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Abbreviations:

IF/TA - interstitial fibrosis and tubular atrophy

MMF - mycophenolate mofetil

BK - polyoma virus BK

CNI - calcineurin inhibitors

DGF - delayed graft function

eCrCl - estimated creatine clearance

SPKT - simultaneous pancreas kidney transplantation

HLA MM – human leukocyte antigen mismatch

ABSTRACT:

Aims - Chronic transplant dysfunction after kidney transplantation is major reason of kidney graft loss and is caused by immunological and non-immunological factors. There is evidence that mycophenolate mofetil (MMF) may exert a positive effect on renal damage in addition to immunosuppression, by its direct antifibrotic properties. The aim of our study was to retrospectively investigate role of MMF dose on progression of chronic allograft dysfunction and IF/TA.

Methods-This is a retrospective cohort study that included 79 patients with kidney and kidney-pancreas transplantation. Immunosuppression consisted of anti-IL2 antibody induction, MMF, a calcineurin inhibitor ± steroids. An association of average MMF dose over 1 year post transplant with progression of interstitial fibrosis (Δci), tubular atrophy (Δct) and estimated creatinine clearance (eCr_{cl}) at 1 year post transplant was evaluated using univariate and multivariate analyses.

Results - Higher average MMF dose was significantly independently associated with better eCr_{cl} at 1 year post transplant (b=0.21 ± 0.1, p=0.04). In multiple regression analysis lower Δci (b=-0.2 ± 0.09, p=0.05) and Δct (b=-0.29 ± 0.1, p=0.02) were independently associated with greater average MMF dose. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Conclusions - Higher average MMF dose over 1 year is associated with better renal function and slower progression of IF/TA, at least partly independent of its immunosuppressive effects.

Strenghts and limitations of this study

Important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF (up to 4 g daily) during 1 year post transplantation had significantly lower progression of graft interstitial fibrosis and tubular atrophy. This is important finding, because of predictive value of graft IF/TA and should translate into better long-term graft survival. Our study has several shortcomings, such as its retrospective aspect and relatively short study period. As it was not aim of the study, we did not report side effects associated with different dosage of MMF.

INTRODUCTION:

Kidney transplantation significantly improves patient survival and quality of life comparing to dialysis. While significant improvements have been made in the treatment of acute rejection and short survival of transplanted kidney, there has not been major improvement in the long-term survival of transplanted kidney.[1] Chronic transplant dysfunction after kidney transplantation is major cause of kidney graft loss and is evoked by immunological and non-immunological factors.[2, 3] Histology changes that determine chronic transplant dysfunction are interstitial fibrosis and tubular atrophy (IF/TA), arteriosclerosis, arteriolar hyalinosis, glomerulopathy and mesangial matrix expansion.[4] IF/TA is the major pathohystology finding that can be verified on graft biopsies after kidney transplantation and is a predictor of long-term allograft function.[4] Clinical factors that affect progression of IF/TA are: recipient age, HLA mismatch, episodes of severe acute rejection, chronic rejection (esp. antibody-mediated), use of calcineurin inhibitors and BK nephropathy. Avoidance of CNI toxicity is considered as an important step to slow progression of IF/TA.[4-7] Mycophenolate mofetil (MMF) may help lowering CNI toxicity, by allowing lower CNI exposure.[7]

MMF reduces the risk of acute allograft rejection, without nephrotoxic side effects and is ideal candidate for long-term calcineurin drug reduction treatment strategies.[7] Retrospective studies of renal recipients who were treated with mycophenolate mofetil comparing azathioprin showed that MMF treated patients had significantly less chronic

allograft dysfunction.[8, 9] Besides being associated with lower acute rejection rates as compared to azathioprin,[10, 11] evidence from animal and human studies suggests that MMF may also exert a direct antifibrotic properties due to its antiproliferative action on nonimmune cells, including renal tubular cells and vascular smooth muscle cells.[12, 13] The aim of our study was to investigate role of mycophenolate mofetil dose on progression of IF/TA in kidney transplant recipients.

PATIENTS AND METHODS:

Patients:

This is a retrospective study conducted at Clinical Hospital "Merkur". This study represents a part of the posttransplant immune monitoring at the Merkur hospital, approved by the Hospital Ethics Committee. The study included 79 patients with kidney and kidney-pancreas transplantation, transplanted between 2003 and 2011. Eligible patients had to have protocol kidney biopsy at the time of implantation and 12 months after transplantation. Exclusion criteria have been: dual kidney transplantation, kidney-liver transplantation, use of antithymocyte immunoglobulin, BK nephropathy and recurrence of glomerulonephritis after transplantation.

Immunosuppression:

Induction immunosupression consisted of an anti-IL2 antibody (daclizumab or basiliximab), calcineurin inhibitor (tacrolimus or cyclosporine), MMF and methylprednisolone. Maintenance immunosuppression consisted of a calcineurin

inhibitor (tacrolimus or cyclosporine), MMF ± steroids. Target cyclosporine trough concentrations were 250-350 during first month posttransplant, 200-300 during second to 6th month and 100-150 µg/L thereafter. Target tacrolimus trough levels were 10-12 during first month, 8-10 during second to 6th month and 5-8 µg/L thereafter. Mycophenolic acid target trough concentration was aimed to be higher than 7.2 µmol/L with tacrolimus and higher than 5 µmol/L with cyclosporine use.

Daclizumab was administered at day 0: 2mg/kg i.v. before opening of vascular anastomosis and at day 14: 2mg/kg i.v.. Basiliximab was administered at day 0: 20 mg i.v. before opening of vascular anastomosis and at day 4: 20 mg i.v..

Steroids have been dosed as follows: day 0: intraoperatively 500 mg of methylprednisolone, day 1: 250 mg, day 2: 125mg, day 3: 80 mg and day 4: 40 mg. In patients with early steroid withdrawal steroids have been withdrawn at day 5 after transplantation. In patients maintained on steroids, nadir dose of prednisone was 5 mg/d, achieved by 6 months. The criteria for early elimination of steroids were low immunological risk of the recipient (absence of, or low degree of HLA sensitization, i.e. PRA <10%) and good immediate renal function, as well as absence of an episode of acute rejection within 5 days after the transplantation. Steroids have been reintroduced in patients who suffered acute rejection episode.

As prophylaxis for viral (HSV, CMV), fungal (Candida spp.) urinary and P. jiroveci infections, low-dose fluconazole (for one year), valganciclovir (universally for three months) and sulfomethoxazol and trimethoprim (for one year) was used.

Renal allograft biopsies:

Protocol kidney biopsies were done at implantation, 1, 3, 6 and 12 months after transplantation. For cause biopsies were done in case of unexplained deterioration of renal function, or once weekly in patients with DGF. All rejection episodes were histologically confirmed. Histopathological analysis was performed by either of two pathologists who were blinded for immunosuppression. Acute rejections and chronic allograft scores have been analyzed using Banff 97 classification and its updates.[14, 15] All protocol and indication biopsies were analyzed by light microscopy, by immunofluorescence for C4d, and if indicated by immunohistochemistry for BK virus. Biopsies at 1 year post transplant have been also analyzed by electron microscopy for signs of chronic antibody-mediated rejection (transplant glomerulopathy, peritubular capillary basement membrane multilayering).[16]

Clinical outcome parameters:

Progression of chronic allograft scores during 1 year posttransplant was calculated by subtracting implantation chronic scores from chronic allograft scores 12 months posttransplant: interstitial fibrosis (Δci), tubular atrophy (Δct), glomerulosclerosis (Δcg), mesangial matrix increase (Δmm), vasculopathy (Δcv) and arteriolar hyalinosis (Δah). Estimated creatinine clearance (eCr_{cl}) at 3, 6 and 12 months posttransplant was calculated using Cockroft-Gault formula. Acute rejections with Banff grade IA and IB were treated with three 500 mg methylprednisolone pulses. In case of acute rejection grade IIA or

greater, patients have been treated with antithymocyte globulin. Antibody-mediated rejections were treated with steroid pulse and plasmapheresis.

Average dose of MMF during 1 year posttransplant was calculated from MMF dose at month 1, 3, 6 and 12.

Statistical analysis:

Numerical data are presented as mean ± SD or median with range in case of not normal distribution. Normality of distribution has been tested with Kolmogorov-Smirnov test. Correlation between two continuous variables has been tested using Spearman nonparametric correlation. Difference between two groups in continuous variables has been tested with student t-test or with Mann–Whitney test in non-normally distributed variables. The significance of the progression in chronic scores was analyzed using Wilcoxon Matched Pairs test. Univariate and multiple linear regression analysis were performed to determine predictive factors for progression of chronic allograft scores and kidney function at 12 months after transplantation. All variables that were associated with respective outcome in bivariate analysis (at $p \leq 0.1$) were included in multivariate analysis. Because of colinearity between ci and ct score, only one score was included in each multivariate analysis. Statistical significance was considered at $p<0.05$. All statistical analyses were performed using Statistica 10 (StatSoft, Tulsa, OK, USA).

RESULTS:

Patient and transplant characteristics:

Patient characteristics are shown in Table 1. Recipients were a mean of 44.67 ± 12.03 years old at the time of transplantation, 68 percent of them were male and all were Caucasians. 33 percent of recipients had DGF after transplantation. Donors were a mean of 43.89 ± 15.55 years old and 54 percent of them were male. Number of living donor transplantations was 24 (30 percent). Average daily MMF dose during 1 year posttransplant was 2244 ± 585 mg (1062 – 4000) (Table 5). As expected, there was no correlation of MMF dose with MMF trough concentration (R=-0.13; p=0.28). Also, there was no correlation between MMF dose with tacrolimus concentration (R=-0.04; p=0.79). Early steroid withdrawal was done in 46 percent of patients after transplantation. Incidence of subclinical and clinical acute rejections greater then borderline was 30 percent in first year. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Factors associating with eCr_{cl}:

Kidney function increased during 1st year post transplant. eCr_{cl} at month 3 was 56.98 ± 15.78 ml/min, at 6 month 58.94 ± 16.94 ml/min and at 12 month 61.47 ± 16.75 ml/min (p<0.001; 12 months *vs*. 3 months) (Figure 1.) eCr_{cl} at 1 year post transplant was greater in SPKT recipients (71.38 ± 13.45 ml/min *vs*. 57.88 ± 16.47 ml/min; p=0.001) and in patients who did not have DGF (64.08 ± 15.87 ml/min *vs*. 56.15 ± 17.55 ml/min; p=0.05). Donor age (R=-0.46; p<0.001) and recipient age (R=-0.46; p<0.001) negatively correlated with eCr_{cl} at 1 year post transplant, while there was no correlation of renal

function with donor and recipient gender, type of donation (deceased *vs.* living), HLA MM, average CNI concentration, steroid-free regimen of immunosuppression, or history of acute rejection (Table 6). In univariate analysis allograft function at 12 month post Tx was also negatively correlated with ci (R=-0.34; p=0.002) and ct (R=-0.35; p=0.002) at 12 month (Figure 2A, Figure 2B). Although MMF dose was positively correlated with renal function with borderline significance in univariate analysis, in multivariate analysis there was a significant positive association between greater average MMF dose and better eCr_{cl} at 12 month post transplant (b=0.21 ± 0.1; p=0.04) (Table 2.).

Factors affecting IF/TA:

The average ci score increased from 0.16 ± 0.44 to 0.94 ± 0.86 between implantation and month 12 (p<0.001). Average progression of this and other chronic scores during 1 year post transplant is shown in suppl. data (Table 7). In univariate analysis Δci (R=-0.37; p=0.001) and Δct (R=-0.38; p=0.001) significantly negatively correlated with average MMF dose (Figure 3A and 3B, Table 3). There was lower progression of ci score in patients on steroid-free immunosuppression (0.47 ± 0.7 *vs*. 1.09 ± 0.87; p=0.002) and in those who did not have DGF (0.62 ± 0.74 *vs* 1.19 ± 0.98; p=0.02). Acute cellular rejection, recipient and donor gender, recipient and donor age, HLA MM, deceased *vs.* living donor, as well as average concentration of tacrolimus had no significant effect on progression of chronic allograft scores. Factors that remained significantly associated with progression of ci score in multivariate analysis were ci0 score, donor age, average MMF dose, DGF and steroid-free immunosuppression (Table 4.). In multivariate analysis

only ct0 score, average MMF dose and DGF remained independently associated with 12-month progression of ct score (Table 4.).

Discussion:

The most important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF during 1 year post transplantation had significantly lower progression of ci and ct scores. To our knowledge this is first study demonstrating that there is a dose-dependent protective effect of MMF on graft IF/TA. Lower progression of IF/TA could not be explained with lower concentration of CNI, because there was not correlation between tacrolimus concentration with IF/TA. Similarly, there was no correlation between average MMF dose and tacrolimus (R=-0.04; p=0.79) or cyclosporine concentration (R=-0.07, p=0.79). In addition, higher average MMF dose was not associated with decreased incidence of biopsy proven acute rejection, which suggests that antifibrotic properties of higher MMF dose was at least partly independent of its immunosuppressive effects. Higher MMF dose had only moderate effect on 1-year renal function, which is consistent with previous reports showing that transplanted kidneys undergo pathohystology changes without significant early change in kidney function.[17]

In the present retrospective study we have confirmed that IF/TA progression occurs in first year after kidney transplantation. Several studies have shown that progression of IF/TA is correlated with type of immunosuppression.[18] In most transplant centers in

the United States and Europe immunosuppression consists of induction with an anti-IL2R antibody or antithymocyte immunoglobulin and maintenance with a calcineurin inhibitor, MMF and steroids.[19] Studies have reported significant improvement in kidney function in patients on MMF with lower exposition to CNIs, esp. tacrolimus.[20] Recently, in the paper of Kamar e*t al*. it has been reported that maintenance kidney transplant patients converted to a higher dose of the mycophenolate sodium (1440 mg daily) with lower tacrolimus concentration had borderline higher eCr_{cl} on month 6 *vs*. those treated with lower dose of mycophenolate sodium, with usual tacrolimus concentration (eCr_{cl} 49.1 ± 11.1 *vs*. 44.7 ± 11.5 ml/min; p=0.07).[21] Although there was only borderline significance, increased mycophenolate dosing with lower tacrolimus concentration was safe with potential benefit on kidney function.

Our study also corroborates recently published findings of a *post hoc* joint analysis of the Symphony, FDCC and OptiCept trials, where a a lower tacrolimus level and a higher MMF dose were associated with significantly better kidney function at 1 year post transplant.[22] Shortcoming of these studies[17,18] is lack of protocol biopsies. The optimal MMF dosing in patients maintained on contemporary low-dose CNI is still undetermined. However, some results of early MMF registration trials suggest that higher MMF exposure might be beneficial; having in mind that there was no antibody induction in these studies and that CNI was standard dose cyclosporine. Thus, in the Tri-continental study, group treated with 3 g MMF compared with 2 g of MMF showed lower incidence of biopsy proven acute rejection episodes (15.9% vs. 19.7%) within 6 month period selected for the primary efficacy analysis. Similarly, serum creatinine level at 1 year was 1.42 ± 0.07 mg/dL in the MMF 3 g group *vs.* 1.64 ± 0.07 mg/dL in MMF 2 g group.[12]

In the European mycophenolate mofetil study same trends regarding higher MMF dose were observed.[11] As mentioned before, in these studies there was no antibody induction that could have allowed lower dose of cyclosporin with higher dose of MMF and there were no protocol biopsies. In a more recent MYSS trial, there was no difference in acute rejection rate and renal function between MMF and azathioprine in a cyclosporine-based protocol.[19] However in that study only one MMF dose was compared to azathioprine[23] and again there were no protocol biopsies.

Unfortunately adequate prospective MMF dose comparison studies in tacrolimus-based protocols with antibody induction are missing. In the Symphony study it was reported that patients on tacrolimus-MMF-prednisone maintenance imunosuppression after kidney transplantation had better kidney function and graft survival with lower number of acute rejection episodes. Patients in that group had highest MMF exposure.[24] Protocols with even higher MMF exposure might allow additional CNI sparing, that would decrease side effect of CNI (hypertension, diabetes, hyperlipidemia, neurotoxicity).[25]

Clinical relevance of IF/TA without other concomitant pathology (i.e. recurrent disease and chronic antibody-mediated rejection) for prediction of graft deterioration and loss is controversial. In El-Zoghby et al. study there was attempt to identify specific causes of late kidney allograft failure. The authors found that transplant glomerulopathy was responsible for 37 percent loss of functioning grafts, while graft loss due to IF/TA was present in 31 percent of cases (with higher frequency in deceased-donor transplants).[26] At first glance, these results seem at odd with ours, where there were no signs of chronic antibody-mediated rejection. An explanation for this discrepancy in the results of the two studies is not completely clear, but the former study included high number of living

transplants (72.5 percent) with glomerulonephritis as primary disease and with follow-up up to 10 years. Transplant glomerulopathy is more frequently seen late posttransplant, generally with low incidence. Nevertheless, ours and El-Zoghby study, both demonstrated that IF/TA even in absence of other pathology is associated with adverse graft outcome. Another important study, the DeKaf study, tried to use various histopathologic clusters to differentiate subgroups within diagnosis of IF/TA. They found that cluster with more severe fibrosis plus inflammation and arterial lesions had the worst prognosis.[27] Although incidence of acute rejection in our study did not vary with MMF exposure, increased MMF exposure might suppress mild graft inflammation, below the threshold for diagnosing acute rejection. This is subject of our ongoing investigation and will be reported separately. An interesting finding of the present study was that early steroid withdrawal was not associated with worse IF/TA. At first glance this is at odd with the Astellas trial.[23] However, according to our protocol, patients with DGF were not included in early steroid withdrawal and Astellas trial, which did not have protocol biopsies, reported increased IF/TA in early steroid withdrawal group based on indication biopsies performed early posttransplant, thus more likely reflecting donor-derived histology changes, rather than effect of steroid withdrawal.[28]

In our study there was only borderline significance of positive association of 1-year eCr_{cl} with MMF in univariate analysis. This result is not very surprising since decreased renal function is not a very sensitive marker of incipient IF/TA.

Mechanisms by which an average higher exposure to MMF was associated with slower progression of IF/TA may be both immune and nonimmune. Because there was no difference in incidence of acute rejection with respect to increased MMF exposure in our

study, we believe that there may be a significant contribution of nonimmune mechanisms in retardation of IF/TA in patients with higher MMF. In line with this, in many experimental models it has been shown that MMF has antiproliferative and antifibrotic effect.[29-31] In the study of Jiang at al. using rat renal ischemia reperfusion injury, a time- and dose-dependent correlation of higher MMF dose with better renal function and lower interstitial fibrosis was demonstrated. Suggested potential mechanism was lower expression of TGF-β1 and MCP-1with lower macrophage infiltration.[32] In recent clinical trials MMF was shown as a safe drug that could be a good candidate for treatment of interstitial lung disease in systemic sclerosis.[33] Experimental model of encapsulated peritoneal sclerosis in rats proved beneficial effect of MMF as an inhibitor of neovascularisation.[34] Also, MMF monotherapy was associated with a positive effect on hepatic fibrosis progression in HCV liver transplant recipients.[35]

Our study has several shortcomings, such as its retrospective aspect and relatively short study period. Although study period was limited to 12 months post transplantation, a clear correlation of slower progression of IF/TA with higher average MMF dose underlines potential benefit of these findings. As mentioned before, in current study we did not analyze inflammation outside Banff acute rejection threshold in kidney biopsies with respect to MMF dose. As inflammation in areas of IF/TA is an important predictor of renal function and graft loss, this is subject of an ongoing work.

