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Synergistic effect of mycophenolate mofetil and angiotensin-converting enzyme inhibitor in patients with chronic allograft nephropathy

G.T. Moscoso-Solorzano¹, G. Mastroianni-Kirsztajn¹, K.S. Ozaki¹, M.F. Franco²,
A. Pacheco-Silva¹ and N.O.S. Câmara^{1,3}

¹Laboratório de Imunologia Clínica e Experimental, Disciplina de Nefrologia, Hospital do Rim e Hipertensão, ²Departamento de Patologia, Universidade Federal de São Paulo, São Paulo, SP, Brasil
³Laboratório de Imunobiologia de Transplantes, Departamento de Imunologia, Instituto de Ciências Biomédicas IV, Universidade de São Paulo, São Paulo, SP, Brasil

Correspondence to: N.O.S. Câmara, Disciplina de Nefrologia, UNIFESP, Rua Botucatu, 740, 04023-900 São Paulo, SP, Brasil
Fax: +55-11-5573-9652. E-mail: niels@nefro.epm.br

Experimental data and few clinical non-randomized studies have shown that inhibition of the renin-angiotensin system by angiotensin-converting enzyme (ACE) associated or not with the use of mycophenolate mofetil (MMF) could delay or even halt the progression of chronic allograft nephropathy (CAN). In this retrospective historical study, we investigated whether ACE inhibition (ACEI) associated or not with the use of MMF has the same effect in humans as in experimental studies and what factors are associated with a clinical response. A total of 160 transplant patients with biopsy-proven CAN were enrolled. Eighty-one of them were on ACE therapy (G1) and 80 on ACEI-free therapy (G2). Patients were further stratified for the use of MMF. G1 patients showed a marked decrease in proteinuria and stabilized serum creatinine with time. Five-year graft survival after CAN diagnosis was more frequent in G1 (86.9 vs 67.7%; $P < 0.05$). In patients on ACEI-free therapy, the use of MMF was associated with better graft survival. The use of ACEI therapy protected 79% of the patients against graft loss (OR = 0.079, 95%CI = 0.015-0.426; $P = 0.003$). ACEI and MMF or the use of MMF alone after CAN diagnosis conferred protection against graft loss. This finding is well correlated with experimental studies in which ACEI and MMF interrupt the progression of chronic allograft dysfunction and injury. The use of ACEI alone or in combination with MMF significantly reduced proteinuria and stabilized serum creatinine, consequently improving renal allograft survival.

Key words: Renin-angiotensin system; Mycophenolate mofetil; Renal allograft survival; Kidney transplantation

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Introduction

Chronic allograft nephropathy (CAN) is present in 40-60% of allograft biopsies 24 months after transplantation and is the most common cause of late renal graft failure (1,2). CAN is characterized by progressive deterioration of renal function produced by sclerotic changes affecting blood vessels, glomeruli, interstitium, and tubules and is

graded according to the degree of damage to the last two parameters (3). Even though the functional and morphologic findings of CAN have been well characterized, the progressive mechanisms leading to deterioration of graft function are poorly understood (4). Apart from the classical immune events, many other risk factors are implicated in the loss of nephron mass that might involve activation of the renin-angiotensin system (RAS) (5,6).

In this respect, the pharmacological inhibition of angiotensin II synthesis and/or the blockade of its biological activity remarkably ameliorate the renal interstitial abnormalities, reducing the accumulation of protein casts and ultimately slowing the progression of renal dysfunction in many renal disease models (7-13). Furthermore, non-randomized clinical trials have identified the blockade of RAS as a cardinal means of arresting the progression of renal disease, delaying the progression of glomerulosclerosis and interstitial fibrosis in proteinuric diseases as well as CAN (14-21). Some non-randomized clinical studies on renal transplantation have shown that the blockade of RAS was safe and beneficial by reducing proteinuria, besides increasing renal allograft survival (19-21).

