CNI withdrawal and subsequent rapamycin introduction improved significantly graft survival even if the follow-up of our study population was only of 2 yr. Conversely, we could not show a significant improvement in graft function in rapamycin-treated compared with CsA-treated patients, most likely because patients who reached the primary outcome (graft loss) were not included in this analysis. In addition, as reported also by Nankivell et al. (22), sCr is not a reliable markers of the degree of graft histologic lesions.

Interstitial fibrosis and vasculopathy or graft vessel disease is a prominent histologic feature of CAN. Renal transplant vasculopathy typically begins when alloimmune-dependent and/or alloimmune-independent factors cause damage to the graft vessels. Arterial vascular injury invariably leads to vascular remodeling. Both immune- and drug-induced vascular lesions are characterized by an inflammatory response accompanied by the release of cytokines and growth factors that stimulates smooth muscle cells (SMC) to migrate to the intima. Once in the intima, SMC are driven by the inflammatory milieu to a further activation, leading to proliferation and deposition of extracellular matrix. The final result is intimal thickening, flow obstruction, and tissue ischemia (23). CNI have the potential to initiate and sustain vascular injury and tissue ischemia, by an array of acute and chronic effects ranging from vasoconstriction to activation of endothelial cells, resulting in a significant increase in growth factors and cytokine synthesis and release, inhibition of endothelial nitric production, extracellular matrix accumulation, cell migration, and proliferation (24,25). The striking increase of α-SMA–positive cells in the vessel walls of renal biopsies of CNI-treated patients confirms the ability of this class of drugs to induce a significant increase in the proliferation rate of vascular SMC. However, the observation of a significant reduction of α-SMA staining further supports several observations on the beneficial effect of this immunosuppressive drug on the development of atherosclerotic lesions. Indeed, in vitro studies showed that rapamycin, compared with CsA, tacrolimus, mycophenolic acid, and deoxyspergualin, is the only immunosuppressive agent with pronounced inhibitory effects on growth factor–stimulated SMC proliferation (26). In vivo studies demonstrated that rapamycin suppressed luminal narrowing in both arterial allografts and balloon-injured carotid arteries of nonhuman primates (27). Ikonen et al. (27) demonstrated that high doses of rapamycin block the proliferative responses to cytokines by vascular cells and SMC after mechanical injury, such as balloon angioplasty, or allorejection.
In addition, Poston et al. (28) suggested that rapamycin stabilizes and possibly reverses chronic graft vascular disease in a cardiac allograft model. This evidence supported the introduction in the clinical setting of rapamycin-mediated vascular stent for coronary artery disease to reduce the incidence of poststent stenosis (29).

α-SMA is also a specific marker of activated fibroblasts, also known as myofibroblast (30). These cells are characterized by a strong fibrogenic potential. Indeed, the transdifferentiation of epithelial cells into fibroblast and their subsequent activation as myofibroblasts is one of the key early cellular events that initiates the development of kidney fibrosis (30,31). Renal myofibroblast infiltration has been shown to be strongly associated with renal function decline in several chronic diseases in the native kidneys (30,32). However, very little is known about the role of interstitial myofibroblast in the pathogenesis and progression of renal allograft fibrosis. Badid et al. (33) suggested that interstitial α-SMA expression in pretransplant biopsies can strongly predict chronic renal allograft dysfunction. Recently, the presence of α-SMA–expressing interstitial myofibroblasts in the development of chronic allograft nephropathy was suggested by Djamali et al. (34) in an experimental model of chronic allograft dysfunction. However, so far, no data are available on the presence of these cells in human CAN and on the possibility to modulate their influx at the interstitial level. This is the first report to demonstrate the possibility to modulate pharmacologically the number of interstitial myofibroblasts in a progressive form of chronic kidney disease. Although done in transplanted kidneys, this observation might be relevant also for the treatment of chronic progressive diseases of native kidneys. Although the akt axis might be involved in some of the cellular events featuring epithelial to mesenchymal transdifferentiations and subsequent fibroblast activation, the molecular mechanisms of this rapamycin effect needs further evaluation (35).

Rapamycin-induced hyperdyslipidemia often requires pharmacologic treatment with statins. Several studies suggested a potential beneficial effect of these drugs on graft function and structure (36–39). At the moment, there is no study that has demonstrated definitively a beneficial role for statin on graft function and/or structure. Fellstrom et al. (40) recently suggested that fluvastatin treatment significantly improves lipid value in renal transplant recipients but has no effect on graft loss or doubling of serum creatinine, but the sample size of their study was inadequate to address this issue. Although it is conceivable that statin might have played a role in the beneficial effects that we observed in the patients who were switched to rapamycin, our study does not allow us to confirm it. Indeed, virtually all patients on rapamycin were on statins as well.

In conclusion, our study confirms that rapamycin exhibits an efficacy similar to CsA in terms of patient survival, with superimposable rates of acute rejection episodes, as previously demonstrated by multicenter trials. Moreover, it demonstrates for the first time that rapamycin introduction/CNI withdrawal reduces the intragraft α-SMA expression, a surrogate marker of progression of renal damage, and slows the progression of chronic allograft injury and vascular damage. This is clinically reflected by the improvement of graft survival at 2 yr after rapamycin introduction/CNI withdrawal in patients with histologic diagnosis of CAN. A larger, multicenter, and randomized trial is warranted to confirm our observation.

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References


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