In summary, higher MMF dose after kidney transplantation might slower progression of IF/TA, which might lead to better long-term survival of transplanted kidney. Our study may serve as a platform for a prospective, randomized, long-term trial with different MMF doses to evaluate benefit of higher MMF dose in renal transplant recipients.

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Contributorship Statement:

Karlo Mihovilović – participated in research design, collecting data, analyzing data and wrote the paper,

Bojana Maksimović - participated in collecting and analyzing data

Ivana Kovačević Vojtušek - participated in collecting data

Sonja Gracin - participated in collecting data

Branislav Kocman - participated in collecting data

Denis Guštin - participated in collecting data

Željko Vidas – participated in collecting data

Stela Bulimbašić - participated in collecting and analyzing data

Danica Galešić Ljubanović – participated in collecting and analyzing data

Mirjana Sabljar Matovinović – participated in collecting data

Mladen Knotek- proposed research design, analyzed data and participated in writing the paper

Competing Interests

None

Data Sharing Statement

There is no additional data

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34 Hur E, Bozkurt D, Timur O, Bicak S, Sarsik B, Akcicek F, et al. The effects of mycophenolate mofetil on encapsulated peritoneal sclerosis model in rats. *Clin Nephrol* 2012; 77:1-7.

35 Manzia TM, Angelico R, Toti L et al. Long-term, maintenance MMF monotherapy improves the fibrosis progression in liver transplant recipients with recurrent hepatitis C. *Transpl Int* 2011; 24:461-468.

Table 1. Baseline characteristics

RECIPIENT CHARACTERISTICS	AGE (years)	44.67 ± 12.03
	GENDER (f/m)	25/54
	PRIMARY RENAL DISEASE (diabetes mellitus, polycistic kidney disease, glomerulonephritis, pyelonephritis/interstitial nephritis, other/unknown)	24/8/19/6/22
DONOR CHARACTERISTICS	DONOR SOURCE (decased/living)	55/24
	AGE (years)	43.89 ± 15.55
	GENDER (f/m)	36/43
TRANSPLANTATION CHARACTERISTICS	TRANSPLANTED ORGAN (KIDNEY/SPKT)	58/21
	INITIAL IMMUNOSUPRESSION (anti-IL2,TAC,MMF/anti-IL2, CyA,MMF)	53/26
	DELAYED GRAFT FUNCTION (no/yes)	53/26
	STEROID FREE (yes/no)	36/43
	HLA MM	3.33 ± 1.51

Table 2. Multiple regression analysis of factors associated with kidney function

	Beta (β)	St.Err. β	p value
Tx (kidney)	-0.17	0.13	0.19
DGF (no)	0.04	0.1	0.71
Recipient age	-0.41	0.1	<0.001
Donor age	-0.1	0.14	0.45
ci at 12 months	-0.18	0.11	0.09
Average MMF dose	0.21	0.1	0.04

Table 3. Correlation of factors associated with progression of ci and ct scores

	Δci		Δct	
	mean ± SD	p	mean ± SD	p
Kidney *vs.* SPKT	0.86 ± 0.91 *vs*. 0.67 ± 0.73	0.51	0.85 ± 0.87 vs. 0.86 ± 0.65	0.74
DGF (yes *vs.* no)	1.19 ± 0.98 *vs*. 0.62 ± 0.74	0.02	1.15 ± 0.92 vs. 0.69 ± 0.72	0.05
Recipient gender (m *vs.* f)	0.83 ± 0.88 *vs*. 0.76 ± 0.83	0.78	0.91 ± 0.83 vs. 0.72 ± 0.79	0.35
Donor gender (m *vs*. f)	0.91 ± 0.95 *vs*. 0.69 ± 0.75	0.43	0.88 ± 0.93 vs. 0.81 ± 0.67	0.96
Donor source (D *vs*. L)	0.84 ± 0.88 *vs*. 0.75 ± 0.85	0.73	0.87 ± 0.82 vs. 0.79 ± 0.83	0.71
Steroid free (yes *vs*. no)	1.09 ± 0.87 *vs*. 0.47 ± 0.74	0.002	1.07 ± 0.83 vs. 0.58 ± 0.73	0.01
Acute rejection (yes *vs*. no)	0.8 ± 0.89 *vs*. 0.83 ± 0.82	0.78	0.93 ± 0.84 vs. 0.67 ± 0.76	0.23
	R	p	R	p
Recipient age	-0.11	0.33	-0.11	0.32
Donor age	0.17	0.13	0.04	0.73
HLA MM	-0.09	0.43	-0.002	0.99
Average tacrolimus conc.	-0.009	0.95	0.003	0.98
Average MMF dose	-0.37	<0.001	-0.38	<0.001
ci at implantation	-0.32	0.003		
ct at implantation			-0.45	<0.001

Table 4. Multivariate general regression analysis for factors related to progression of ci and ct score

Δci			
	Beta (β)	Std. Err. β	p
ci0	-0.43	0.09	<0.001
DGF (no)	-0.22	0.11	<0.05
Average MMF dose	-0.20	0.09	<0.05
Donor age	0.32	0.09	<0.05
Steroid free (yes)	-0.25	0.11	0.02
Δct			
	Beta (β)	Std. Err. β	p
ct0	-0.44	0.09	<0.001
Average MMF dose	-0.29	0.1	<0.05
DGF (no)	-0.29	0.1	<0.05
Steroid free (yes)	-0.09	0.11	0.39

Table 5. eCr_{cl}, MMF dose and CNI concentration during first year post transplant

	Month posttransplant			
	1	3	6	12
eCr_{cl} (ml/min)		56.98 ± 15.79	58.94 ± 16.94	61.47 ± 16.75
MMF dose (mg)	2500 (750 – 4000) 2427 ± 643.17	2000 (750 – 4000) 2167.72 ± 733.49	2000 (1000-4000) 2188.29 ± 716.91	2000 (1000- 4000) 2193.04 ± 642.95
Tacrolimus conc. (µg/L) (n=53)	10.79 ± 4.16	9.69 ± 3.00	9.03 ± 5.52	7.83 ± 2.45
Cyclosporin conc. (µg/L) (n=26)	335.07 (274 – 413)	231.05 (181-265)	206 (170 – 257)	131 (125 – 171)

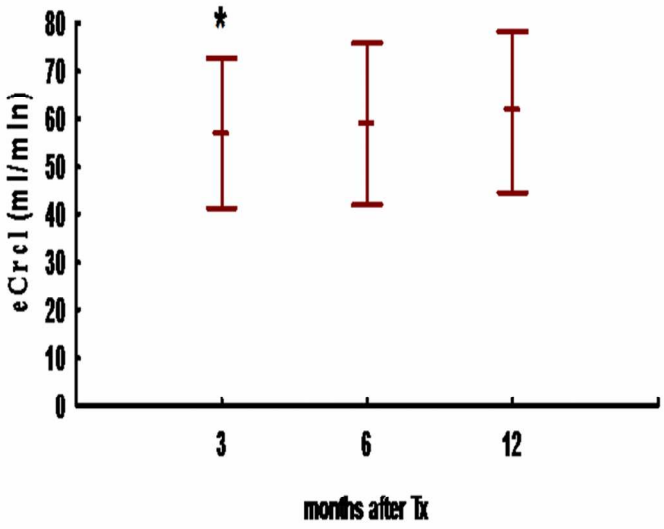
Table 6. Association of variables with eCr_{cl} on 1 year

	Estimated creatinine clearance (ml/min)	p value
Kidney *vs.* SPKT	57.88 ± 15.47 *vs*. 71.38 ± 13.45	0.001
DGF (yes *vs.* no)	56.15 ± 17.55 *vs*. 64.08 ± 15.87	0.05
Recipient gender (m *vs.* f)	59.83 ± 16.02 *vs*. 65 ± 18.07	0.2
Donor gender (m *vs*. f)	63.87 ± 16.71 *vs*. 58.60 ± 16.58	0.17
Donor source (D *vs*. L)	62.36 ± 17.85 *vs*. 59.43 ± 14.05	0.47
Steroid-free (yes *vs*. no)	63.94 ± 17.73 *vs*. 59.39 ± 15.81	0.23
Acute rejection (yes *vs*. no)	61.64 ± 16.59 *vs*. 61.39 ± 16.97	0.95
	R	p value
Recipient age	-0.45	<0.001
Donor age	-0.46	<0.001
HLA MM	0.07	0.52
Average tacrolimus concentration	-0.02	0.9
Average MMF dose	0.18	0.1
ci at 1 year post Tx	-0.34	0.002
ct at 1 year post Tx	-0.35	0.002
cv at 1 year post Tx	-0.20	0.07

Table 7. One-year progression of chronic allograft scores

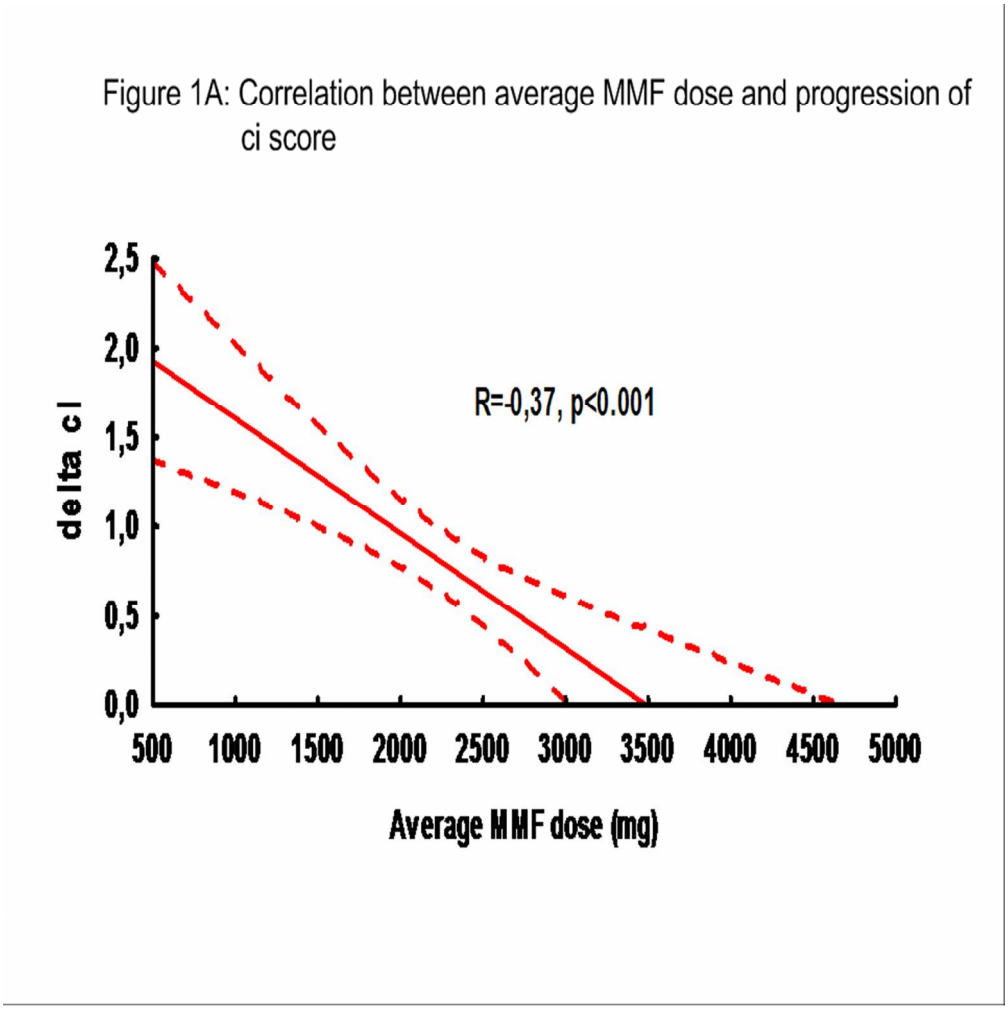
Banff score	N	At transplantation	N	12 month	p
Interstitial fibrosis (ci)	79	0.16 ± 0.44	79	0.94 ± 0.85	<0.001
Tubular atrophy (ct)	79	0.24 ± 0.46	79	1.05 ± 0.77	<0.001
Chronic glomerulopathy (cg)	79	0	79	0	
Mesangial matrix (mm)	79	0.01 ± 0.11	79	0.09 ± 0.36	0.09
Fibrointimal thickening (cv)	76	0.37 ± 0.83	78	0.29 ± 0.70	0.47
Arteriolar hyalinosis (ah)	78	0.68 ± 1,04	79	0.79 ± 1.04	0.26

Figure 1: Estimated creatinine clearance during first year posttransplant



*, p<0.001; 3 month vs. 12 months eCrcl. Values are shown as mean ± SD

108x108mm (300 x 300 DPI)



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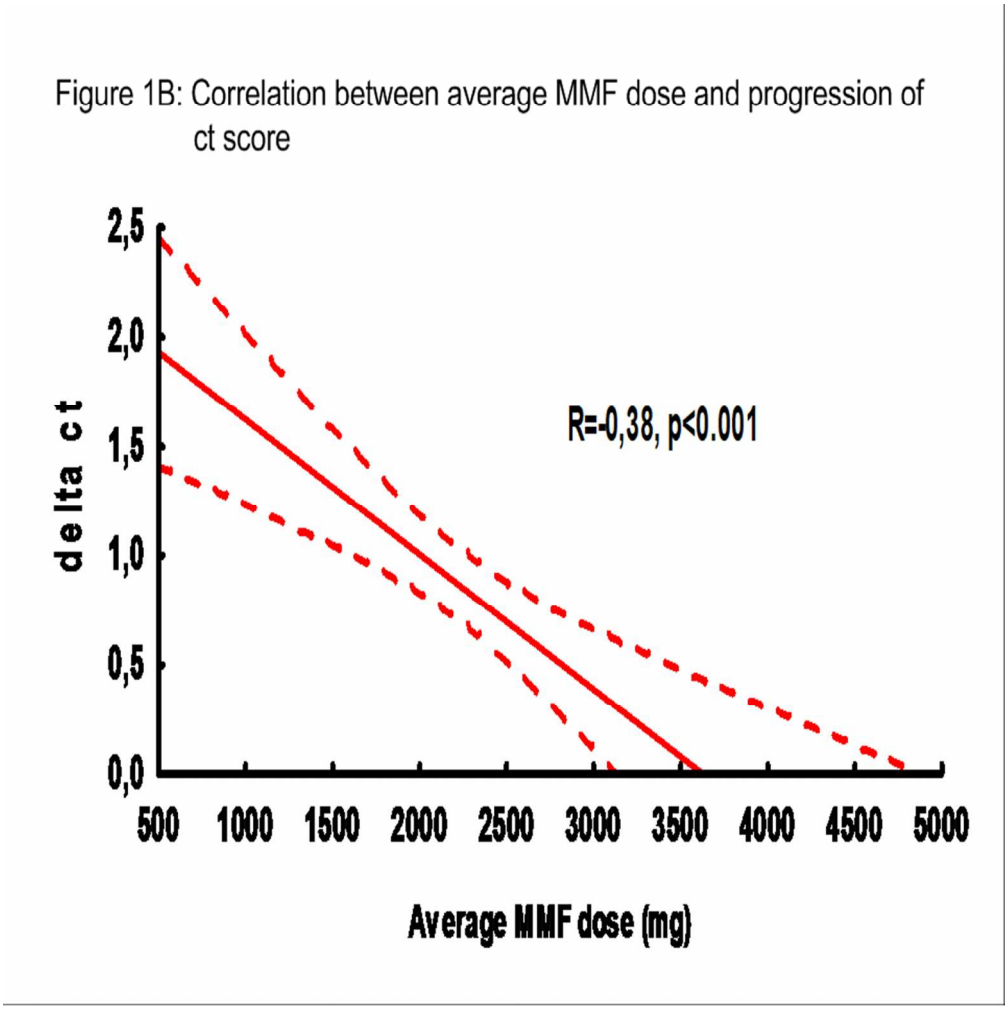

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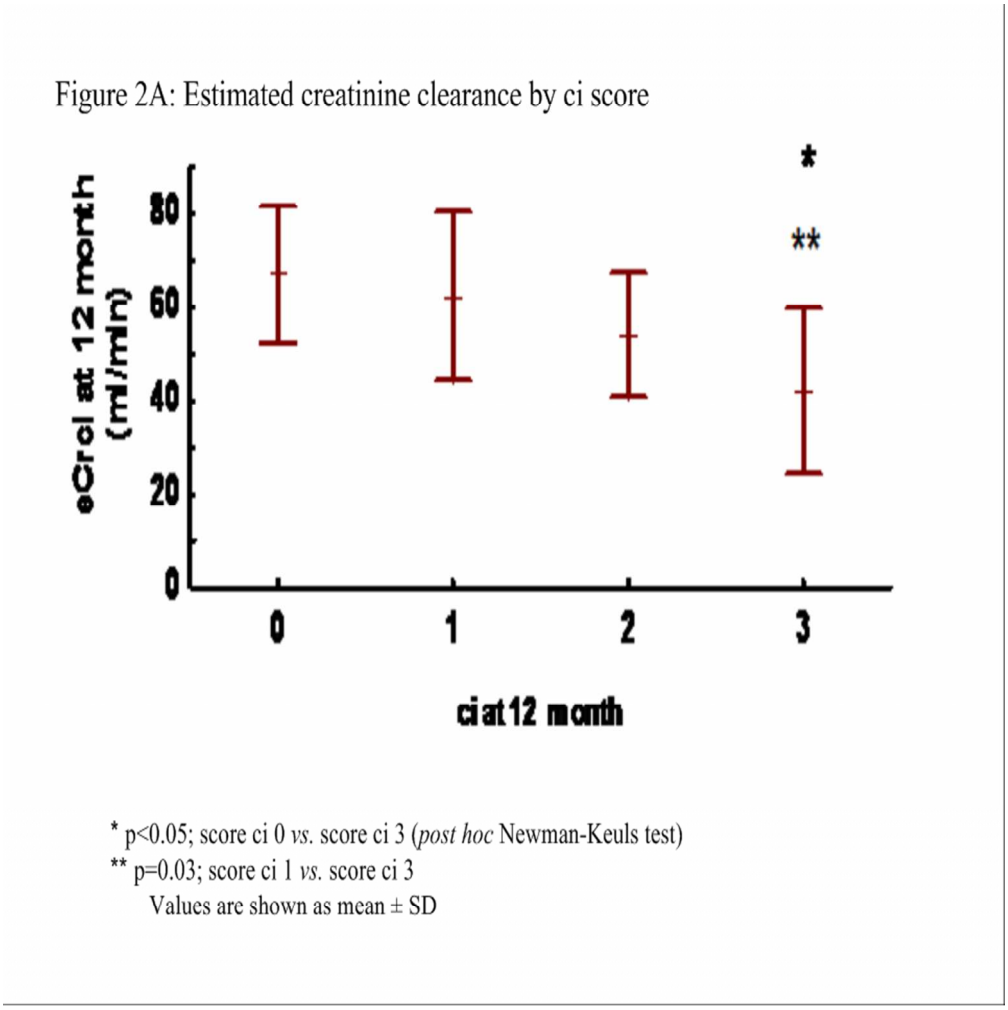