Mycophenolate mofetil (MMF) has been used in solid organ transplants with good results on the reduction of acute rejection episodes and promoting good graft survival (22-27). The effect of combined therapy with an angiotensin-converting enzyme inhibitor (ACEI) and MMF has only been analyzed in experimental studies, which showed a synergistic effect in reducing renal damage (28-30). So far, no studies have confirmed or denied these findings in humans. To answer the question of whether ACEI in combination with MMF has the same effect in humans, we designed this study with patients with biopsy-proven CAN under ACEI therapy or not, in combination or not with MMF. Factors associated with a better clinical response and with improved graft outcomes were investigated. We anticipated that the use of ACEI would delay the progression of chronic allograft nephropathy and the use of MMF would enhance the renoprotective action of ACEI, consequently decreasing renal allograft loss in patients with CAN.

Subjects and Methods

Subjects

For this retrospective study, a total of 250 consecutive renal transplant patients with a previous biopsy-proven CAN were enrolled between 2000 and 2002. The study was approved by the local Ethics Committee of São Paulo University. Patients were excluded if: 1) they were the recipients of a previous organ transplant (N = 9); 2) they were the recipients of a multiple organ transplant (N = 4); 3) their biopsy samples could not be recovered for re-analysis (N = 12); 4) the results of biopsy re-analysis did not meet the minimal Banff criteria for CAN (N = 4); 5) had recurrence or *de novo* occurrence of glomerulopathies post-transplantation (N = 51), or 6) had neoplasias or polyomavirus infections (N = 9). Thus, 161 transplant patients with biopsy-proven CAN were evaluated.

Study design

In this retrospective study, after a biopsy-proven diagnosis of CAN, the enrolled patients were divided into two groups defined by the use or not of ACEI therapy for at least 16 weeks before the diagnosis of CAN. The characteristics of the ACEI therapy group (N = 81, G1) and the non-ACEI therapy group (N = 80, G2) were then compared. The main clinical indication of ACEI therapy was hypertension and captopril was the drug used for all G1 patients. The groups were matched for gender, type of donor (deceased versus living donor), pre-transplant disease and time of CAN diagnosis and CAN Banff grade.

Stratification according to MMF use

The two groups (G1 and G2) were also subdivided based on MMF use and the ACEI group (G1) was subdivided into three subgroups: patients who received ACEI alone (N = 12), patients who received ACEI and MMF before a biopsy-proven diagnosis of CAN (N = 14), and patients who received ACEI and MMF after a biopsy-proven diagnosis of CAN (N = 55). Similarly, patients who did not use ACEI (G2) were also subdivided into three subgroups: patients who did not receive ACEI or MMF (N = 19), patients who did not receive ACEI but received MMF before a biopsy-proven diagnosis of CAN (N = 18), and patients who did not receive ACEI, but received MMF after a biopsy-proven diagnosis of CAN (N = 43).

Operational definitions

Delayed graft function was defined as the requirement of dialysis during the first week after transplantation in the absence of rejection and/or technical problems. Acute rejection confirmed by biopsy was defined according to Banff 97 criteria. Any rejection before the 3rd month of transplantation was classified as early acute rejection, and as late rejection after this period. Systemic arterial hypertension was defined as a repeatedly elevated blood pressure exceeding 140 x 90 mmHg or when patients were using at least one antihypertensive drug. New-onset diabetes after transplantation was defined when fasting plasma glucose was ≥ 126 mg/dL (≥ 7 mmol/L), when a random blood sugar level ≥ 200 mg/dL (≥ 11.1 mmol/L) accompanied by symptoms was observed, or when an oral glucose tolerance test showed a value ≥ 200 mg/dL.

Loss of renal function was considered to be one endpoint in our study. For this purpose, we assessed the increase in serum creatinine during follow-up. Patients who had an increase of serum creatinine higher than 30% compared to baseline level (time of CAN diagnosis) at the end of follow-up were defined as non-responders. Conversely, all patients who did not lose graft function or those

who showed a less than 30% increase in serum creatinine over time were defined as responders. Thus, the two patient groups were subdivided into responders (G1a, N = 71, and G2a, N = 51) and non-responders (G1b, N = 10, and G2b, N = 29).

Histological analysis

Biopsy specimens were evaluated and graded according to the Banff 97 criteria and the histological chronic allograft damage index (CADi) (31,32). At least four slides were used to quantitate the histological changes in each biopsy stained with hematoxylin-eosin, Masson's trichrome, periodic acid-Schiff, and silver, respectively. All biopsy specimens were reviewed by three independent observers (pathologists) before inclusion of the patient in the study. The final result regarding the severity of acute and chronic lesions in each renal compartment was calculated by applying concordance criteria among these observers. In the case of discrepancy among the three pathologists, the mode of the values for each variable was used as the final grade of lesion severity.

Statistical analyses

Pre-transplant demographic characteristics used for covariate-adjusted analysis included the type of dialysis therapy (hemodialysis vs peritoneal dialysis) and the source of the graft (deceased vs living donor). The age of the donor (above or below 39 years) and the age of the recipient (above or below 34 years) were dichotomized. The cut-off was obtained based on the mean values. The post-transplant variables included the presence of proteinuria in 24-h collected samples, delayed graft function, early and late acute rejection, and cold ischemia time (above or below 60 min). We examined the proportional-hazard assumption by plotting the graft survival curves for each group of a covariate on a log-log scale. Since the curves appeared to be reasonably parallel, we regarded the model as appropriate.

A CADi score of 2.3 or higher, a Banff 97 grade higher than II, a serum creatinine level of 2.5 mg/dL or higher, creatinine clearance of 60 mL/min, proteinuria of 1 g/24 h or higher, were considered as cut-off points for statistical comparison among groups. The cut-off was obtained based on its best value to predict renal allograft loss. In order to assess the effect of ACEI therapy alone or in combination with MMF on erythropoiesis, we determined hematocrit levels immediately before the use of ACEI and at the end of follow-up.

The Kaplan-Meier method was used to estimate graft survival after CAN diagnosis. Graft loss was defined by the requirement of permanent dialysis after graft failure, and

death of patients with functioning grafts was not considered as an end-point event. We determined statistical significance by log-rank comparisons of survival curves using two-sided P values. Values are reported as means \pm SD and, when appropriate, as median and ranges. Parametric and non-parametric tests, the chi-square test and Fisher's exact test were applied to compare demographic covariates between groups when appropriate. A P value of less than 0.05 was considered to be significant.

A multivariate Cox proportional hazards model was used to analyze the relation between graft loss and other covariates that were significant in univariate analysis or were well-known to be involved in graft loss. The Cox proportional hazard model-enter stepwise procedure was used to obtain the final model of significant predictors. Confounding variables were analyzed when required and were considered to be absent when the exponential coefficient (OR) did not change by more than 10%. The Stata statistical software 12.0 was used for all statistical analyses (Stata Corporation, USA).

Results

Demographic data of the study population

The demographic characteristics of the population are reported in Tables 1 and 2. The initial immunosuppression therapy was cyclosporine for 137 patients (85%), tacrolimus for 18 (11%), MMF for 32 (20%), azathioprine for 115 (71%), and sirolimus for 11 (6%). Univariate analysis revealed that dialysis time ($P = 0.014$), recipient age ($P = 0.000$), hepatitis (B or C virus-positive serology; $P = 0.014$), and post-transplant diabetes mellitus ($P = 0.007$) were significantly higher in G1. At the time of ACEI therapy initiation there were no differences in CAN grade between the groups or, interestingly, in serum creatinine levels (Table 2).

ACEI and/or MMF therapy in patients with CAN

Patients with CAN who used ACEI (G1) showed a marked reduction in proteinuria compared to the group that did not use ACEI (G2). Furthermore, their serum creatinine levels stabilized with time (Table 2).

The two groups (G1 and G2) were subdivided into responders and non-responders on the basis of an increase in baseline serum creatinine of more than 30% at the end of ACEI therapy. Responder patients on ACEI therapy not only showed stabilized renal function but also decreased proteinuria, whereas responder patients on ACEI-free therapy presented increased proteinuria. Some patients considered as non-responders in the ACEI therapy group showed increased serum creatinine during fol-

low-up, although their proteinuria levels were reduced (Table 2).