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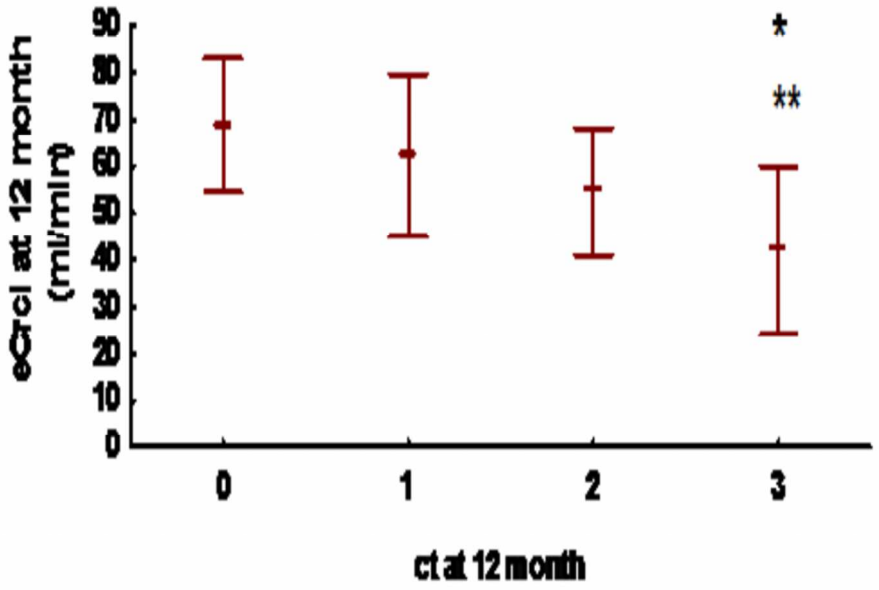
Figure 2B: Estimated creatinine clearance by ct score



* p<0.05; score ct 0 *vs.* score ct 3 (post hoc Newman-Keuls test)
** p=0.03; score ct 1 *vs.* score ct 3
Values are shown as mean ± SD

108x108mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No/page number	Recommendation
Title and abstract	1/1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2/5	Explain the scientific background and rationale for the investigation being reported
Objectives	3/5,6	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4/6	Present key elements of study design early in the paper
Setting	5/6,7, 8,9	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6/6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7/6,7, 8	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*/8	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10/6	Explain how the study size was arrived at
Quantitative variables	11/9	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12/9	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*/9, 10	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*/9, 10	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*/11	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16/10, 11	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18/12	Summarise key results with reference to study objectives
Limitations	19/16	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20/12-16	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21/12-16	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22/2	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Effect of Mycophenolate Mofetil on Progression of Interstitial Fibrosis and Tubular Atrophy after Kidney Transplantation- A Retrospective Study

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Effect of Mycophenolate Mofetil on Progression of Interstitial Fibrosis and Tubular Atrophy after Kidney Transplantation - A Retrospective Study

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ABSTRACT:

Objectives - Chronic transplant dysfunction after kidney transplantation is major reason of kidney graft loss and is caused by immunological and non-immunological factors. There is evidence that mycophenolate mofetil (MMF) may exert a positive effect on renal damage in addition to immunosuppression, by its direct antifibrotic properties. The aim of our study was to retrospectively investigate role of MMF dose on progression of chronic allograft dysfunction and IF/TA.

Setting - Retrospective, cohort study.

Participants - Kidney transplant patients in tertiary care institution. This is a retrospective cohort study that included 79 patients with kidney and kidney-pancreas transplantation. Immunosuppression consisted of anti-IL2 antibody induction, MMF, a calcineurin inhibitor ± steroids.

Primary outcome measures - An association of average MMF dose over 1 year post transplant with progression of interstitial fibrosis (Δci), tubular atrophy (Δct) and estimated creatinine clearance (eCr_{cl}) at 1 year post transplant was evaluated using univariate and multivariate analyses.

Results - Higher average MMF dose was significantly independently associated with better eCr_{cl} at 1 year post transplant (b=0.21 ± 0.1, p=0.04). In multiple regression analysis lower Δci (b=-0.2 ± 0.09, p=0.05) and Δct (b=-0.29 ± 0.1, p=0.02) were independently associated with greater average MMF dose. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Conclusions - Higher average MMF dose over 1 year is associated with better renal function and slower progression of IF/TA, at least partly independent of its immunosuppressive effects.

Strenghts and limitations of this study

Important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF (up to 4 g daily) during 1 year post transplantation had significantly lower progression of graft interstitial fibrosis and tubular atrophy. This is important finding, because of predictive value of graft IF/TA and should translate into better long-term graft survival. Our study has several shortcomings, such as its retrospective aspect and relatively short study period. As it was not aim of the study, we did not report side effects associated with different dosage of MMF.

INTRODUCTION:

Kidney transplantation significantly improves patient survival and quality of life comparing to dialysis. While significant improvements have been made in the treatment of acute rejection and short survival of transplanted kidney, there has not been major improvement in the long-term survival of transplanted kidney.[1] Chronic transplant dysfunction after kidney transplantation is major cause of kidney graft loss and is evoked by immunological and non-immunological factors.[2, 3] Histology changes that determine chronic transplant dysfunction are interstitial fibrosis and tubular atrophy (IF/TA), arteriosclerosis, arteriolar hyalinosis, glomerulopathy and mesangial matrix expansion.[4] IF/TA is the major pathohystology finding that can be verified on graft biopsies after kidney transplantation and is a predictor of long-term allograft function.[4] Clinical factors that affect progression of IF/TA are: recipient age, HLA mismatch, episodes of severe acute rejection, chronic rejection (esp. antibody-mediated), use of calcineurin inhibitors and BK nephropathy. Avoidance of CNI toxicity is considered as an important step to slow progression of IF/TA.[4-7] Mycophenolate mofetil (MMF) may help lowering CNI toxicity, by allowing lower CNI exposure.[7]

MMF reduces the risk of acute allograft rejection, without nephrotoxic side effects and is ideal candidate for long-term calcineurin drug reduction treatment strategies.[7] Retrospective studies of renal recipients who were treated with mycophenolate mofetil comparing azathioprin showed that MMF treated patients had significantly less chronic allograft dysfunction.[8, 9] Besides being associated with lower acute rejection rates as compared to azathioprin,[10, 11] evidence from animal and human studies suggests that

MMF may also exert a direct antifibrotic properties due to its antiproliferative action on nonimmune cells, including renal tubular cells and vascular smooth muscle cells.[12, 13] The aim of our study was to investigate role of mycophenolate mofetil dose on progression of IF/TA in kidney transplant recipients.

PATIENTS AND METHODS:

Patients:

This is a retrospective study conducted at Clinical Hospital "Merkur". This study represents a part of the posttransplant immune monitoring at the Merkur hospital, approved by the Hospital Ethics Committee. Patients gave there informed written consent for anonymized transplant data collection for research purposes. The study included 79 patients with kidney and kidney-pancreas transplantation, transplanted between 2003 and 2011. Eligible patients had to have protocol kidney biopsy at the time of implantation and 12 months after transplantation. Exclusion criteria have been: dual kidney transplantation, kidney-liver transplantation, use of antithymocyte immunoglobulin, BK nephropathy and recurrence of glomerulonephritis after transplantation.

Immunosuppression:

Induction immunosupression consisted of an anti-IL2 antibody (daclizumab or basiliximab), calcineurin inhibitor (tacrolimus or cyclosporine), MMF and methylprednisolone. Maintenance immunosuppression consisted of a calcineurin inhibitor (tacrolimus or cyclosporine), MMF ± steroids. Target cyclosporine trough concentrations were 250-350 during first month posttransplant, 200-300 during second to 6th month and 100-150 µg/L thereafter. Target tacrolimus trough levels were 10-12 during first month, 8-10 during second to 6th month and 5-8 µg/L thereafter. Mycophenolic acid target trough concentration was aimed to be higher than 7.2 µmol/L with tacrolimus and higher than 5 µmol/L with cyclosporine use.

Daclizumab was administered at day 0: 2mg/kg i.v. before opening of vascular anastomosis and at day 14: 2mg/kg i.v.. Basiliximab was administered at day 0: 20 mg i.v. before opening of vascular anastomosis and at day 4: 20 mg i.v..

Steroids have been dosed as follows: day 0: intraoperatively 500 mg of methylprednisolone, day 1: 250 mg, day 2: 125mg, day 3: 80 mg and day 4: 40 mg. In patients with early steroid withdrawal steroids have been withdrawn at day 5 after transplantation. In patients maintained on steroids, nadir dose of prednisone was 5 mg/d, achieved by 6 months. The criteria for early elimination of steroids were low immunological risk of the recipient (absence of, or low degree of HLA sensitization, i.e. PRA <10%) and good immediate renal function, as well as absence of an episode of acute rejection within 5 days after the transplantation. Steroids have been reintroduced in patients who suffered acute rejection episode.

As prophylaxis for viral (HSV, CMV), fungal (Candida spp.) urinary and P. jiroveci infections, low-dose fluconazole (for one year), valganciclovir (universally for three months) and sulfomethoxazol and trimethoprim (for one year) was used.

Renal allograft biopsies:

Protocol kidney biopsies were done at implantation, 1, 3, 6 and 12 months after transplantation. For cause biopsies were done in case of unexplained deterioration of renal function, or once weekly in patients with DGF. All rejection episodes were histologically confirmed. Histopathological analysis was performed by either of two pathologists who were blinded for immunosuppression. Acute rejections and chronic allograft scores have been analyzed using Banff 97 classification and its updates.[14, 15] All protocol and indication biopsies were analyzed by light microscopy, by immunofluorescence for C4d, and if indicated by immunohistochemistry for BK virus. Biopsies at 1 year post transplant have been also analyzed by electron microscopy for signs of chronic antibody-mediated rejection (transplant glomerulopathy, peritubular capillary basement membrane multilayering).[16]

Clinical outcome parameters:

Progression of chronic allograft scores during 1 year posttransplant was calculated by subtracting implantation chronic scores from chronic allograft scores 12 months

posttransplant: interstitial fibrosis (Δci), tubular atrophy (Δct), glomerulosclerosis (Δcg), mesangial matrix increase (Δmm), vasculopathy (Δcv) and arteriolar hyalinosis (Δah). Estimated creatinine clearance (eCr_{cl}) at 3, 6 and 12 months posttransplant was calculated using Cockroft-Gault formula. Acute rejections with Banff grade IA and IB were treated with three 500 mg methylprednisolone pulses. In case of acute rejection grade IIA or greater, patients have been treated with antithymocyte globulin. Antibody-mediated rejections were treated with steroid pulse and plasmapheresis.

Average dose of MMF during 1 year posttransplant was calculated from MMF dose at month 1, 3, 6 and 12.

Adverse effects analysed were clinicaly significant leucopenia, defined as white blood cell count less than 3000/ml, time to first symptomatic infection and number of symptomatic infection episodes per patient during first post transplant year.

Statistical analysis:

Numerical data are presented as mean ± SD or median with range in case of not normal distribution. Normality of distribution has been tested with Kolmogorov-Smirnov test. Correlation between two continuous variables has been tested using Spearman nonparametric correlation. Difference between two groups in continuous variables has been tested with student t-test or with Mann–Whitney test in non-normally distributed variables. The significance of the progression in chronic scores was analyzed using Wilcoxon Matched Pairs test. Univariate and multiple linear regression analysis were performed to determine predictive factors for progression of chronic allograft scores and kidney function at 12 months after transplantation. All variables that were associated with

respective outcome in bivariate analysis (at $p \leq 0.1$) were included in multivariate analysis. Because of colinearity between ci and ct score, only one score was included in each multivariate analysis. Statistical significance was considered at $p<0.05$. All statistical analyses were performed using Statistica 10 (StatSoft, Tulsa, OK, USA).

RESULTS:

Patient and transplant characteristics:

Patient characteristics are shown in Table 1. Recipients were a mean of 44.67 ± 12.03 years old at the time of transplantation, 68 percent of them were male and all were Caucasians. 33 percent of recipients had DGF after transplantation. Donors were a mean of 43.89 ± 15.55 years old and 54 percent of them were male. Number of living donor transplantations was 24 (30 percent). Average daily MMF dose during 1 year posttransplant was 2244 ± 585 mg (1062 – 4000) (Table 2). As expected, there was no correlation of MMF dose with MMF trough concentration (R=-0.13; p=0.28). Also, there was no correlation between MMF dose with tacrolimus concentration (R=-0.04; p=0.79). Early steroid withdrawal was done in 46 percent of patients after transplantation. Incidence of subclinical and clinical acute rejections greater then borderline was 30 percent in first year. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Factors associating with eCrcl:

Kidney function increased during 1st year post transplant. eCrcl at month 3 was 56.98 ± 15.78 ml/min, at 6 month 58.94 ± 16.94 ml/min and at 12 month 61.47 ± 16.75 ml/min ($p<0.001$; 12 months *vs*. 3 months) (Figure 1.) eCrcl at 1 year post transplant was greater in SPKT recipients (71.38 ± 13.45 ml/min *vs.* 57.88 ± 16.47 ml/min; $p=0.001$) and in patients who did not have DGF (64.08 ± 15.87 ml/min *vs*. 56.15 ± 17.55 ml/min; $p=0.05$). Donor age ($R=-0.46$; $p<0.001$) and recipient age ($R=-0.46$; $p<0.001$) negatively correlated with eCrcl at 1 year post transplant, while there was no correlation of renal function with donor and recipient gender, type of donation (deceased *vs.* living), HLA MM, average CNI concentration, steroid-free regimen of immunosuppression, or history of acute rejection (Table 3). In univariate analysis allograft function at 12 month post Tx was also negatively correlated with ci ($R=-0.34$; $p=0.002$) and ct ($R=-0.35$; $p=0.002$) at 12 month (Figure 2A, Figure 2B). Although MMF dose was positively correlated with renal function with borderline significance in univariate analysis, in multivariate analysis there was a significant positive association between greater average MMF dose and better eCrcl at 12 month post transplant ($b=0.21 \pm 0.1$; $p=0.04$) (Table 4).

Factors affecting IF/TA:

The average ci score increased from 0.16 ± 0.44 to 0.94 ± 0.86 between implantation and month 12 ($p<0.001$). Average progression of this and other chronic scores during 1 year post transplant is shown in Table 5. In univariate analysis Δci ($R=-0.37$; $p=0.001$) and Δct ($R=-0.38$; $p=0.001$) significantly negatively correlated with average MMF dose (Figure 3A and 3B, Table 6). There was lower progression of ci score in patients on steroid-free

immunosuppression (0.47 ± 0.7 *vs*. 1.09 ± 0.87; p=0.002) and in those who did not have DGF (0.62 ± 0.74 *vs* 1.19 ± 0.98; p=0.02). Acute cellular rejection, recipient and donor gender, recipient and donor age, HLA MM, deceased *vs.* living donor, as well as average concentration of tacrolimus had no significant effect on progression of chronic allograft scores. Factors that remained significantly associated with progression of ci score in multivariate analysis were ci0 score, donor age, average MMF dose, DGF and steroid-free immunosuppression (Table 7.). In multivariate analysis only ct0 score, average MMF dose and DGF remained independently associated with 12-month progression of ct score (Table 7.). Selected AE are shown in Table 8. There was no difference in AE (leucopenia and infections) with respect to average median MMF dose.

Discussion:

The most important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF during 1 year post transplantation had significantly lower progression of ci and ct scores. To our knowledge this is first study demonstrating that there is a dose-dependent protective effect of MMF on graft IF/TA. Lower progression of IF/TA could not be explained with lower concentration of CNI, because there was not correlation between tacrolimus concentration with IF/TA. Similarly, there was no correlation between average MMF dose and tacrolimus (R=-0.04; p=0.79) or cyclosporine concentration (R=-0.07, p=0.79). In addition, higher average MMF dose was not associated with decreased incidence of

biopsy proven acute rejection, which suggests that antifibrotic properties of higher MMF dose was at least partly independent of its immunosuppressive effects. Higher MMF dose had only moderate effect on 1-year renal function, which is consistent with previous reports showing that transplanted kidneys undergo pathohystology changes without significant early change in kidney function.[17]

In the present retrospective study we have confirmed that IF/TA progression occurs in first year after kidney transplantation. Several studies have shown that progression of IF/TA is correlated with type of immunosuppression.[18] In most transplant centers in the United States and Europe immunosuppression consists of induction with an anti-IL2R antibody or antithymocyte immunoglobulin and maintenance with a calcineurin inhibitor, MMF and steroids.[19] Studies have reported significant improvement in kidney function in patients on MMF with lower exposition to CNIs, esp. tacrolimus.[20] Recently, in the paper of Kamar e*t al*. it has been reported that maintenance kidney transplant patients converted to a higher dose of the mycophenolate sodium (1440 mg daily) with lower tacrolimus concentration had borderline higher eCr_{cl} on month 6 *vs*. those treated with lower dose of mycophenolate sodium, with usual tacrolimus concentration (eCr_{cl} 49.1 ± 11.1 *vs*. 44.7 ± 11.5 ml/min; p=0.07).[21] Although there was only borderline significance, increased mycophenolate dosing with lower tacrolimus concentration was safe with potential benefit on kidney function.