The two groups were further subdivided on the basis of MMF use as described in Subjects and Methods. In general, patients with CAN who used ACEI combined or not with MMF showed decreased proteinuria. However, we only observed a statistically significant difference between patients who used ACEI and MMF after biopsy-proven CAN and the subgroup that did not use MMF or even those who were not treated with ACEI (Table 2). In contrast, among the patients who did not use ACEI, serum creati-

nine and proteinuria were significantly increased only in the group who never used ACEI or MMF (serum creatinine: 2.0 ± 0.8 vs 2.4 ± 1.6 mg/dL; $P = 0.001$, proteinuria: 0.4 ± 0.5 vs 1.1 ± 1.2 g/24 h, $P < 0.05$; Table 2).

In order to assess the effect of ACEI alone or in combination with MMF on erythropoiesis, we determined the hematocrit levels immediately before the use of ACEI and at the end of follow-up. In general, ACEI therapy was associated with a significant decrease in hematocrit levels. Patients on ACEI-free therapy, although presenting a reduction in hematocrit levels, only showed a statistically significant difference when also taking MMF. The association with MMF aggravated anemia (Table 3).

Table 1. Demographic data of the study population.

Variables	With ACEI (G1, N = 81)	Without ACEI (G2, N = 80)
Recipient age (years)	37 ± 12	30 ± 12*
Donor age (years)	35 ± 13	39 ± 14
Gender (male)	54%	46%
Deceased donor	38%	37%
HLA matching (living-related donor)		
I (identical HLA, O mismatch)	20	2*
II (50% mismatch HLA)	66	76
III (different HLA)	14	22
Hemodialysis therapy	95%	75%
Hemodialysis time (months)	47 ± 41	33 ± 30*
Cold ischemia time (min)	698 ± 1580	564 ± 747
Early acute renal rejection	40%	44%
Later acute renal rejection	11%	19%
Delayed graft function	31%	38%
Diabetes mellitus after transplantation	18%	5%*
Anti-cytomegalovirus antibodies (IgG)	18%	16%
Hepatitis (B or C virus)	28%	20%*
CAN grade at first biopsy		
CAN grade 1	50%	63%
CAN grade 2	39%	27%
CAN grade 3	11%	10%
CADI score >2.3	59%	46%
Initial immunosuppressive therapy, N (%)		
Cyclosporine	75 (54%)	62 (45%)*
Tacrolimus	2 (11%)	16 (89%)*
Rapamycin	4 (49%)	7 (51%)
Azathioprine	62 (54%)	53 (46%)
MMF	14 (44%)	18 (56%)
Final immunosuppressive therapy, N (%)		
Cyclosporine	65 (52%)	61 (48%)
Tacrolimus	4 (22%)	19 (78%)*
Rapamycin	2 (40%)	3 (60%)
Azathioprine	13 (45%)	16 (55%)
MMF	55 (56%)	43 (44%)*

Data are reported as means ± SD or percent. ACEI = angiotensin-converting enzyme inhibitor; HLA = human leukocyte antigen; CAN = chronic allograft nephropathy; CADI = chronic allograft damage index; MMF = mycophenolate mofetil. * $P < 0.05$ compared with ACEI (Student *t*-test for independent samples and chi-square test in categorical variables, both used in normal distribution of variables).

Allograft survival and multivariate analyses

Figure 1 shows the Kaplan-Meier estimate of graft survival for patients receiving ACEI therapy (G1) and ACEI-free therapy (G2). ACEI therapy was associated with better graft survival after CAN diagnosis. Multiple ACEI and MMF combinations were analyzed in a graft survival model. The use of MMF increased graft survival in patients who did not use ACEI. In this respect, the group that did not use ACEI or MMF had a median 65% censored population, the shortest survival time among the groups ($P = 0.02$; Figure 1).