Our study also corroborates recently published findings of a *post hoc* joint analysis of the Symphony, FDCC and OptiCept trials, where a a lower tacrolimus level and a higher MMF dose were associated with significantly better kidney function at 1 year post transplant.[22] Shortcoming of these studies[17,18] is lack of protocol biopsies. The

optimal MMF dosing in patients maintained on contemporary low-dose CNI is still undetermined. However, some results of early MMF registration trials suggest that higher MMF exposure might be beneficial; having in mind that there was no antibody induction in these studies and that CNI was standard dose cyclosporine. Thus, in the Tri-continental study, group treated with 3 g MMF compared with 2 g of MMF showed lower incidence of biopsy proven acute rejection episodes (15.9% vs. 19.7%) within 6 month period selected for the primary efficacy analysis. Similarly, serum creatinine level at 1 year was 1.42 ± 0.07 mg/dL in the MMF 3 g group *vs.* 1.64 ± 0.07 mg/dL in MMF 2 g group.[12] In the European mycophenolate mofetil study same trends regarding higher MMF dose were observed.[11] As mentioned before, in these studies there was no antibody induction that could have allowed lower dose of cyclosporin with higher dose of MMF and there were no protocol biopsies. In a more recent MYSS trial, there was no difference in acute rejection rate and renal function between MMF and azathioprine in a cyclosporine-based protocol.[19] However in that study only one MMF dose was compared to azathioprine[23] and again there were no protocol biopsies. Unfortunately adequate prospective MMF dose comparison studies in tacrolimus-based protocols with antibody induction are missing. In the Symphony study it was reported that patients on tacrolimus-MMF-prednisone maintenance imunosuppression after kidney transplantation had better kidney function and graft survival with lower number of acute rejection episodes. Patients in that group had highest MMF exposure.[24] Protocols with even higher MMF exposure might allow additional CNI sparing, that would decrease side effect of CNI (hypertension, diabetes, hyperlipidemia, neurotoxicity).[25]

Clinical relevance of IF/TA without other concomitant pathology (i.e. recurrent disease and chronic antibody-mediated rejection) for prediction of graft deterioration and loss is controversial. In El-Zoghby et al. study there was attempt to identify specific causes of late kidney allograft failure. The authors found that transplant glomerulopathy was responsible for 37 percent loss of functioning grafts, while graft loss due to IF/TA was present in 31 percent of cases (with higher frequency in deceased-donor transplants).[26] At first glance, these results seem at odd with ours, where there were no signs of chronic antibody-mediated rejection. An explanation for this discrepancy in the results of the two studies is not completely clear, but the former study included high number of living transplants (72.5 percent) with glomerulonephritis as primary disease and with follow-up up to 10 years. Transplant glomerulopathy is more frequently seen late posttransplant, generally with low incidence. Nevertheless, ours and El-Zoghby study, both demonstrated that IF/TA even in absence of other pathology is associated with adverse graft outcome. Another important study, the DeKaf study, tried to use various histopathologic clusters to differentiate subgroups within diagnosis of IF/TA. They found that cluster with more severe fibrosis plus inflammation and arterial lesions had the worst prognosis.[27] Although incidence of acute rejection in our study did not vary with MMF exposure, increased MMF exposure might suppress mild graft inflammation, below the threshold for diagnosing acute rejection. This is subject of our ongoing investigation and will be reported separately. An interesting finding of the present study was that early steroid withdrawal was not associated with worse IF/TA. At first glance this is at odd with the Astellas trial.[23] However, according to our protocol, patients with DGF were not included in early steroid withdrawal and Astellas trial, which did not have protocol

biopsies, reported increased IF/TA in early steroid withdrawal group based on indication biopsies performed early posttransplant, thus more likely reflecting donor-derived histology changes, rather than effect of steroid withdrawal.[28]

In our study there was only borderline significance of positive association of 1-year eCr_{cl} with MMF in univariate analysis. This result is not very surprising since decreased renal function is not a very sensitive marker of incipient IF/TA.

Mechanisms by which an average higher exposure to MMF was associated with slower progression of IF/TA may be both immune and nonimmune. Because there was no difference in incidence of acute rejection with respect to increased MMF exposure in our study, we believe that there may be a significant contribution of nonimmune mechanisms in retardation of IF/TA in patients with higher MMF. In line with this, in many experimental models it has been shown that MMF has antiproliferative and antifibrotic effect.[29-31] In the study of Jiang at al. using rat renal ischemia reperfusion injury, a time- and dose-dependent correlation of higher MMF dose with better renal function and lower interstitial fibrosis was demonstrated. Suggested potential mechanism was lower expression of TGF-β1 and MCP-1with lower macrophage infiltration.[32] In recent clinical trials MMF was shown as a safe drug that could be a good candidate for treatment of interstitial lung disease in systemic sclerosis.[33] Experimental model of encapsulated peritoneal sclerosis in rats proved beneficial effect of MMF as an inhibitor of neovascularisation.[34] Also, MMF monotherapy was associated with a positive effect on hepatic fibrosis progression in HCV liver transplant recipients.[35]

Our study has several shortcomings, such as its retrospective aspect and relatively short study period. Although study period was limited to 12 months post transplantation, a

clear correlation of slower progression of IF/TA with higher average MMF dose underlines potential benefit of these findings. As mentioned before, in current study we did not analyze inflammation outside Banff acute rejection threshold in kidney biopsies with respect to MMF dose. As inflammation in areas of IF/TA is an important predictor of renal function and graft loss, this is subject of an ongoing work.

In summary, higher MMF dose after kidney transplantation might slower progression of IF/TA, which might lead to better long-term survival of transplanted kidney. Our study serves as a platform for a prospective, randomized, long-term trial with different MMF doses to evaluate benefit of higher MMF dose in renal transplant recipients. (NCT018600183).

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Contributorship Statement

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Abbreviations:

IF/TA - interstitial fibrosis and tubular atrophy

MMF - mycophenolate mofetil

BK - polyoma virus BK

CNI - calcineurin inhibitors

DGF - delayed graft function

eCrCl - estimated creatine clearance

SPKT - simultaneous pancreas kidney transplantation

HLA MM – human leukocyte antigen mismatch

AE - adverse events

Figure Legends

Figure 1: Estimated creatinine clearance during first year posttransplant

Figure 2A: Correlation between average MMF dose and progression of ci score

Figure 2B: Correlation between average MMF dose and progression of ct score

Figure 3A: Estimated creatinine clearance by ci score

Figure 3B: Estimated creatinine clearance by ct score

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Table 1. Baseline characteristics

RECIPIENT CHARACTERISTICS	AGE (years)	44.67 ± 12.03
	GENDER (f/m)	25/54
	PRIMARY RENAL DISEASE (diabetes mellitus, polycistic kidney disease, glomerulonephritis, pyelonephritis/interstitial nephritis, other/unknown)	24/8/19/6/22
DONOR CHARACTERISTICS	DONOR SOURCE (decased/living)	55/24
	AGE (years)	43.89 ± 15.55
	GENDER (f/m)	36/43
TRANSPLANTATION CHARACTERISTICS	TRANSPLANTED ORGAN (KIDNEY/SPKT)	58/21
	INITIAL IMMUNOSUPRESSION (anti-IL2,TAC,MMF/anti-IL2, CyA,MMF)	53/26
	DELAYED GRAFT FUNCTION (no/yes)	53/26
	STEROID FREE (yes/no)	36/43
	HLA MM	3.33 ± 1.51

Table 2. eCr_{cl}, MMF dose and CNI concentration during first year post transplant

	Month posttransplant			
	1	3	6	12
eCr_{cl} (ml/min)		56.98 ± 15.79	58.94 ± 16.94	61.47 ± 16.75
MMF dose (mg)	2500 (750 – 4000) 2427 ± 643.17	2000 (750 – 4000) 2167.72 ± 733.49	2000 (1000-4000) 2188.29 ± 716.91	2000 (1000-4000) 2193.04 ± 642.95
Tacrolimus conc. (µg/L) (n=53)	10.79 ± 4.16	9.69 ± 3.00	9.03 ± 5.52	7.83 ± 2.45
Cyclosporin conc. (µg/L) (n=26)	335.07 (274 – 413)	231.05 (181-265)	206 (170 – 257)	131 (125 – 171)

Table 3. Association of variables with eCr_{cl} on 1 year

	Estimated creatinine clearance (ml/min)	p value
Kidney *vs.* SPKT	57.88 ± 15.47 *vs*. 71.38 ± 13.45	0.001
DGF (yes *vs.* no)	56.15 ± 17.55 *vs*. 64.08 ± 15.87	0.05
Recipient gender (m *vs.* f)	59.83 ± 16.02 *vs*. 65 ± 18.07	0.2
Donor gender (m *vs*. f)	63.87 ± 16.71 *vs*. 58.60 ± 16.58	0.17
Donor source (D *vs*. L)	62.36 ± 17.85 *vs*. 59.43 ± 14.05	0.47
Steroid-free (yes *vs*. no)	63.94 ± 17.73 *vs*. 59.39 ± 15.81	0.23
Acute rejection (yes *vs*. no)	61.64 ± 16.59 *vs*. 61.39 ± 16.97	0.95
	R	p value
Recipient age	-0.45	<0.001
Donor age	-0.46	<0.001
HLA MM	0.07	0.52
Average tacrolimus concentration	-0.02	0.9
Average MMF dose	0.18	0.1
ci at 1 year post Tx	-0.34	0.002
ct at 1 year post Tx	-0.35	0.002
cv at 1 year post Tx	-0.20	0.07

Table 4. Multiple regression analysis of factors associated with kidney function

	Beta (β)	St.Err. β	p value
Tx (kidney)	-0.17	0.13	0.19
DGF (no)	0.04	0.1	0.71
Recipient age	-0.41	0.1	<0.001
Donor age	-0.1	0.14	0.45
ci at 12 months	-0.18	0.11	0.09
Average MMF dose	0.21	0.1	0.04

Table 5. One-year progression of chronic allograft scores

Banff score	N	At transplantation	N	12 month	p
Interstitial fibrosis (ci)	79	0.16 ± 0.44	79	0.94 ± 0.85	<0.001
Tubular atrophy (ct)	79	0.24 ± 0.46	79	1.05 ± 0.77	<0.001
Chronic glomerulopathy (cg)	79	0	79	0	
Mesangial matrix (mm)	79	0.01 ± 0.11	79	0.09 ± 0.36	0.09
Fibrointimal thickening (cv)	76	0.37 ± 0.83	78	0.29 ± 0.70	0.47
Arteriolar hyalinosis (ah)	78	0.68 ± 1,04	79	0.79 ± 1.04	0.26

Table 6. Correlation of factors associated with progression of ci and ct scores

	Δci		Δct	
	mean ± SD	p	mean ± SD	p
Kidney *vs.* SPKT	0.86 ± 0.91 *vs*. 0.67 ± 0.73	0.51	0.85 ± 0.87 vs. 0.86 ± 0.65	0.74
DGF (yes *vs.* no)	1.19 ± 0.98 *vs*. 0.62 ± 0.74	0.02	1.15 ± 0.92 vs. 0.69 ± 0.72	0.05
Recipient gender (m *vs.* f)	0.83 ± 0.88 *vs*. 0.76 ± 0.83	0.78	0.91 ± 0.83 vs. 0.72 ± 0.79	0.35
Donor gender (m *vs*. f)	0.91 ± 0.95 *vs*. 0.69 ± 0.75	0.43	0.88 ± 0.93 vs. 0.81 ± 0.67	0.96
Donor source (D *vs*. L)	0.84 ± 0.88 *vs*. 0.75 ± 0.85	0.73	0.87 ± 0.82 vs. 0.79 ± 0.83	0.71
Steroid free (yes *vs*. no)	1.09 ± 0.87 *vs*. 0.47 ± 0.74	0.002	1.07 ± 0.83 vs. 0.58 ± 0.73	0.01
Acute rejection (yes *vs*. no)	0.8 ± 0.89 *vs*. 0.83 ± 0.82	0.78	0.93 ± 0.84 vs. 0.67 ± 0.76	0.23
	R	p	R	p
Recipient age	-0.11	0.33	-0.11	0.32
Donor age	0.17	0.13	0.04	0.73
HLA MM	-0.09	0.43	-0.002	0.99
Average tacrolimus conc.	-0.009	0.95	0.003	0.98
Average MMF dose	-0.37	<0.001	-0.38	<0.001
ci at implantation	-0.32	0.003		
ct at implantation			-0.45	<0.001

Table 7. Multivariate general regression analysis for factors related to progression of ci and ct score

Δci			
	Beta (β)	Std. Err. β	p
ci0	-0.43	0.09	<0.001
DGF (no)	-0.22	0.11	<0.05
Average MMF dose	-0.20	0.09	<0.05
Donor age	0.32	0.09	<0.05
Steroid free (yes)	-0.25	0.11	0.02
Δct			
	Beta (β)	Std. Err. β	p
ct0	-0.44	0.09	<0.001
Average MMF dose	-0.29	0.1	<0.05
DGF (no)	-0.29	0.1	<0.05
Steroid free (yes)	-0.09	0.11	0.39

Table 8. Adverse events with respect to 1 year average median MMF dose.

	MMF dose < median	MMF dose > median	p
Average number of infection episodes per patient	1.16 ± 0.97	1.23 ± 1.22	0.88
Mean time to first infection (days)	157±138	175±143	0.76
Proportion of patients with leucopenia	6 /31	7 /48	0.58

Effect of Mycophenolate Mofetil on Progression of Interstitial Fibrosis and Tubular Atrophy after Kidney Transplantation - A Retrospective Study

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Abbreviations:

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DGF - delayed graft function

eCrCl - estimated creatine clearance

SPKT - simultaneous pancreas kidney transplantation

HLA MM – human leukocyte antigen mismatch

AE - adverse events

ABSTRACT:

Objectives - Chronic transplant dysfunction after kidney transplantation is major reason of kidney graft loss and is caused by immunological and non-immunological factors. There is evidence that mycophenolate mofetil (MMF) may exert a positive effect on renal damage in addition to immunosuppression, by its direct antifibrotic properties. The aim of our study was to retrospectively investigate role of MMF dose on progression of chronic allograft dysfunction and IF/TA.

Setting - Retrospective, cohort study.

Participants - Kidney transplant patients in tertiary care institution. This is a retrospective cohort study that included 79 patients with kidney and kidney-pancreas transplantation. Immunosuppression consisted of anti-IL2 antibody induction, MMF, a calcineurin inhibitor ± steroids.

Primary outcome measures - An association of average MMF dose over 1 year post transplant with progression of interstitial fibrosis (Δci), tubular atrophy (Δct) and estimated creatinine clearance (eCr_{cl}) at 1 year post transplant was evaluated using univariate and multivariate analyses.

Results - Higher average MMF dose was significantly independently associated with better eCr_{cl} at 1 year post transplant (b=0.21 ± 0.1, p=0.04). In multiple regression analysis lower Δci (b=-0.2 ± 0.09, p=0.05) and Δct (b=-0.29 ± 0.1, p=0.02) were independently associated with greater average MMF dose. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Conclusions - Higher average MMF dose over 1 year is associated with better renal function and slower progression of IF/TA, at least partly independent of its immunosuppressive effects.

Strenghts and limitations of this study

Important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF (up to 4 g daily) during 1 year post transplantation had significantly lower progression of graft interstitial fibrosis and tubular atrophy. This is important finding, because of predictive value of graft IF/TA and should translate into better long-term graft survival. Our study has several shortcomings, such as its retrospective aspect and relatively short study period. As it was not aim of the study, we did not report side effects associated with different dosage of MMF.

INTRODUCTION:

Kidney transplantation significantly improves patient survival and quality of life comparing to dialysis. While significant improvements have been made in the treatment of acute rejection and short survival of transplanted kidney, there has not been major improvement in the long-term survival of transplanted kidney.[1] Chronic transplant dysfunction after kidney transplantation is major cause of kidney graft loss and is evoked by immunological and non-immunological factors.[2, 3] Histology changes that determine chronic transplant dysfunction are interstitial fibrosis and tubular atrophy (IF/TA), arteriosclerosis, arteriolar hyalinosis, glomerulopathy and mesangial matrix expansion.[4] IF/TA is the major pathohystology finding that can be verified on graft biopsies after kidney transplantation and is a predictor of long-term allograft function.[4] Clinical factors that affect progression of IF/TA are: recipient age, HLA mismatch, episodes of severe acute rejection, chronic rejection (esp. antibody-mediated), use of calcineurin inhibitors and BK nephropathy. Avoidance of CNI toxicity is considered as an important step to slow progression of IF/TA.[4-7] Mycophenolate mofetil (MMF) may help lowering CNI toxicity, by allowing lower CNI exposure.[7]

MMF reduces the risk of acute allograft rejection, without nephrotoxic side effects and is ideal candidate for long-term calcineurin drug reduction treatment strategies.[7] Retrospective studies of renal recipients who were treated with mycophenolate mofetil comparing azathioprin showed that MMF treated patients had significantly less chronic

allograft dysfunction.[8, 9] Besides being associated with lower acute rejection rates as compared to azathioprin,[10, 11] evidence from animal and human studies suggests that MMF may also exert a direct antifibrotic properties due to its antiproliferative action on nonimmune cells, including renal tubular cells and vascular smooth muscle cells.[12, 13] The aim of our study was to investigate role of mycophenolate mofetil dose on progression of IF/TA in kidney transplant recipients.

PATIENTS AND METHODS:

Patients:

This is a retrospective study conducted at Clinical Hospital "Merkur". This study represents a part of the posttransplant immune monitoring at the Merkur hospital, approved by the Hospital Ethics Committee. Patients gave there informed written consent for anonymized transplant data collection for research purposes. The study included 79 patients with kidney and kidney-pancreas transplantation, transplanted between 2003 and 2011. Eligible patients had to have protocol kidney biopsy at the time of implantation and 12 months after transplantation. Exclusion criteria have been: dual kidney transplantation, kidney-liver transplantation, use of antithymocyte immunoglobulin, BK nephropathy and recurrence of glomerulonephritis after transplantation.

Immunosuppression:

Induction immunosupression consisted of an anti-IL2 antibody (daclizumab or basiliximab), calcineurin inhibitor (tacrolimus or cyclosporine), MMF and methylprednisolone. Maintenance immunosuppression consisted of a calcineurin inhibitor (tacrolimus or cyclosporine), MMF ± steroids. Target cyclosporine trough concentrations were 250-350 during first month posttransplant, 200-300 during second to 6th month and 100-150 µg/L thereafter. Target tacrolimus trough levels were 10-12 during first month, 8-10 during second to 6th month and 5-8 µg/L thereafter. Mycophenolic acid target trough concentration was aimed to be higher than 7.2 µmol/L with tacrolimus and higher than 5 µmol/L with cyclosporine use.

Daclizumab was administered at day 0: 2mg/kg i.v. before opening of vascular anastomosis and at day 14: 2mg/kg i.v.. Basiliximab was administered at day 0: 20 mg i.v. before opening of vascular anastomosis and at day 4: 20 mg i.v..

Steroids have been dosed as follows: day 0: intraoperatively 500 mg of methylprednisolone, day 1: 250 mg, day 2: 125mg, day 3: 80 mg and day 4: 40 mg. In patients with early steroid withdrawal steroids have been withdrawn at day 5 after transplantation. In patients maintained on steroids, nadir dose of prednisone was 5 mg/d, achieved by 6 months. The criteria for early elimination of steroids were low immunological risk of the recipient (absence of, or low degree of HLA sensitization, i.e. PRA <10%) and good immediate renal function, as well as absence of an episode of acute rejection within 5 days after the transplantation. Steroids have been reintroduced in patients who suffered acute rejection episode.

As prophylaxis for viral (HSV, CMV), fungal (Candida spp.) urinary and P. jiroveci infections, low-dose fluconazole (for one year), valganciclovir (universally for three months) and sulfomethoxazol and trimethoprim (for one year) was used.

Renal allograft biopsies:

Protocol kidney biopsies were done at implantation, 1, 3, 6 and 12 months after transplantation. For cause biopsies were done in case of unexplained deterioration of renal function, or once weekly in patients with DGF. All rejection episodes were histologically confirmed. Histopathological analysis was performed by either of two pathologists who were blinded for immunosuppression. Acute rejections and chronic allograft scores have been analyzed using Banff 97 classification and its updates.[14, 15] All protocol and indication biopsies were analyzed by light microscopy, by immunofluorescence for C4d, and if indicated by immunohistochemistry for BK virus. Biopsies at 1 year post transplant have been also analyzed by electron microscopy for signs of chronic antibody-mediated rejection (transplant glomerulopathy, peritubular capillary basement membrane multilayering).[16]

Clinical outcome parameters:

Progression of chronic allograft scores during 1 year posttransplant was calculated by subtracting implantation chronic scores from chronic allograft scores 12 months

posttransplant: interstitial fibrosis (Δci), tubular atrophy (Δct), glomerulosclerosis (Δcg), mesangial matrix increase (Δmm), vasculopathy (Δcv) and arteriolar hyalinosis (Δah). Estimated creatinine clearance (eCr_{cl}) at 3, 6 and 12 months posttransplant was calculated using Cockroft-Gault formula. Acute rejections with Banff grade IA and IB were treated with three 500 mg methylprednisolone pulses. In case of acute rejection grade IIA or greater, patients have been treated with antithymocyte globulin. Antibody-mediated rejections were treated with steroid pulse and plasmapheresis.