When we analyzed the survival model using other variables, such as acute rejection, co-morbidities, CAN grade, CADI score ≥ 2.3 , deceased donor, recipient and donor ages, cold ischemic time, hemodialysis therapy, delayed graft function, and anti-CMV antibodies, we did not observe any correlations with graft survival rate. The use of ACEI or MMF therapy was positively associated with graft survival (Table 4) even when some of these variables were tested in multivariate analyses.

Cox regression analyses demonstrated that the use of ACEI therapy protected 79% of the patients against graft loss (OR = 0.079, 95%CI = 0.015-0.426; $P = 0.003$), showing that ACEI therapy is a protective factor. The same trend was observed, but to a lesser extent, for those patients who did not use ACEI therapy but who were on MMF therapy after biopsy-proven CAN, and in

Table 2. Renal function (serum creatinine, Scr, mg/dL) and proteinuria levels (Prot, g/24 h) at enrollment (Scr 1 and Prot 1) and at the end of follow-up (Scr 2 and Prot 2) in patients with or without ACEI therapy (responders = patients who did not lose graft function or those who showed less than a 30% increase in serum creatinine over time and non-responders), stratified or not for the use of MMF.

Subgroups	Scr 1	Scr 2	Prot 1	Prot 2
ACEI therapy (G1)	1.81 ± 0.56	1.87 ± 0.52	1.31 ± 1.94	0.53 ± 0.68*
No use of MMF (N = 12)	1.8 ± 0.7	1.8 ± 0.6	1.2 ± 1.2	0.6 ± 0.7
MMF pre (N = 14)	2.0 ± 0.6	2.0 ± 0.5	0.9 ± 1.6	0.3 ± 0.4
MMF post (N = 55)	1.8 ± 0.5	1.8 ± 0.5	1.4 ± 2.0	0.6 ± 0.7*
ACEI-free therapy (G2)	2.07 ± 0.73	2.65 ± 1.64*	0.51 ± 0.74	0.85 ± 1.01*
No use of MMF (N = 19)	2.0 ± 0.8*	2.4 ± 1.6*	0.4 ± 0.5*	1.1 ± 1.2*
MMF pre (N = 18)	2.1 ± 0.7	2.9 ± 2.1	0.6 ± 1.1	0.8 ± 1.1
MMF post (N = 43)	2.0 ± 0.7	2.4 ± 1.3	0.5 ± 0.7	0.7 ± 0.9
ACEI treatment				
Responders	1.84 ± 0.57	1.83 ± 0.51	1.33 ± 2.00	0.52 ± 0.68*
Non-responders	1.52 ± 0.32	2.27 ± 0.52*	1.10 ± 1.12	0.71 ± 0.70
ACEI-free treatment				
Responders	1.94 ± 0.54	1.85 ± 0.55	0.38 ± 0.41	0.54 ± 0.56*
Non-responders	2.28 ± 0.40	4.05 ± 1.96*	0.74 ± 1.08	1.40 ± 1.37*

Data are reported as mean ± SD. ACEI = angiotensin converting enzyme inhibitor; MMF = mycophenolate mofetil. *P < 0.05, Scr1 vs Scr2 and Prot1 vs Prot2 (Student *t*-test).

Table 3. Hematocrit values immediately before the introduction of ACEI and at the end of follow-up in patients with or without ACEI therapy, stratified or not for the use of MMF.

Subgroups	Before	After
ACEI therapy (G1)		
No use of MMF (N = 12)	39 ± 6.8	34 ± 7.4*
MMF pre (N = 14)	42 ± 6.9	36 ± 4.2*
MMF post (N = 55)	41 ± 7.4	35 ± 4.2*
ACEI-free therapy (G2)		
No use of MMF (N = 19)	39 ± 5.8	36 ± 6.1
MMF pre (N = 18)	40 ± 6.4	38 ± 6.5
MMF post (N = 43)	39 ± 7.0	35 ± 6.1*

Data are reported as means ± SD. ACEI = angiotensin-converting enzyme inhibitor; MMF = mycophenolate mofetil. *P < 0.05 compared to "before" (Student *t*-test for the same sample).