Average dose of MMF during 1 year posttransplant was calculated from MMF dose at month 1, 3, 6 and 12.

Adverse effects analysed were clinicaly significant leucopenia, defined as white blood cell count less than 3000/ml, time to first symptomatic infection and number of symptomatic infection episodes per patient during first post transplant year.

Statistical analysis:

Numerical data are presented as mean ± SD or median with range in case of not normal distribution. Normality of distribution has been tested with Kolmogorov-Smirnov test. Correlation between two continuous variables has been tested using Spearman nonparametric correlation. Difference between two groups in continuous variables has been tested with student t-test or with Mann–Whitney test in non-normally distributed variables. The significance of the progression in chronic scores was analyzed using Wilcoxon Matched Pairs test. Univariate and multiple linear regression analysis were performed to determine predictive factors for progression of chronic allograft scores and kidney function at 12 months after transplantation. All variables that were associated with

respective outcome in bivariate analysis (at $p \leq 0.1$) were included in multivariate analysis. Because of colinearity between ci and ct score, only one score was included in each multivariate analysis. Statistical significance was considered at $p<0.05$. All statistical analyses were performed using Statistica 10 (StatSoft, Tulsa, OK, USA).

RESULTS:

Patient and transplant characteristics:

Patient characteristics are shown in Table 1. Recipients were a mean of 44.67 ± 12.03 years old at the time of transplantation, 68 percent of them were male and all were Caucasians. 33 percent of recipients had DGF after transplantation. Donors were a mean of 43.89 ± 15.55 years old and 54 percent of them were male. Number of living donor transplantations was 24 (30 percent). Average daily MMF dose during 1 year posttransplant was 2244 ± 585 mg (1062 – 4000) (Table 2~~5~~). As expected, there was no correlation of MMF dose with MMF trough concentration (R=-0.13; p=0.28). Also, there was no correlation between MMF dose with tacrolimus concentration (R=-0.04; p=0.79). Early steroid withdrawal was done in 46 percent of patients after transplantation. Incidence of subclinical and clinical acute rejections greater then borderline was 30 percent in first year. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Factors associating with eCrcl:

Kidney function increased during 1st year post transplant. eCrcl at month 3 was 56.98 ± 15.78 ml/min, at 6 month 58.94 ± 16.94 ml/min and at 12 month 61.47 ± 16.75 ml/min ($p<0.001$; 12 months *vs*. 3 months) (Figure 1.) eCrcl at 1 year post transplant was greater in SPKT recipients (71.38 ± 13.45 ml/min *vs.* 57.88 ± 16.47 ml/min; p=0.001) and in patients who did not have DGF (64.08 ± 15.87 ml/min *vs*. 56.15 ± 17.55 ml/min; p=0.05). Donor age (R=-0.46; $p<0.001$) and recipient age (R=-0.46; $p<0.001$) negatively correlated with eCrcl at 1 year post transplant, while there was no correlation of renal function with donor and recipient gender, type of donation (deceased *vs.* living), HLA MM, average CNI concentration, steroid-free regimen of immunosuppression, or history of acute rejection (Table 3~~6~~). In univariate analysis allograft function at 12 month post Tx was also negatively correlated with ci (R=-0.34; p=0.002) and ct (R=-0.35; p=0.002) at 12 month (Figure 2~~1~~2A, Figure 2~~1~~2B). Although MMF dose was positively correlated with renal function with borderline significance in univariate analysis, in multivariate analysis there was a significant positive association between greater average MMF dose and better eCrcl at 12 month post transplant (b=0.21 ± 0.1; p=0.04) (Table 4~~2.~~).

Factors affecting IF/TA:

The average ci score increased from 0.16 ± 0.44 to 0.94 ± 0.86 between implantation and month 12 ($p<0.001$). Average progression of this and other chronic scores during 1 year post transplant is shown in ~~suppl. data~~ (Table 5~~7~~). In univariate analysis Δci (R=-0.37; p=0.001) and Δct (R=-0.38; p=0.001) significantly negatively correlated with average MMF dose (Figure 3~~2~~3A and 3~~2~~3B, Table 6~~3~~). There was lower progression of ci score

in patients on steroid-free immunosuppression (0.47 ± 0.7 *vs*. 1.09 ± 0.87; p=0.002) and in those who did not have DGF (0.62 ± 0.74 *vs* 1.19 ± 0.98; p=0.02). Acute cellular rejection, recipient and donor gender, recipient and donor age, HLA MM, deceased *vs.* living donor, as well as average concentration of tacrolimus had no significant effect on progression of chronic allograft scores. Factors that remained significantly associated with progression of ci score in multivariate analysis were ci0 score, donor age, average MMF dose, DGF and steroid-free immunosuppression (Table 74.). In multivariate analysis only ct0 score, average MMF dose and DGF remained independently associated with 12-month progression of ct score (Table 74.). Selected AE are shown in Table 8. There was no difference in AE (leucopenia and infections) with respect to average median MMF dose.

Discussion:

The most important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF during 1 year post transplantation had significantly lower progression of ci and ct scores. To our knowledge this is first study demonstrating that there is a dose-dependent protective effect of MMF on graft IF/TA. Lower progression of IF/TA could not be explained with lower concentration of CNI, because there was not correlation between tacrolimus concentration with IF/TA. Similarly, there was no correlation between average MMF dose and tacrolimus (R=-0.04; p=0.79) or cyclosporine concentration (R=-0.07, p=0.79).

In addition, higher average MMF dose was not associated with decreased incidence of biopsy proven acute rejection, which suggests that antifibrotic properties of higher MMF dose was at least partly independent of its immunosuppressive effects. Higher MMF dose had only moderate effect on 1-year renal function, which is consistent with previous reports showing that transplanted kidneys undergo pathohystology changes without significant early change in kidney function.[17]

In the present retrospective study we have confirmed that IF/TA progression occurs in first year after kidney transplantation. Several studies have shown that progression of IF/TA is correlated with type of immunosuppression.[18] In most transplant centers in the United States and Europe immunosuppression consists of induction with an anti-IL2R antibody or antithymocyte immunoglobulin and maintenance with a calcineurin inhibitor, MMF and steroids.[19] Studies have reported significant improvement in kidney function in patients on MMF with lower exposition to CNIs, esp. tacrolimus.[20] Recently, in the paper of Kamar e*t al*. it has been reported that maintenance kidney transplant patients converted to a higher dose of the mycophenolate sodium (1440 mg daily) with lower tacrolimus concentration had borderline higher eCr_{cl} on month 6 *vs*. those treated with lower dose of mycophenolate sodium, with usual tacrolimus concentration (eCr_{cl} 49.1 ± 11.1 *vs*. 44.7 ± 11.5 ml/min; p=0.07).[21] Although there was only borderline significance, increased mycophenolate dosing with lower tacrolimus concentration was safe with potential benefit on kidney function.

Our study also corroborates recently published findings of a *post hoc* joint analysis of the Symphony, FDCC and OptiCept trials, where a a lower tacrolimus level and a higher MMF dose were associated with significantly better kidney function at 1 year post

transplant.[22] Shortcoming of these studies[17,18] is lack of protocol biopsies. The optimal MMF dosing in patients maintained on contemporary low-dose CNI is still undetermined. However, some results of early MMF registration trials suggest that higher MMF exposure might be beneficial; having in mind that there was no antibody induction in these studies and that CNI was standard dose cyclosporine. Thus, in the Tri-continental study, group treated with 3 g MMF compared with 2 g of MMF showed lower incidence of biopsy proven acute rejection episodes (15.9% vs. 19.7%) within 6 month period selected for the primary efficacy analysis. Similarly, serum creatinine level at 1 year was 1.42 ± 0.07 mg/dL in the MMF 3 g group *vs.* 1.64 ± 0.07 mg/dL in MMF 2 g group.[12] In the European mycophenolate mofetil study same trends regarding higher MMF dose were observed.[11] As mentioned before, in these studies there was no antibody induction that could have allowed lower dose of cyclosporin with higher dose of MMF and there were no protocol biopsies. In a more recent MYSS trial, there was no difference in acute rejection rate and renal function between MMF and azathioprine in a cyclosporine-based protocol.[19] However in that study only one MMF dose was compared to azathioprine[23] and again there were no protocol biopsies.

Unfortunately adequate prospective MMF dose comparison studies in tacrolimus-based protocols with antibody induction are missing. In the Symphony study it was reported that patients on tacrolimus-MMF-prednisone maintenance imunosuppression after kidney transplantation had better kidney function and graft survival with lower number of acute rejection episodes. Patients in that group had highest MMF exposure.[24] Protocols with even higher MMF exposure might allow additional CNI sparing, that would decrease side effect of CNI (hypertension, diabetes, hyperlipidemia, neurotoxicity).[25]

Clinical relevance of IF/TA without other concomitant pathology (i.e. recurrent disease and chronic antibody-mediated rejection) for prediction of graft deterioration and loss is controversial. In El-Zoghby et al. study there was attempt to identify specific causes of late kidney allograft failure. The authors found that transplant glomerulopathy was responsible for 37 percent loss of functioning grafts, while graft loss due to IF/TA was present in 31 percent of cases (with higher frequency in deceased-donor transplants).[26] At first glance, these results seem at odd with ours, where there were no signs of chronic antibody-mediated rejection. An explanation for this discrepancy in the results of the two studies is not completely clear, but the former study included high number of living transplants (72.5 percent) with glomerulonephritis as primary disease and with follow-up up to 10 years. Transplant glomerulopathy is more frequently seen late posttransplant, generally with low incidence. Nevertheless, ours and El-Zoghby study, both demonstrated that IF/TA even in absence of other pathology is associated with adverse graft outcome. Another important study, the DeKaf study, tried to use various histopathologic clusters to differentiate subgroups within diagnosis of IF/TA. They found that cluster with more severe fibrosis plus inflammation and arterial lesions had the worst prognosis.[27] Although incidence of acute rejection in our study did not vary with MMF exposure, increased MMF exposure might suppress mild graft inflammation, below the threshold for diagnosing acute rejection. This is subject of our ongoing investigation and will be reported separately. An interesting finding of the present study was that early steroid withdrawal was not associated with worse IF/TA. At first glance this is at odd with the Astellas trial.[23] However, according to our protocol, patients with DGF were not included in early steroid withdrawal and Astellas trial, which did not have protocol

biopsies, reported increased IF/TA in early steroid withdrawal group based on indication biopsies performed early posttransplant, thus more likely reflecting donor-derived histology changes, rather than effect of steroid withdrawal.[28]

In our study there was only borderline significance of positive association of 1-year eCr_{cl} with MMF in univariate analysis. This result is not very surprising since decreased renal function is not a very sensitive marker of incipient IF/TA.

Mechanisms by which an average higher exposure to MMF was associated with slower progression of IF/TA may be both immune and nonimmune. Because there was no difference in incidence of acute rejection with respect to increased MMF exposure in our study, we believe that there may be a significant contribution of nonimmune mechanisms in retardation of IF/TA in patients with higher MMF. In line with this, in many experimental models it has been shown that MMF has antiproliferative and antifibrotic effect.[29-31] In the study of Jiang at al. using rat renal ischemia reperfusion injury, a time- and dose-dependent correlation of higher MMF dose with better renal function and lower interstitial fibrosis was demonstrated. Suggested potential mechanism was lower expression of TGF-β1 and MCP-1with lower macrophage infiltration.[32] In recent clinical trials MMF was shown as a safe drug that could be a good candidate for treatment of interstitial lung disease in systemic sclerosis.[33] Experimental model of encapsulated peritoneal sclerosis in rats proved beneficial effect of MMF as an inhibitor of neovascularisation.[34] Also, MMF monotherapy was associated with a positive effect on hepatic fibrosis progression in HCV liver transplant recipients.[35]

Our study has several shortcomings, such as its retrospective aspect and relatively short study period. Although study period was limited to 12 months post transplantation, a

clear correlation of slower progression of IF/TA with higher average MMF dose underlines potential benefit of these findings. As mentioned before, in current study we did not analyze inflammation outside Banff acute rejection threshold in kidney biopsies with respect to MMF dose. As inflammation in areas of IF/TA is an important predictor of renal function and graft loss, this is subject of an ongoing work.

In summary, higher MMF dose after kidney transplantation might slower progression of IF/TA, which might lead to better long-term survival of transplanted kidney. Our study ~~may~~ serves as a platform for a prospective, randomized, long-term trial with different MMF doses to evaluate benefit of higher MMF dose in renal transplant recipients (NCT018600183).~~.~~

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Figure 1: Estimated creatinine clearance during first year posttransplant

Figure 2A: Correlation between average MMF dose and progression of ci score

Figure 2B: Correlation between average MMF dose and progression of ct score

Figure 3A: Estimated creatinine clearance by ci score

Figure 3B: Estimated creatinine clearance by ct score

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Table 1. Baseline characteristics

RECIPIENT CHARACTERISTICS	AGE (years)	44.67 ± 12.03
	GENDER (f/m)	25/54
	PRIMARY RENAL DISEASE (diabetes mellitus, polycistic kidney disease, glomerulonephritis, pyelonephritis/interstitial nephritis, other/unknown)	24/8/19/6/22
DONOR CHARACTERISTICS	DONOR SOURCE (decased/living)	55/24
	AGE (years)	43.89 ± 15.55
	GENDER (f/m)	36/43
TRANSPLANTATION CHARACTERISTICS	TRANSPLANTED ORGAN (KIDNEY/SPKT)	58/21
	INITIAL IMMUNOSUPRESSION (anti-IL2,TAC,MMF/anti-IL2, CyA,MMF)	53/26
	DELAYED GRAFT FUNCTION (no/yes)	53/26
	STEROID FREE (yes/no)	36/43
	HLA MM	3.33 ± 1.51

Table 2. eCr_{cl}, MMF dose and CNI concentration during first year post transplant

	Month posttransplant			
	1	3	6	12
eCr_{cl} (ml/min)		56.98 ± 15.79	58.94 ± 16.94	61.47 ± 16.75
MMF dose (mg)	2500 (750 – 4000) 2427 ± 643.17	2000 (750 – 4000) 2167.72 ± 733.49	2000 (1000-4000) 2188.29 ± 716.91	2000 (1000-4000) 2193.04 ± 642.95
Tacrolimus conc. (µg/L) (n=53)	10.79 ± 4.16	9.69 ± 3.00	9.03 ± 5.52	7.83 ± 2.45
Cyclosporin conc. (µg/L) (n=26)	335.07 (274 – 413)	231.05 (181-265)	206 (170 – 257)	131 (125 – 171)

Table 3. Association of variables with eCr_{cl} on 1 year

	Estimated creatinine clearance (ml/min)	p value
Kidney *vs.* SPKT	57.88 ± 15.47 *vs*. 71.38 ± 13.45	0.001
DGF (yes *vs.* no)	56.15 ± 17.55 *vs*. 64.08 ± 15.87	0.05
Recipient gender (m *vs.* f)	59.83 ± 16.02 *vs*. 65 ± 18.07	0.2
Donor gender (m *vs*. f)	63.87 ± 16.71 *vs*. 58.60 ± 16.58	0.17
Donor source (D *vs*. L)	62.36 ± 17.85 *vs*. 59.43 ± 14.05	0.47
Steroid-free (yes *vs*. no)	63.94 ± 17.73 *vs*. 59.39 ± 15.81	0.23
Acute rejection (yes *vs*. no)	61.64 ± 16.59 *vs*. 61.39 ± 16.97	0.95
	R	p value
Recipient age	-0.45	<0.001
Donor age	-0.46	<0.001
HLA MM	0.07	0.52
Average tacrolimus concentration	-0.02	0.9
Average MMF dose	0.18	0.1
ci at 1 year post Tx	-0.34	0.002
ct at 1 year post Tx	-0.35	0.002
cv at 1 year post Tx	-0.20	0.07

Table 4. Multiple regression analysis of factors associated with kidney function

	Beta (β)	St.Err. β	p value
Tx (kidney)	-0.17	0.13	0.19
DGF (no)	0.04	0.1	0.71
Recipient age	-0.41	0.1	<0.001
Donor age	-0.1	0.14	0.45
ci at 12 months	-0.18	0.11	0.09
Average MMF dose	0.21	0.1	0.04

Table 5. One-year progression of chronic allograft scores

Banff score	N	At transplantation	N	12 month	p
Interstitial fibrosis (ci)	79	0.16 ± 0.44	79	0.94 ± 0.85	<0.001
Tubular atrophy (ct)	79	0.24 ± 0.46	79	1.05 ± 0.77	<0.001
Chronic glomerulopathy (cg)	79	0	79	0	
Mesangial matrix (mm)	79	0.01 ± 0.11	79	0.09 ± 0.36	0.09
Fibrointimal thickening (cv)	76	0.37 ± 0.83	78	0.29 ± 0.70	0.47
Arteriolar hyalinosis (ah)	78	0.68 ± 1,04	79	0.79 ± 1.04	0.26

Table 6. Correlation of factors associated with progression of ci and ct scores

	Δci		Δct	
	mean ± SD	p	mean ± SD	p
Kidney *vs.* SPKT	0.86 ± 0.91 *vs*. 0.67 ± 0.73	0.51	0.85 ± 0.87 vs. 0.86 ± 0.65	0.74
DGF (yes *vs.* no)	1.19 ± 0.98 *vs*. 0.62 ± 0.74	0.02	1.15 ± 0.92 vs. 0.69 ± 0.72	0.05
Recipient gender (m *vs.* f)	0.83 ± 0.88 *vs*. 0.76 ± 0.83	0.78	0.91 ± 0.83 vs. 0.72 ± 0.79	0.35
Donor gender (m *vs*. f)	0.91 ± 0.95 *vs*. 0.69 ± 0.75	0.43	0.88 ± 0.93 vs. 0.81 ± 0.67	0.96
Donor source (D *vs*. L)	0.84 ± 0.88 *vs*. 0.75 ± 0.85	0.73	0.87 ± 0.82 vs. 0.79 ± 0.83	0.71
Steroid free (yes *vs*. no)	1.09 ± 0.87 *vs*. 0.47 ± 0.74	0.002	1.07 ± 0.83 vs. 0.58 ± 0.73	0.01
Acute rejection (yes *vs*. no)	0.8 ± 0.89 *vs*. 0.83 ± 0.82	0.78	0.93 ± 0.84 vs. 0.67 ± 0.76	0.23
	R	p	R	p
Recipient age	-0.11	0.33	-0.11	0.32
Donor age	0.17	0.13	0.04	0.73
HLA MM	-0.09	0.43	-0.002	0.99
Average tacrolimus conc.	-0.009	0.95	0.003	0.98
Average MMF dose	-0.37	<0.001	-0.38	<0.001
ci at implantation	-0.32	0.003		
ct at implantation			-0.45	<0.001

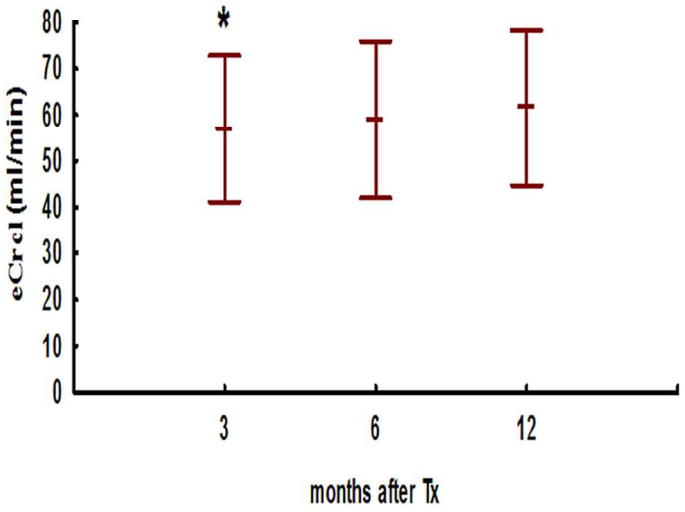
Table 7. Multivariate general regression analysis for factors related to progression of ci and ct score

Δci			
	Beta (β)	Std. Err. β	p
ci0	-0.43	0.09	<0.001
DGF (no)	-0.22	0.11	<0.05
Average MMF dose	-0.20	0.09	<0.05
Donor age	0.32	0.09	<0.05
Steroid free (yes)	-0.25	0.11	0.02
Δct			
	Beta (β)	Std. Err. β	p
ct0	-0.44	0.09	<0.001
Average MMF dose	-0.29	0.1	<0.05
DGF (no)	-0.29	0.1	<0.05
Steroid free (yes)	-0.09	0.11	0.39

Table 8. Adverse events with respect to 1 year average median MMF dose.