Figure 1. Graft survival curves for G1 (patient with ACEI) and G2 (patient without ACEI) renal transplanted patients. A, Graft survival was analyzed after the diagnosis of chronic allograft nephropathy (CAN) in G1 and G2. B, Graft survival was also analyzed in both groups stratified for the use of mycophenolate mofetil (MMF) before (pre) or after (post) CAN diagnosis, and not. All survival curves showed significant differences.

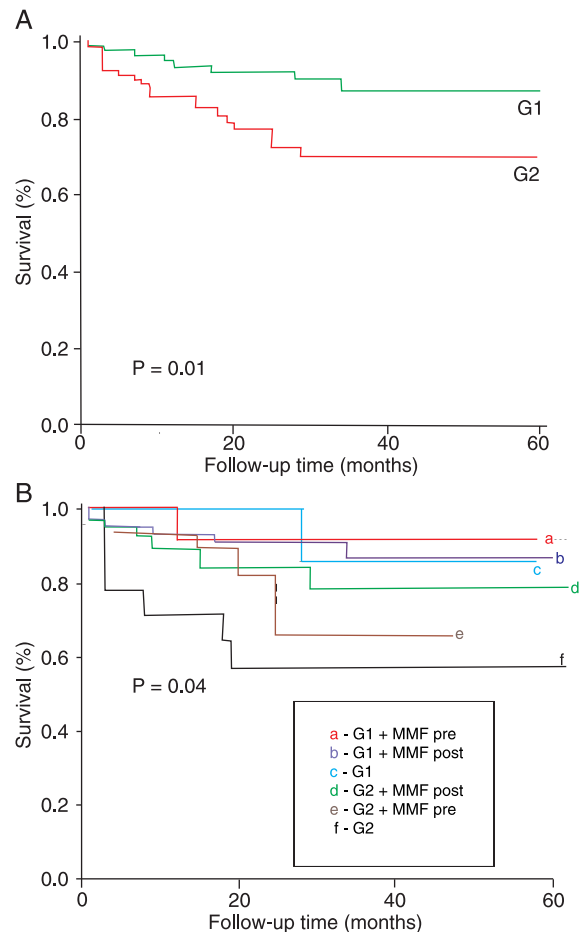


Table 4. Univariate analyses of patients with graft loss.

Variables	No graft loss (N = 132)	Graft loss (N = 29)
Recipient age (years)	32 ± 13	34 ± 12
Donor age (years)	36 ± 15	37 ± 14
Gender (male)	85 (82%)	14 (18%)
Deceased donor	44 (37%)	12 (41%)
Hemodialysis therapy	123 (93)	29 (100)
Cold ischemic time (min)	660 ± 823	634 ± 1309
Early acute renal rejection	54 (41%)	13 (45%)
Delayed graft function	44 (33%)	11 (38%)
Diabetes mellitus post-transplantation	15 (11%)	4 (14%)
Anti-cytomegalovirus antibodies (IgG)	22 (17%)	6 (21%)
ACEI use	71 (54%)*	10 (35%)*
Use of MMF after CAN diagnosis	102 (77%)	17 (58.6%)*

Data are reported as means ± SD or N (%). ACEI = angiotensin-converting enzyme inhibitor; MMF = mycophenolate mofetil; CAN = chronic allograft nephropathy. *P < 0.05 compared to no graft loss (Student *t*-test for independent samples and chi-square test in categorical variables, both used in normal distribution of variables).

Table 5. COX-adjusted analysis model for graft loss in the population studied.

Variables	P	OR	95%CI
Use of ACEI	0.003	0.079	0.015-0.426
No use of ACEI, but use of MMF post-CAN	0.024	0.108	0.016-0.743
Use of MMF post-CAN	0.001	0.196	0.072-0.529
Donor age (≥39 years)	0.339	1.556	0.628-3.856
Acute rejection	0.360	1.448	0.656-3.194
Delayed graft function	0.451	1.415	0.574-3.488
Recipient age (≥34 years)	0.495	1.340	0.579-3.102
HLA matching (living-related donor)	0.190	1.297	0.879-1.915
Gender (male)	0.571	1.270	0.555-2.909

ACEI = angiotensin-converting enzyme inhibitor; MMF = mycophenolate mofetil; CAN = chronic allograft nephropathy.

those who were on ACEI therapy and who initiated MMF after biopsy-proven CAN (Table 5).

Discussion

The renoprotective effect of ACEI has been established in a wide variety of progressive diabetic and non-diabetic renal diseases (14-21). In renal transplantation, few clinical studies have shown the beneficial effects of ACEI therapy (19-21). Interestingly, a reduction in end-stage renal disease was observed even in clinical cardiovascular studies evaluating the benefit of ACEI therapy in reducing mortality and cardiovascular effects (33).

Our results showed that ACEI therapy in patients with CAN could slow its progression, stabilizing serum creatinine and decreasing and limiting the progression of proteinuria as well as histological lesions. Patients who received ACEI maintained stable renal function with decreasing proteinuria at the end of therapy, in contrast to the ACEI-free group who presented a progressive loss of renal function with increasing proteinuria. These findings correlated with the results of other renal disease progression studies such as the Ramipril Efficacy in Nephropathy study of proteinuric non-diabetic nephropathies (16). ACEI therapy was associated with increased baseline glomerular filtration rate, possibly indicating a functional regression of renal disease in some patients.

However, recent data reported by Opelz et al. (34) demonstrated that no improvement in patient or graft survival outcome was found in kidney and heart transplant recipients on ACEI/angiotensin receptor blocker (ARB) treatment. Another retrospective study evaluated the patient and graft survival rates of 2031 recipients of a first kidney transplant with or without ACEI and/or ARB therapy. Heinze et al. (35) found a marked improvement in ten-year graft survival in patients on ACEI/ARB treatment. In these two recent retrospective studies, the authors did not specifically analyze the impact on patients with CAN diagnosed by biopsy, for whom the impact of ACEI/ARB therapy could be more pronounced.

In this context, an elegant experimental study from the group of Remuzzi (14) showed that ACEI therapy could stop proteinuria and glomerulosclerosis, interrupting the progression of chronic allograft dysfunction and injury.

After MMF was introduced in renal transplantation, some reports have demonstrated its benefit in CAN (22-24). Ojo et al. (22), in a study on 66,774 human renal allograft recipients, showed that MMF reduces late allograft loss independently of its effects on acute rejection. Experimental studies have long evaluated the impact of MMF in rodent models of end-stage renal disease. In experimental diabetes involving chronic nitric oxide synthase inhibition in rat models, the use of MMF prevented the development of glomerular atrophy and the progression of tubular atrophy and tubulointerstitial fibrosis (36,37).

In the present study, MMF had a synergistic effect with ACEI since the combination of ACEI and MMF maintained serum creatinine stable throughout the study and decreased proteinuria more than when MMF was used alone.

The combination of MMF and ACEI was a good prognostic factor for graft survival even in patients with established CAN. The death-censored graft survival analysis was much better for the group treated with ACEI alone, following the group treated with ACEI in combination with MMF, whereas the group receiving neither ACEI nor MMF showed the worst renal allograft outcomes.

The main limitation of our study was the influence of ACEI and MMF therapy on erythropoiesis. Both therapies were associated with a decrease in hematocrit levels. However, this side effect should not limit the use of ACEI in combination with MMF, with anemia being carefully monitored and the administration of an erythropoietin supplement being considered whenever necessary.

The present study was not randomized and has some of the limitations of retrospective studies. Many other con-

foundings factors could not be eliminated by the statistical approach used, and should be kept in mind. Randomized clinical trials would be ideal. However, they involve important ethical issues since experimental studies and meta-analyses of non-transplanted patients have demonstrated the benefit of both drugs.

Our results indicate that the use of ACEI alone or in combination with MMF significantly reduces proteinuria and stabilizes serum creatinine over time, consequently improving renal allograft survival.

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