	MMF dose < median	**MMF dose > median**	**p**
Average number of infection episodes per patient	1.16 ± 0.97	1.23 ± 1.22	0.88
Mean time to first infection (days)	157±138	175±143	0.76
Proportion of patients with leucopenia	6 /31	7 /48	0.58

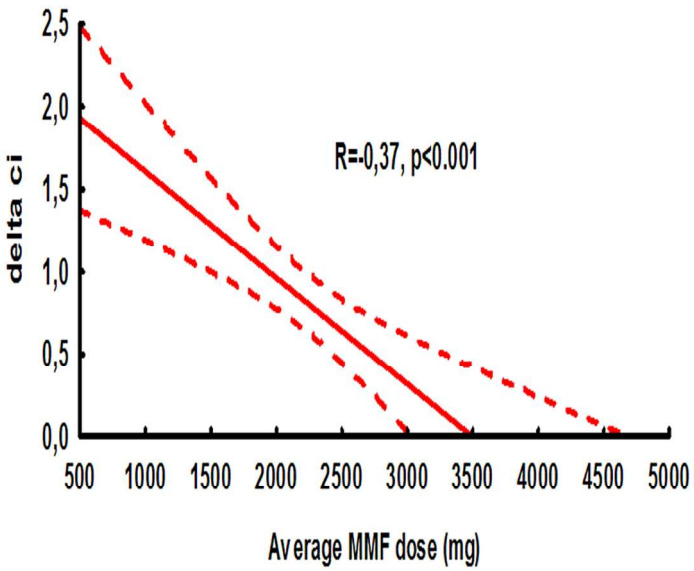
Figure 1: Estimated creatinine clearance during first year posttransplant



*, p<0.001; 3 month *vs.* 12 months eCr_{cl}. Values are shown as mean ± SD

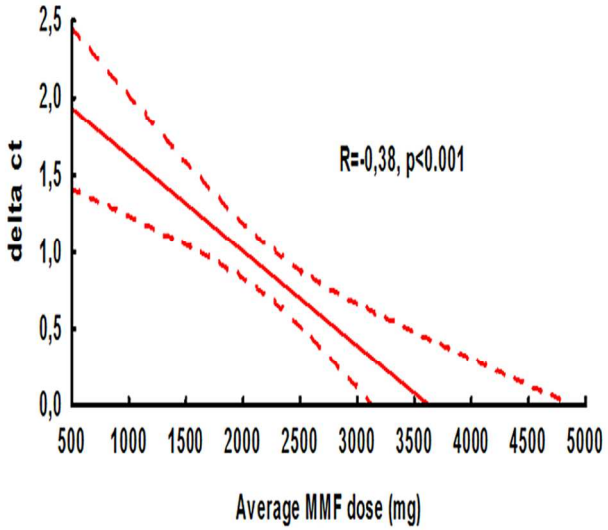
90x105mm (300 x 300 DPI)

Figure 2A: Correlation between average MMF dose and progression of ci score



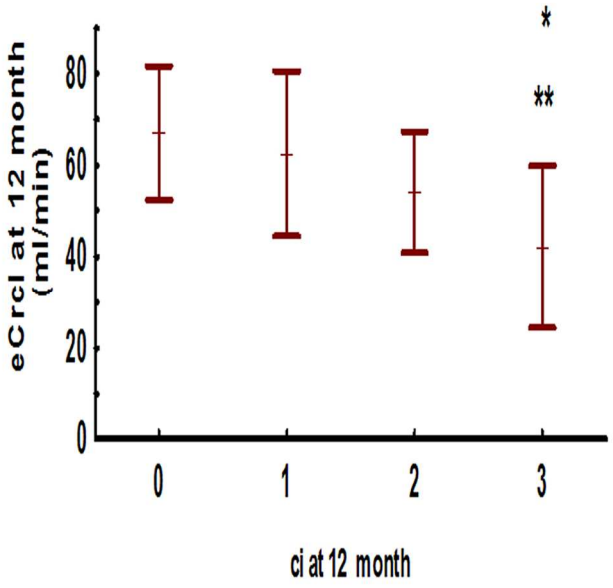
90x121mm (300 x 300 DPI)

Figure 2B: Correlation between average MMF dose and progression of ct score



90x121mm (300 x 300 DPI)

Figure 3A: Estimated creatinine clearance by ci score



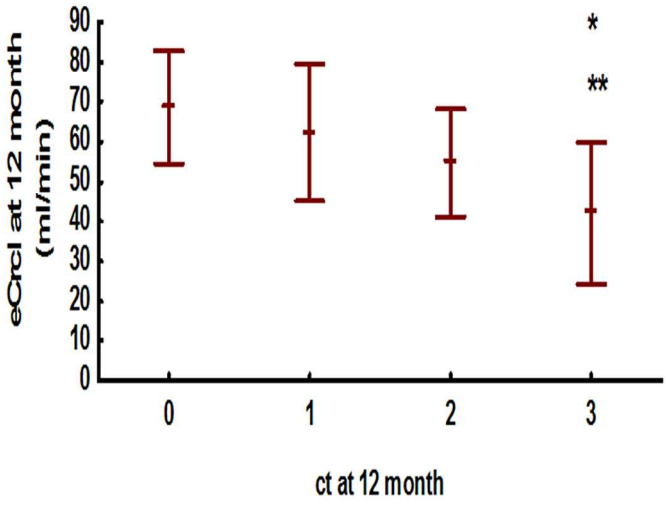
* p<0.05; score ci 0 *vs.* score ci 3 (*post hoc* Newman-Keuls test)

** p=0.03; score ci 1 *vs.* score ci 3

Values are shown as mean ± SD

90x121mm (300 x 300 DPI)

Figure 3B: Estimated creatinine clearance by ct score



* p<0.05; score ct 0 *vs.* score ct 3 (post hoc Newman-Keuls test)

** p=0.03; score ct 1 *vs.* score ct 3

Values are shown as mean ± SD

90x121mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No/page number	Recommendation
Title and abstract	1/1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2/5	Explain the scientific background and rationale for the investigation being reported
Objectives	3/5,6	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4/6	Present key elements of study design early in the paper
Setting	5/6,7, 8,9	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6/6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7/6,7, 8	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*/8	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10/6	Explain how the study size was arrived at
Quantitative variables	11/9	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12/9	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*/9, 10	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*/9, 10	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*/11	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16/10, 11	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18/12	Summarise key results with reference to study objectives
Limitations	19/16	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20/12-16	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21/12-16	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22/2	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

BMJ Open

Effect of Mycophenolate Mofetil on Progression of Interstitial Fibrosis and Tubular Atrophy after Kidney Transplantation- A Retrospective Study

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1

Effect of Mycophenolate Mofetil on Progression of Interstitial Fibrosis and Tubular Atrophy after Kidney Transplantation - A Retrospective Study

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ABSTRACT:

Objectives - Chronic transplant dysfunction after kidney transplantation is major reason of kidney graft loss and is caused by immunological and non-immunological factors. There is evidence that mycophenolate mofetil (MMF) may exert a positive effect on renal damage in addition to immunosuppression, by its direct antifibrotic properties. The aim of our study was to retrospectively investigate role of MMF dose on progression of chronic allograft dysfunction and IF/TA.

Setting - Retrospective, cohort study.

Participants - Kidney transplant patients in tertiary care institution. This is a retrospective cohort study that included 79 patients with kidney and kidney-pancreas transplantation. Immunosuppression consisted of anti-IL2 antibody induction, MMF, a calcineurin inhibitor ± steroids.

Primary outcome measures - An association of average MMF dose over 1 year post transplant with progression of interstitial fibrosis (Δci), tubular atrophy (Δct) and estimated creatinine clearance (eCr_{cl}) at 1 year post transplant was evaluated using univariate and multivariate analyses.

Results - Higher average MMF dose was significantly independently associated with better eCr_{cl} at 1 year post transplant (b=0.21 ± 0.1, p=0.04). In multiple regression analysis lower Δci (b=-0.2 ± 0.09, p=0.05) and Δct (b=-0.29 ± 0.1, p=0.02) were independently associated with greater average MMF dose. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Conclusions - Higher average MMF dose over 1 year is associated with better renal function and slower progression of IF/TA, at least partly independent of its immunosuppressive effects.

Strenghts and limitations of this study

Important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF (up to 4 g daily) during 1 year post transplantation had significantly lower progression of graft interstitial fibrosis and tubular atrophy. This is important finding, because of predictive value of graft IF/TA and should translate into better long-term graft survival. Our study has several shortcomings, such as its retrospective aspect and relatively short study period. As it was not aim of the study, we did not report side effects associated with different dosage of MMF.

INTRODUCTION:

Kidney transplantation significantly improves patient survival and quality of life comparing to dialysis. While significant improvements have been made in the treatment of acute rejection and short survival of transplanted kidney, there has not been major improvement in the long-term survival of transplanted kidney.[1] Chronic transplant dysfunction after kidney transplantation is major cause of kidney graft loss and is evoked by immunological and non-immunological factors.[2, 3] Histology changes that determine chronic transplant dysfunction are interstitial fibrosis and tubular atrophy

(IF/TA), arteriosclerosis, arteriolar hyalinosis, glomerulopathy and mesangial matrix expansion.[4] IF/TA is the major pathohystology finding that can be verified on graft biopsies after kidney transplantation and is a predictor of long-term allograft function.[4] Clinical factors that affect progression of IF/TA are: recipient age, HLA mismatch, episodes of severe acute rejection, chronic rejection (esp. antibody-mediated), use of calcineurin inhibitors and BK nephropathy. Avoidance of CNI toxicity is considered as an important step to slow progression of IF/TA.[4-7] Mycophenolate mofetil (MMF) may help lowering CNI toxicity, by allowing lower CNI exposure.[7]

MMF reduces the risk of acute allograft rejection, without nephrotoxic side effects and is ideal candidate for long-term calcineurin drug reduction treatment strategies.[7] Retrospective studies of renal recipients who were treated with mycophenolate mofetil comparing azathioprin showed that MMF treated patients had significantly less chronic allograft dysfunction.[8, 9] Besides being associated with lower acute rejection rates as compared to azathioprin,[10, 11] evidence from animal and human studies suggests that MMF may also exert a direct antifibrotic properties due to its antiproliferative action on nonimmune cells, including renal tubular cells and vascular smooth muscle cells.[12, 13] The aim of our study was to investigate role of mycophenolate mofetil dose on progression of IF/TA in kidney transplant recipients.

PATIENTS AND METHODS:

Patients:

This is a retrospective study conducted at Clinical Hospital “Merkur”. This study represents a part of the posttransplant immune monitoring at the Merkur hospital, approved by the Hospital Ethics Committee. Patients gave there informed written consent for anonymized transplant data collection for research purposes. The study included 79 patients

with kidney and kidney-pancreas transplantation, transplanted between 2003 and 2011. Eligible patients had to have protocol kidney biopsy at the time of implantation and 12 months after transplantation. Exclusion criteria have been: dual kidney transplantation, kidney-liver transplantation, use of antithymocyte immunoglobulin, BK nephropathy and recurrence of glomerulonephritis after transplantation.

Immunosuppression:

Induction immunosupression consisted of an anti-IL2 antibody (daclizumab or basiliximab), calcineurin inhibitor (tacrolimus or cyclosporine), MMF and methylprednisolone. Maintenance immunosuppression consisted of a calcineurin inhibitor (tacrolimus or cyclosporine), MMF ± steroids. Target cyclosporine trough concentrations were 250-350 during first month posttransplant, 200-300 during second to 6th month and 100-150 µg/L thereafter. Target tacrolimus trough levels were 10-12 during first month, 8-10 during second to 6th month and 5-8 µg/L thereafter. Mycophenolic acid target trough concentration was aimed to be higher than 7.2 µmol/L with tacrolimus and higher than 5 µmol/L with cyclosporine use.

Daclizumab was administered at day 0: 2mg/kg i.v. before opening of vascular anastomosis and at day 14: 2mg/kg i.v.. Basiliximab was administered at day 0: 20 mg i.v. before opening of vascular anastomosis and at day 4: 20 mg i.v..

Steroids have been dosed as follows: day 0: intraoperatively 500 mg of methylprednisolone, day 1: 250 mg, day 2: 125mg, day 3: 80 mg and day 4: 40 mg. In patients with early steroid withdrawal steroids have been withdrawn at day 5 after transplantation. In patients maintained on steroids, nadir dose of prednisone was 5 mg/d,

achieved by 6 months. The criteria for early elimination of steroids were low immunological risk of the recipient (absence of, or low degree of HLA sensitization, i.e. PRA <10%) and good immediate renal function, as well as absence of an episode of acute rejection within 5 days after the transplantation. Steroids have been reintroduced in patients who suffered acute rejection episode.

As prophylaxis for viral (HSV, CMV), fungal (Candida spp.) urinary and P. jiroveci infections, low-dose fluconazole (for one year), valganciclovir (universally for three months) and sulfomethoxazol and trimethoprim (for one year) was used.

Renal allograft biopsies:

Protocol kidney biopsies were done at implantation, 1, 3, 6 and 12 months after transplantation. For cause biopsies were done in case of unexplained deterioration of renal function, or once weekly in patients with DGF. All rejection episodes were histologically confirmed. Histopathological analysis was performed by either of two pathologists who were blinded for immunosuppression. Acute rejections and chronic allograft scores have been analyzed using Banff 97 classification and its updates.[14, 15] All protocol and indication biopsies were analyzed by light microscopy, by immunofluorescence for C4d, and if indicated by immunohistochemistry for BK virus. Biopsies at 1 year post transplant have been also analyzed by electron microscopy for signs of chronic antibody-mediated rejection (transplant glomerulopathy, peritubular capillary basement membrane multilayering).[16]

Clinical outcome parameters:

Progression of chronic allograft scores during 1 year posttransplant was calculated by subtracting implantation chronic scores from chronic allograft scores 12 months posttransplant: interstitial fibrosis (Δci), tubular atrophy (Δct), glomerulosclerosis (Δcg), mesangial matrix increase (Δmm), vasculopathy (Δcv) and arteriolar hyalinosis (Δah). Estimated creatinine clearance (eCr_{cl}) at 3, 6 and 12 months posttransplant was calculated using Cockroft-Gault formula. Acute rejections with Banff grade IA and IB were treated with three 500 mg methylprednisolone pulses. In case of acute rejection grade IIA or greater, patients have been treated with antithymocyte globulin. Antibody-mediated rejections were treated with steroid pulse and plasmapheresis.

Average dose of MMF during 1 year posttransplant was calculated from MMF dose at month 1, 3, 6 and 12.

Adverse effects analysed were clinicaly significant leucopenia, defined as white blood cell count less than 3000/ml, time to first symptomatic infection and number of symptomatic infection episodes per patient during first post transplant year.

Statistical analysis:

Numerical data are presented as mean ± SD or median with range in case of not normal distribution. Normality of distribution has been tested with Kolmogorov-Smirnov test. Correlation between two continuous variables has been tested using Spearman nonparametric correlation. Difference between two groups in continuous variables has been tested with student t-test or with Mann–Whitney test in non-normally distributed variables. The significance of the progression in chronic scores was analyzed using Wilcoxon Matched Pairs test. Univariate and multiple linear regression analysis were performed to determine predictive factors for progression of chronic allograft scores and kidney function at 12 months after transplantation. All variables that were associated with respective outcome in bivariate analysis (at p= 0.1) were included in multivariate

analysis. Because of colinearity between ci and ct score, only one score was included in each multivariate analysis. Statistical significance was considered at $p<0.05$. All statistical analyses were performed using Statistica 10 (StatSoft, Tulsa, OK, USA).

RESULTS:

Patient and transplant characteristics:

Patient characteristics are shown in Table 1. Recipients were a mean of 44.67 ± 12.03 years old at the time of transplantation, 68 percent of them were male and all were Caucasians. 33 percent of recipients had DGF after transplantation. Donors were a mean of 43.89 ± 15.55 years old and 54 percent of them were male. Number of living donor transplantations was 24 (30 percent). Average daily MMF dose during 1 year posttransplant was 2244 ± 585 mg (1062 – 4000) (Table 2). As expected, there was no correlation of MMF dose with MMF trough concentration (R=-0.13; p=0.28). Also, there was no correlation between MMF dose with tacrolimus concentration (R=-0.04; p=0.79). Early steroid withdrawal was done in 46 percent of patients after transplantation. Incidence of subclinical and clinical acute rejections greater then borderline was 30 percent in first year. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Factors associating with eCr_{cl}:

Kidney function increased during 1st year post transplant. eCr_{cl} at month 3 was 56.98 ± 15.78 ml/min, at 6 month 58.94 ± 16.94 ml/min and at 12 month 61.47 ± 16.75 ml/min ($p<0.001$; 12 months *vs*. 3 months) (Figure 1.) eCr_{cl} at 1 year post transplant was greater

in SPKT recipients (71.38 ± 13.45 ml/min *vs.* 57.88 ± 16.47 ml/min; p=0.001) and in patients who did not have DGF (64.08 ± 15.87 ml/min *vs*. 56.15 ± 17.55 ml/min; p=0.05). Donor age (R=-0.46; p<0.001) and recipient age (R=-0.46; p<0.001) negatively correlated with eCr_{cl} at 1 year post transplant, while there was no correlation of renal function with donor and recipient gender, type of donation (deceased *vs.* living), HLA MM, average CNI concentration, steroid-free regimen of immunosuppression, or history of acute rejection (Table 3). In univariate analysis allograft function at 12 month post Tx was also negatively correlated with ci (R=-0.34; p=0.002) and ct (R=-0.35; p=0.002) at 12 month (Figure 2A, Figure 2B). Although MMF dose was positively correlated with renal function with borderline significance in univariate analysis, in multivariate analysis there was a significant positive association between greater average MMF dose and better eCr_{cl} at 12 month post transplant (b=0.21 ± 0.1; p=0.04) (Table 4).

Factors affecting IF/TA:

The average ci score increased from 0.16 ± 0.44 to 0.94 ± 0.86 between implantation and month 12 (p<0.001). Average progression of this and other chronic scores during 1 year post transplant is shown in Table 5. In univariate analysis Δci (R=-0.37; p=0.001) and Δct (R=-0.38; p=0.001) significantly negatively correlated with average MMF dose (Figure 3A and 3B, Table 6). There was lower progression of ci score in patients on steroid-free immunosuppression (0.47 ± 0.7 *vs*. 1.09 ± 0.87; p=0.002) and in those who did not have DGF (0.62 ± 0.74 *vs* 1.19 ± 0.98; p=0.02). Acute cellular rejection, recipient and donor gender, recipient and donor age, HLA MM, deceased *vs.* living donor, as well as average concentration of tacrolimus had no significant effect on progression of chronic allograft scores. Higher average MMF dose was associated with lower progression of ci and ct score regardless CNI type (data not shown). Factors that remained significantly associated with progression of ci score in multivariate analysis were ci0 score, donor age,

average MMF dose, DGF and steroid-free immunosuppression (Table 7.). In multivariate analysis only ct0 score, average MMF dose and DGF remained independently associated with 12-month progression of ct score (Table 7.). Selected AE are shown in Table 8. There was no difference in AE (leucopenia and infections) with respect to average median MMF dose.

Discussion:

The most important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF during 1 year post transplantation had significantly lower progression of ci and ct scores. To our knowledge this is first study demonstrating that there is a dose-dependent protective effect of MMF on graft IF/TA. Lower progression of IF/TA could not be explained with lower concentration of CNI, because there was not correlation between tacrolimus concentration with IF/TA. Similarly, there was no correlation between average MMF dose and tacrolimus (R=-0.04; p=0.79) or cyclosporine concentration (R=-0.07, p=0.79). In addition, higher average MMF dose was not associated with decreased incidence of biopsy proven acute rejection, which suggests that antifibrotic properties of higher MMF dose was at least partly independent of its immunosuppressive effects. Higher MMF dose had only moderate effect on 1-year renal function, which is consistent with previous reports showing that transplanted kidneys undergo pathohystology changes without significant early change in kidney function.[17]

In the present retrospective study we have confirmed that IF/TA progression occurs in first year after kidney transplantation. Several studies have shown that progression of IF/TA is correlated with type of immunosuppression.[18] In most transplant centers in

the United States and Europe immunosuppression consists of induction with an anti-IL2R antibody or antithymocyte immunoglobulin and maintenance with a calcineurin inhibitor, MMF and steroids.[19] Studies have reported significant improvement in kidney function in patients on MMF with lower exposition to CNIs, esp. tacrolimus.[20] Recently, in the paper of Kamar e*t al*. it has been reported that maintenance kidney transplant patients converted to a higher dose of the mycophenolate sodium (1440 mg daily) with lower tacrolimus concentration had borderline higher eCr_{cl} on month 6 *vs*. those treated with lower dose of mycophenolate sodium, with usual tacrolimus concentration (eCr_{cl} 49.1 ± 11.1 *vs*. 44.7 ± 11.5 ml/min; p=0.07).[21] Although there was only borderline significance, increased mycophenolate dosing with lower tacrolimus concentration was safe with potential benefit on kidney function.

Our study also corroborates recently published findings of a *post hoc* joint analysis of the Symphony, FDCC and OptiCept trials, where a a lower tacrolimus level and a higher MMF dose were associated with significantly better kidney function at 1 year post transplant.[22] Shortcoming of these studies[17,18] is lack of protocol biopsies. The optimal MMF dosing in patients maintained on contemporary low-dose CNI is still undetermined. However, some results of early MMF registration trials suggest that higher MMF exposure might be beneficial; having in mind that there was no antibody induction in these studies and that CNI was standard dose cyclosporine. Thus, in the Tri-continental study, group treated with 3 g MMF compared with 2 g of MMF showed lower incidence of biopsy proven acute rejection episodes (15.9% vs. 19.7%) within 6 month period selected for the primary efficacy analysis. Similarly, serum creatinine level at 1 year was 1.42 ± 0.07 mg/dL in the MMF 3 g group *vs.* 1.64 ± 0.07 mg/dL in MMF 2 g group.[12] In the European mycophenolate mofetil study same trends regarding higher MMF dose were observed.[11] As mentioned before, in these studies there was no antibody induction that could have allowed lower dose of cyclosporin with higher dose of MMF

and there were no protocol biopsies. In a more recent MYSS trial, there was no difference in acute rejection rate and renal function between MMF and azathioprine in a cyclosporine-based protocol.[19] However in that study only one MMF dose was compared to azathioprine[23] and again there were no protocol biopsies.

Unfortunately adequate prospective MMF dose comparison studies in tacrolimus-based protocols with antibody induction are missing. In the Symphony study it was reported that patients on tacrolimus-MMF-prednisone maintenance imunosuppression after kidney transplantation had better kidney function and graft survival with lower number of acute rejection episodes. Patients in that group had highest MMF exposure.[24] Protocols with even higher MMF exposure might allow additional CNI sparing, that would decrease side effect of CNI (hypertension, diabetes, hyperlipidemia, neurotoxicity).[25]

Clinical relevance of IF/TA without other concomitant pathology (i.e. recurrent disease and chronic antibody-mediated rejection) for prediction of graft deterioration and loss is controversial. In El-Zoghby et al. study there was attempt to identify specific causes of late kidney allograft failure. The authors found that transplant glomerulopathy was responsible for 37 percent loss of functioning grafts, while graft loss due to IF/TA was present in 31 percent of cases (with higher frequency in deceased-donor transplants).[26] At first glance, these results seem at odd with ours, where there were no signs of chronic antibody-mediated rejection. An explanation for this discrepancy in the results of the two studies is not completely clear, but the former study included high number of living transplants (72.5 percent) with glomerulonephritis as primary disease and with follow-up up to 10 years. Transplant glomerulopathy is more frequently seen late posttransplant, generally with low incidence. Nevertheless, ours and El-Zoghby study, both demonstrated that IF/TA even in absence of other pathology is associated with adverse graft outcome. Another important study, the DeKaf study, tried to use various histopathologic clusters to differentiate subgroups within diagnosis of IF/TA. They found

that cluster with more severe fibrosis plus inflammation and arterial lesions had the worst prognosis.[27] Although incidence of acute rejection in our study did not vary with MMF exposure, increased MMF exposure might suppress mild graft inflammation, below the threshold for diagnosing acute rejection. This is subject of our ongoing investigation and will be reported separately. An interesting finding of the present study was that early steroid withdrawal was not associated with worse IF/TA. At first glance this is at odd with the Astellas trial.[23] However, according to our protocol, patients with DGF were not included in early steroid withdrawal and Astellas trial, which did not have protocol biopsies, reported increased IF/TA in early steroid withdrawal group based on indication biopsies performed early posttransplant, thus more likely reflecting donor-derived histology changes, rather than effect of steroid withdrawal.[28]

In our study there was only borderline significance of positive association of 1-year eCr_{cl} with MMF in univariate analysis. This result is not very surprising since decreased renal function is not a very sensitive marker of incipient IF/TA.

Mechanisms by which an average higher exposure to MMF was associated with slower progression of IF/TA may be both immune and nonimmune. Because there was no difference in incidence of acute rejection with respect to increased MMF exposure in our study, we believe that there may be a significant contribution of nonimmune mechanisms in retardation of IF/TA in patients with higher MMF. In line with this, in many experimental models it has been shown that MMF has antiproliferative and antifibrotic effect.[29-31] In the study of Jiang at al. using rat renal ischemia reperfusion injury, a time- and dose-dependent correlation of higher MMF dose with better renal function and lower interstitial fibrosis was demonstrated. Suggested potential mechanism was lower expression of TGF-β1 and MCP-1with lower macrophage infiltration.[32] In recent clinical trials MMF was shown as a safe drug that could be a good candidate for treatment of interstitial lung disease in systemic sclerosis.[33] Experimental model of

encapsulated peritoneal sclerosis in rats proved beneficial effect of MMF as an inhibitor of neovascularisation.[34] Also, MMF monotherapy was associated with a positive effect on hepatic fibrosis progression in HCV liver transplant recipients.[35]

Our study has several shortcomings, such as its retrospective aspect and relatively short study period. Although study period was limited to 12 months post transplantation, a clear correlation of slower progression of IF/TA with higher average MMF dose underlines potential benefit of these findings. As mentioned before, in current study we did not analyze inflammation outside Banff acute rejection threshold in kidney biopsies with respect to MMF dose. As inflammation in areas of IF/TA is an important predictor of renal function and graft loss, this is subject of an ongoing work.

In summary, higher MMF dose after kidney transplantation might slower progression of IF/TA, which might lead to better long-term survival of transplanted kidney. Our study serves as a platform for a prospective, randomized, long-term trial with different MMF doses to evaluate benefit of higher MMF dose in renal transplant recipients. (NCT018600183).

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Contributorship Statement

Karlo Mihovilović – participated in research design, collecting data, analyzing data and wrote the paper, karlomihovilovic@gmail.com

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Abbreviations:

IF/TA - interstitial fibrosis and tubular atrophy

MMF - mycophenolate mofetil

BK - polyoma virus BK

CNI - calcineurin inhibitors

DGF - delayed graft function

eCrCl - estimated creatine clearance

SPKT - simultaneous pancreas kidney transplantation

HLA MM – human leukocyte antigen mismatch

AE - adverse events

Figure Legends

Figure 1: Estimated creatinine clearance during first year posttransplant

Figure 2A: Correlation between average MMF dose and progression of ci score

Figure 2B: Correlation between average MMF dose and progression of ct score

Figure 3A: Estimated creatinine clearance by ci score

Figure 3B: Estimated creatinine clearance by ct score

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Table 1. Baseline characteristics

RECIPIENT CHARACTERISTICS	AGE (years)	44.67 ± 12.03
	GENDER (f/m)	25/54
	PRIMARY RENAL DISEASE (diabetes mellitus, polycistic kidney disease, glomerulonephritis, pyelonephritis/interstitial nephritis, other/unknown)	24/8/19/6/22
DONOR CHARACTERISTICS	DONOR SOURCE (deceased/living)	55/24
	AGE (years)	43.89 ± 15.55
	GENDER (f/m)	36/43
TRANSPLANTATION CHARACTERISTICS	TRANSPLANTED ORGAN (KIDNEY/SPKT)	58/21
	INITIAL IMMUNOSUPRESSION (anti-IL2,TAC,MMF/anti-IL2, CyA,MMF)	53/26
	DELAYED GRAFT FUNCTION (no/yes)	53/26
	STEROID FREE (yes/no)	36/43
	HLA MM	3.33 ± 1.51

Table 2. eCr_{cl}, MMF dose and CNI concentration during first year post transplant

Month posttransplant				
	1	3	6	12
eCr_{cl} (ml/min)		56.98 ± 15.79	58.94 ± 16.94	61.47 ± 16.75
MMF dose (mg)	2500 (750 – 4000) 2427 ± 643.17	2000 (750 – 4000) 2167.72 ± 733.49	2000 (1000-4000) 2188.29 ± 716.91	2000 (1000-4000) 2193.04 ± 642.95
Tacrolimus conc. (µg/L) (n=53)	10.79 ± 4.16	9.69 ± 3.00	9.03 ± 5.52	7.83 ± 2.45
Cyclosporin conc. (µg/L) (n=26)	335.07 (274 – 413)	231.05 (181-265)	206 (170 – 257)	131 (125 – 171)

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Table 3. Association of variables with eCr_{Cl} on 1 year

	Estimated creatinine clearance (ml/min)	p value
Kidney *vs.* SPKT	57.88 ± 15.47 *vs*. 71.38 ± 13.45	0.001
DGF (yes *vs*. no)	56.15 ± 17.55 *vs*. 64.08 ± 15.87	0.05
Recipient gender (m *vs*. f)	59.83 ± 16.02 *vs*. 65 ± 18.07	0.2
Donor gender (m *vs*. f)	63.87 ± 16.71 *vs*. 58.60 ± 16.58	0.17
Donor source (D *vs*. L)	62.36 ± 17.85 *vs*. 59.43 ± 14.05	0.47
Steroid-free (yes *vs*. no)	63.94 ± 17.73 *vs*. 59.39 ± 15.81	0.23
Acute rejection (yes *vs*. no)	61.64 ± 16.59 *vs*. 61.39 ± 16.97	0.95
	R	p value
Recipient age	-0.45	<0.001
Donor age	-0.46	<0.001
HLA MM	0.07	0.52
Average tacrolimus concentration	-0.02	0.9
Average MMF dose	0.18	0.1
ci at 1 year post Tx	-0.34	0.002
ct at 1 year post Tx	-0.35	0.002
cv at 1 year post Tx	-0.20	0.07

Table 4. Multiple regression analysis of factors associated with kidney function

	Beta (β)	St.Err. β	p value
Tx (kidney)	-0.17	0.13	0.19
DGF (no)	0.04	0.1	0.71
Recipient age	-0.41	0.1	<0.001
Donor age	-0.1	0.14	0.45
ci at 12 months	-0.18	0.11	0.09
Average MMF dose	0.21	0.1	0.04

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Table 5. One-year progression of chronic allograft scores

Banff score	N	At transplantation	N	12 month	p
Interstitial fibrosis (ci)	79	0.16 ± 0.44	79	0.94 ± 0.85	<0.001
Tubular atrophy (ct)	79	0.24 ± 0.46	79	1.05 ± 0.77	<0.001
Chronic glomerulopathy (cg)	79	0	79	0	
Mesangial matrix (mm)	79	0.01 ± 0.11	79	0.09 ± 0.36	0.09
Fibrointimal thickening (cv)	76	0.37 ± 0.83	78	0.29 ± 0.70	0.47
Arteriolar hyalinosis (ah)	78	0.68 ± 1,04	79	0.79 ± 1.04	0.26

Table 6. Correlation of factors associated with progression of ci and ct scores

	Δci		Δct	
	mean ± SD	p	mean ± SD	p
Kidney *vs.* SPKT	0.86 ± 0.91 *vs*. 0.67 ± 0.73	0.51	0.85 ± 0.87 vs. 0.86 ± 0.65	0.74
DGF (yes *vs.* no)	1.19 ± 0.98 *vs*. 0.62 ± 0.74	0.02	1.15 ± 0.92 vs. 0.69 ± 0.72	0.05
Recipient gender (m *vs.* f)	0.83 ± 0.88 *vs*. 0.76 ± 0.83	0.78	0.91 ± 0.83 vs. 0.72 ± 0.79	0.35
Donor gender (m *vs*. f)	0.91 ± 0.95 *vs*. 0.69 ± 0.75	0.43	0.88 ± 0.93 vs. 0.81 ± 0.67	0.96
Donor source (D *vs*. L)	0.84 ± 0.88 *vs*. 0.75 ± 0.85	0.73	0.87 ± 0.82 vs. 0.79 ± 0.83	0.71
Steroid free (no *vs*. yes)	1.09 ± 0.87 *vs*. 0.47 ± 0.74	0.002	1.07 ± 0.83 vs. 0.58 ± 0.73	0.01
Acute rejection (yes *vs*. no)	0.8 ± 0.89 *vs*. 0.83 ± 0.82	0.78	0.93 ± 0.84 vs. 0.67 ± 0.76	0.23
	R	p	R	p
Recipient age	-0.11	0.33	-0.11	0.32
Donor age	0.17	0.13	0.04	0.73
HLA MM	-0.09	0.43	-0.002	0.99
Average tacrolimus conc.	-0.009	0.95	0.003	0.98
Average MMF dose	-0.37	<0.001	-0.38	<0.001
ci at implantation	-0.32	0.003		
ct at implantation			-0.45	<0.001

Table 7. Multivariate general regression analysis for factors related to progression of ci and ct score

	Beta (β)	Std. Err. β	p
ci0	-0.43	0.09	<0.001
DGF (no)	-0.22	0.11	<0.05
Average MMF dose	-0.20	0.09	<0.05
Donor age	0.32	0.09	<0.05
Steroid free (yes)	-0.25	0.11	0.02
Δct			
	Beta (β)	Std. Err. β	p
ct0	-0.44	0.09	<0.001
Average MMF dose	-0.29	0.1	<0.05
DGF (no)	-0.29	0.1	<0.05
Steroid free (yes)	-0.09	0.11	0.39

Table 8. Adverse events with respect to 1 year average median MMF dose.

	MMF dose < median	**MMF dose > median**	**p**
Average number of infection episodes per patient	1.16 ± 0.97	1.23 ± 1.22	0.88
Mean time to first infection (days)	157±138	175±143	0.76
Proportion of patients with leucopenia	6 /31	7 /48	0.58

Effect of Mycophenolate Mofetil on Progression of Interstitial Fibrosis and Tubular Atrophy after Kidney Transplantation - A Retrospective Study

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ABSTRACT:

Objectives - Chronic transplant dysfunction after kidney transplantation is major reason of kidney graft loss and is caused by immunological and non-immunological factors. There is evidence that mycophenolate mofetil (MMF) may exert a positive effect on renal damage in addition to immunosuppression, by its direct antifibrotic properties. The aim of our study was to retrospectively investigate role of MMF dose on progression of chronic allograft dysfunction and IF/TA.

Setting - Retrospective, cohort study.

Participants - Kidney transplant patients in tertiary care institution. This is a retrospective cohort study that included 79 patients with kidney and kidney-pancreas transplantation. Immunosuppression consisted of anti-IL2 antibody induction, MMF, a calcineurin inhibitor ± steroids.

Primary outcome measures - An association of average MMF dose over 1 year post transplant with progression of interstitial fibrosis (Δci), tubular atrophy (Δct) and estimated creatinine clearance (eCr_{cl}) at 1 year post transplant was evaluated using univariate and multivariate analyses.

Results - Higher average MMF dose was significantly independently associated with better eCr_{cl} at 1 year post transplant (b=0.21 ± 0.1, p=0.04). In multiple regression analysis lower Δci (b=-0.2 ± 0.09, p=0.05) and Δct (b=-0.29 ± 0.1, p=0.02) were independently associated with greater average MMF dose. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Conclusions - Higher average MMF dose over 1 year is associated with better renal function and slower progression of IF/TA, at least partly independent of its immunosuppressive effects.

Strenghts and limitations of this study

Important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF (up to 4 g daily) during 1 year post transplantation had significantly lower progression of graft interstitial fibrosis and tubular atrophy. This is important finding, because of predictive value of graft IF/TA and should translate into better long-term graft survival. Our study has several shortcomings, such as its retrospective aspect and relatively short study period. As it was not aim of the study, we did not report side effects associated with different dosage of MMF.

INTRODUCTION:

Kidney transplantation significantly improves patient survival and quality of life comparing to dialysis. While significant improvements have been made in the treatment of acute rejection and short survival of transplanted kidney, there has not been major improvement in the long-term survival of transplanted kidney.[1] Chronic transplant dysfunction after kidney transplantation is major cause of kidney graft loss and is evoked by immunological and non-immunological factors.[2, 3] Histology changes that determine chronic transplant dysfunction are interstitial fibrosis and tubular atrophy (IF/TA), arteriosclerosis, arteriolar hyalinosis, glomerulopathy and mesangial matrix expansion.[4] IF/TA is the major pathohystology finding that can be verified on graft biopsies after kidney transplantation and is a predictor of long-term allograft function.[4] Clinical factors that affect progression of IF/TA are: recipient age, HLA mismatch, episodes of severe acute rejection, chronic rejection (esp. antibody-mediated), use of calcineurin inhibitors and BK nephropathy. Avoidance of CNI toxicity is considered as an important step to slow progression of IF/TA.[4-7] Mycophenolate mofetil (MMF) may help lowering CNI toxicity, by allowing lower CNI exposure.[7]

MMF reduces the risk of acute allograft rejection, without nephrotoxic side effects and is ideal candidate for long-term calcineurin drug reduction treatment strategies.[7] Retrospective studies of renal recipients who were treated with mycophenolate mofetil comparing azathioprin showed that MMF treated patients had significantly less chronic allograft dysfunction.[8, 9] Besides being associated with lower acute rejection rates as compared to azathioprin,[10, 11] evidence from animal and human studies suggests that

MMF may also exert a direct antifibrotic properties due to its antiproliferative action on nonimmune cells, including renal tubular cells and vascular smooth muscle cells.[12, 13] The aim of our study was to investigate role of mycophenolate mofetil dose on progression of IF/TA in kidney transplant recipients.

PATIENTS AND METHODS:

Patients:

This is a retrospective study conducted at Clinical Hospital "Merkur". This study represents a part of the posttransplant immune monitoring at the Merkur hospital, approved by the Hospital Ethics Committee. Patients gave there informed written consent for anonymized transplant data collection for research purposes. The study included 79 patients with kidney and kidney-pancreas transplantation, transplanted between 2003 and 2011. Eligible patients had to have protocol kidney biopsy at the time of implantation and 12 months after transplantation. Exclusion criteria have been: dual kidney transplantation, kidney-liver transplantation, use of antithymocyte immunoglobulin, BK nephropathy and recurrence of glomerulonephritis after transplantation.

Immunosuppression:

Induction immunosupression consisted of an anti-IL2 antibody (daclizumab or basiliximab), calcineurin inhibitor (tacrolimus or cyclosporine), MMF and methylprednisolone. Maintenance immunosuppression consisted of a calcineurin inhibitor (tacrolimus or cyclosporine), MMF ± steroids. Target cyclosporine trough concentrations were 250-350 during first month posttransplant, 200-300 during second to 6th month and 100-150 µg/L thereafter. Target tacrolimus trough levels were 10-12 during first month, 8-10 during second to 6th month and 5-8 µg/L thereafter. Mycophenolic acid target trough concentration was aimed to be higher than 7.2 µmol/L with tacrolimus and higher than 5 µmol/L with cyclosporine use.

Daclizumab was administered at day 0: 2mg/kg i.v. before opening of vascular anastomosis and at day 14: 2mg/kg i.v.. Basiliximab was administered at day 0: 20 mg i.v. before opening of vascular anastomosis and at day 4: 20 mg i.v..

Steroids have been dosed as follows: day 0: intraoperatively 500 mg of methylprednisolone, day 1: 250 mg, day 2: 125mg, day 3: 80 mg and day 4: 40 mg. In patients with early steroid withdrawal steroids have been withdrawn at day 5 after transplantation. In patients maintained on steroids, nadir dose of prednisone was 5 mg/d, achieved by 6 months. The criteria for early elimination of steroids were low immunological risk of the recipient (absence of, or low degree of HLA sensitization, i.e. PRA <10%) and good immediate renal function, as well as absence of an episode of acute rejection within 5 days after the transplantation. Steroids have been reintroduced in patients who suffered acute rejection episode.

As prophylaxis for viral (HSV, CMV), fungal (Candida spp.) urinary and P. jiroveci infections, low-dose fluconazole (for one year), valganciclovir (universally for three months) and sulfomethoxazol and trimethoprim (for one year) was used.

Renal allograft biopsies:

Protocol kidney biopsies were done at implantation, 1, 3, 6 and 12 months after transplantation. For cause biopsies were done in case of unexplained deterioration of renal function, or once weekly in patients with DGF. All rejection episodes were histologically confirmed. Histopathological analysis was performed by either of two pathologists who were blinded for immunosuppression. Acute rejections and chronic allograft scores have been analyzed using Banff 97 classification and its updates.[14, 15] All protocol and indication biopsies were analyzed by light microscopy, by immunofluorescence for C4d, and if indicated by immunohistochemistry for BK virus. Biopsies at 1 year post transplant have been also analyzed by electron microscopy for signs of chronic antibody-mediated rejection (transplant glomerulopathy, peritubular capillary basement membrane multilayering).[16]

Clinical outcome parameters:

Progression of chronic allograft scores during 1 year posttransplant was calculated by subtracting implantation chronic scores from chronic allograft scores 12 months

posttransplant: interstitial fibrosis (Δci), tubular atrophy (Δct), glomerulosclerosis (Δcg), mesangial matrix increase (Δmm), vasculopathy (Δcv) and arteriolar hyalinosis (Δah). Estimated creatinine clearance (eCr_{cl}) at 3, 6 and 12 months posttransplant was calculated using Cockroft-Gault formula. Acute rejections with Banff grade IA and IB were treated with three 500 mg methylprednisolone pulses. In case of acute rejection grade IIA or greater, patients have been treated with antithymocyte globulin. Antibody-mediated rejections were treated with steroid pulse and plasmapheresis.

Average dose of MMF during 1 year posttransplant was calculated from MMF dose at month 1, 3, 6 and 12.

Adverse effects analysed were clinicaly significant leucopenia, defined as white blood cell count less than 3000/ml, time to first symptomatic infection and number of symptomatic infection episodes per patient during first post transplant year.

Statistical analysis:

Numerical data are presented as mean ± SD or median with range in case of not normal distribution. Normality of distribution has been tested with Kolmogorov-Smirnov test. Correlation between two continuous variables has been tested using Spearman nonparametric correlation. Difference between two groups in continuous variables has been tested with student t-test or with Mann–Whitney test in non-normally distributed variables. The significance of the progression in chronic scores was analyzed using Wilcoxon Matched Pairs test. Univariate and multiple linear regression analysis were performed to determine predictive factors for progression of chronic allograft scores and kidney function at 12 months after transplantation. All variables that were associated with

respective outcome in bivariate analysis (at $p \leq 0.1$) were included in multivariate analysis. Because of colinearity between ci and ct score, only one score was included in each multivariate analysis. Statistical significance was considered at $p<0.05$. All statistical analyses were performed using Statistica 10 (StatSoft, Tulsa, OK, USA).

RESULTS:

Patient and transplant characteristics:

Patient characteristics are shown in Table 1. Recipients were a mean of 44.67 ± 12.03 years old at the time of transplantation, 68 percent of them were male and all were Caucasians. 33 percent of recipients had DGF after transplantation. Donors were a mean of 43.89 ± 15.55 years old and 54 percent of them were male. Number of living donor transplantations was 24 (30 percent). Average daily MMF dose during 1 year posttransplant was 2244 ± 585 mg (1062 – 4000) (Table 2). As expected, there was no correlation of MMF dose with MMF trough concentration (R=-0.13; p=0.28). Also, there was no correlation between MMF dose with tacrolimus concentration (R=-0.04; p=0.79). Early steroid withdrawal was done in 46 percent of patients after transplantation. Incidence of subclinical and clinical acute rejections greater then borderline was 30 percent in first year. There was no correlation between average MMF dose and incidence of acute rejection (p=0.68).

Factors associating with eCr_{cl}:

Kidney function increased during 1st year post transplant. eCr_{cl} at month 3 was 56.98 ± 15.78 ml/min, at 6 month 58.94 ± 16.94 ml/min and at 12 month 61.47 ± 16.75 ml/min ($p<0.001$; 12 months *vs*. 3 months) (Figure 1.) eCr_{cl} at 1 year post transplant was greater in SPKT recipients (71.38 ± 13.45 ml/min *vs.* 57.88 ± 16.47 ml/min; p=0.001) and in patients who did not have DGF (64.08 ± 15.87 ml/min *vs*. 56.15 ± 17.55 ml/min; p=0.05). Donor age (R=-0.46; $p<0.001$) and recipient age (R=-0.46; $p<0.001$) negatively correlated with eCr_{cl} at 1 year post transplant, while there was no correlation of renal function with donor and recipient gender, type of donation (deceased *vs.* living), HLA MM, average CNI concentration, steroid-free regimen of immunosuppression, or history of acute rejection (Table 3). In univariate analysis allograft function at 12 month post Tx was also negatively correlated with ci (R=-0.34; p=0.002) and ct (R=-0.35; p=0.002) at 12 month (Figure 2A, Figure 2B). Although MMF dose was positively correlated with renal function with borderline significance in univariate analysis, in multivariate analysis there was a significant positive association between greater average MMF dose and better eCr_{cl} at 12 month post transplant (b=0.21 ± 0.1; p=0.04) (Table 4).

Factors affecting IF/TA:

The average ci score increased from 0.16 ± 0.44 to 0.94 ± 0.86 between implantation and month 12 ($p<0.001$). Average progression of this and other chronic scores during 1 year post transplant is shown in Table 5. In univariate analysis Δci (R=-0.37; p=0.001) and Δct (R=-0.38; p=0.001) significantly negatively correlated with average MMF dose (Figure 3A and 3B, Table 6). There was lower progression of ci score in patients on steroid-free

immunosuppression (0.47 ± 0.7 *vs*. 1.09 ± 0.87; p=0.002) and in those who did not have DGF (0.62 ± 0.74 *vs* 1.19 ± 0.98; p=0.02). Acute cellular rejection, recipient and donor gender, recipient and donor age, HLA MM, deceased *vs.* living donor, as well as average concentration of tacrolimus had no significant effect on progression of chronic allograft scores. Higher average MMF dose was associated with lower progression of ci and ct score regardless CNI type (data not shown). Factors that remained significantly associated with progression of ci score in multivariate analysis were ci0 score, donor age, average MMF dose, DGF and steroid-free immunosuppression (Table 7.). In multivariate analysis only ct0 score, average MMF dose and DGF remained independently associated with 12-month progression of ct score (Table 7.). Selected AE are shown in Table 8. There was no difference in AE (leucopenia and infections) with respect to average median MMF dose.

Discussion:

The most important novel finding in our study is that greater average MMF exposure was strongly negatively correlated with IF/TA progression during first year after kidney transplantation. Patients on higher average dose of MMF during 1 year post transplantation had significantly lower progression of ci and ct scores. To our knowledge this is first study demonstrating that there is a dose-dependent protective effect of MMF on graft IF/TA. Lower progression of IF/TA could not be explained with lower concentration of CNI, because there was not correlation between tacrolimus concentration with IF/TA. Similarly, there was no correlation between average MMF

dose and tacrolimus (R=-0.04; p=0.79) or cyclosporine concentration (R=-0.07, p=0.79). In addition, higher average MMF dose was not associated with decreased incidence of biopsy proven acute rejection, which suggests that antifibrotic properties of higher MMF dose was at least partly independent of its immunosuppressive effects. Higher MMF dose had only moderate effect on 1-year renal function, which is consistent with previous reports showing that transplanted kidneys undergo pathohystology changes without significant early change in kidney function.[17]

In the present retrospective study we have confirmed that IF/TA progression occurs in first year after kidney transplantation. Several studies have shown that progression of IF/TA is correlated with type of immunosuppression.[18] In most transplant centers in the United States and Europe immunosuppression consists of induction with an anti-IL2R antibody or antithymocyte immunoglobulin and maintenance with a calcineurin inhibitor, MMF and steroids.[19] Studies have reported significant improvement in kidney function in patients on MMF with lower exposition to CNIs, esp. tacrolimus.[20] Recently, in the paper of Kamar e*t al*. it has been reported that maintenance kidney transplant patients converted to a higher dose of the mycophenolate sodium (1440 mg daily) with lower tacrolimus concentration had borderline higher eCr_{cl} on month 6 *vs*. those treated with lower dose of mycophenolate sodium, with usual tacrolimus concentration (eCr_{cl} 49.1 ± 11.1 *vs*. 44.7 ± 11.5 ml/min; p=0.07).[21] Although there was only borderline significance, increased mycophenolate dosing with lower tacrolimus concentration was safe with potential benefit on kidney function.

Our study also corroborates recently published findings of a *post hoc* joint analysis of the Symphony, FDCC and OptiCept trials, where a a lower tacrolimus level and a higher

MMF dose were associated with significantly better kidney function at 1 year post transplant.[22] Shortcoming of these studies[17,18] is lack of protocol biopsies. The optimal MMF dosing in patients maintained on contemporary low-dose CNI is still undetermined. However, some results of early MMF registration trials suggest that higher MMF exposure might be beneficial; having in mind that there was no antibody induction in these studies and that CNI was standard dose cyclosporine. Thus, in the Tri-continental study, group treated with 3 g MMF compared with 2 g of MMF showed lower incidence of biopsy proven acute rejection episodes (15.9% vs. 19.7%) within 6 month period selected for the primary efficacy analysis. Similarly, serum creatinine level at 1 year was 1.42 ± 0.07 mg/dL in the MMF 3 g group *vs.* 1.64 ± 0.07 mg/dL in MMF 2 g group.[12] In the European mycophenolate mofetil study same trends regarding higher MMF dose were observed.[11] As mentioned before, in these studies there was no antibody induction that could have allowed lower dose of cyclosporin with higher dose of MMF and there were no protocol biopsies. In a more recent MYSS trial, there was no difference in acute rejection rate and renal function between MMF and azathioprine in a cyclosporine-based protocol.[19] However in that study only one MMF dose was compared to azathioprine[23] and again there were no protocol biopsies. Unfortunately adequate prospective MMF dose comparison studies in tacrolimus-based protocols with antibody induction are missing. In the Symphony study it was reported that patients on tacrolimus-MMF-prednisone maintenance imunosuppression after kidney transplantation had better kidney function and graft survival with lower number of acute rejection episodes. Patients in that group had highest MMF exposure.[24] Protocols with

even higher MMF exposure might allow additional CNI sparing, that would decrease side effect of CNI (hypertension, diabetes, hyperlipidemia, neurotoxicity).[25]

Clinical relevance of IF/TA without other concomitant pathology (i.e. recurrent disease and chronic antibody-mediated rejection) for prediction of graft deterioration and loss is controversial. In El-Zoghby et al. study there was attempt to identify specific causes of late kidney allograft failure. The authors found that transplant glomerulopathy was responsible for 37 percent loss of functioning grafts, while graft loss due to IF/TA was present in 31 percent of cases (with higher frequency in deceased-donor transplants).[26] At first glance, these results seem at odd with ours, where there were no signs of chronic antibody-mediated rejection. An explanation for this discrepancy in the results of the two studies is not completely clear, but the former study included high number of living transplants (72.5 percent) with glomerulonephritis as primary disease and with follow-up up to 10 years. Transplant glomerulopathy is more frequently seen late posttransplant, generally with low incidence. Nevertheless, ours and El-Zoghby study, both demonstrated that IF/TA even in absence of other pathology is associated with adverse graft outcome. Another important study, the DeKaf study, tried to use various histopathologic clusters to differentiate subgroups within diagnosis of IF/TA. They found that cluster with more severe fibrosis plus inflammation and arterial lesions had the worst prognosis.[27] Although incidence of acute rejection in our study did not vary with MMF exposure, increased MMF exposure might suppress mild graft inflammation, below the threshold for diagnosing acute rejection. This is subject of our ongoing investigation and will be reported separately. An interesting finding of the present study was that early steroid withdrawal was not associated with worse IF/TA. At first glance this is at odd

with the Astellas trial.[23] However, according to our protocol, patients with DGF were not included in early steroid withdrawal and Astellas trial, which did not have protocol biopsies, reported increased IF/TA in early steroid withdrawal group based on indication biopsies performed early posttransplant, thus more likely reflecting donor-derived histology changes, rather than effect of steroid withdrawal.[28]

In our study there was only borderline significance of positive association of 1-year eCr_{cl} with MMF in univariate analysis. This result is not very surprising since decreased renal function is not a very sensitive marker of incipient IF/TA.

Mechanisms by which an average higher exposure to MMF was associated with slower progression of IF/TA may be both immune and nonimmune. Because there was no difference in incidence of acute rejection with respect to increased MMF exposure in our study, we believe that there may be a significant contribution of nonimmune mechanisms in retardation of IF/TA in patients with higher MMF. In line with this, in many experimental models it has been shown that MMF has antiproliferative and antifibrotic effect.[29-31] In the study of Jiang at al. using rat renal ischemia reperfusion injury, a time- and dose-dependent correlation of higher MMF dose with better renal function and lower interstitial fibrosis was demonstrated. Suggested potential mechanism was lower expression of TGF-β1 and MCP-1with lower macrophage infiltration.[32] In recent clinical trials MMF was shown as a safe drug that could be a good candidate for treatment of interstitial lung disease in systemic sclerosis.[33] Experimental model of encapsulated peritoneal sclerosis in rats proved beneficial effect of MMF as an inhibitor of neovascularisation.[34] Also, MMF monotherapy was associated with a positive effect on hepatic fibrosis progression in HCV liver transplant recipients.[35]

Our study has several shortcomings, such as its retrospective aspect and relatively short study period. Although study period was limited to 12 months post transplantation, a clear correlation of slower progression of IF/TA with higher average MMF dose underlines potential benefit of these findings. As mentioned before, in current study we did not analyze inflammation outside Banff acute rejection threshold in kidney biopsies with respect to MMF dose. As inflammation in areas of IF/TA is an important predictor of renal function and graft loss, this is subject of an ongoing work.

In summary, higher MMF dose after kidney transplantation might slower progression of IF/TA, which might lead to better long-term survival of transplanted kidney. Our study serves as a platform for a prospective, randomized, long-term trial with different MMF doses to evaluate benefit of higher MMF dose in renal transplant recipients. (NCT018600183).

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Contributorship Statement

Karlo Mihovilović – participated in research design, collecting data, analyzing data and wrote the paper, karlomihovilovic@gmail.com

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Abbreviations:

IF/TA - interstitial fibrosis and tubular atrophy

MMF - mycophenolate mofetil

BK - polyoma virus BK

CNI - calcineurin inhibitors

DGF - delayed graft function

eCrCl - estimated creatine clearance

SPKT - simultaneous pancreas kidney transplantation

HLA MM – human leukocyte antigen mismatch

AE - adverse events

Figure Legends

Figure 1: Estimated creatinine clearance during first year posttransplant

Figure 2A: Correlation between average MMF dose and progression of ci score

Figure 2B: Correlation between average MMF dose and progression of ct score

Figure 3A: Estimated creatinine clearance by ci score

Figure 3B: Estimated creatinine clearance by ct score

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Table 1. Baseline characteristics

RECIPIENT CHARACTERISTICS	AGE (years)	44.67 ± 12.03
	GENDER (f/m)	25/54
	PRIMARY RENAL DISEASE (diabetes mellitus, polycistic kidney disease, glomerulonephritis, pyelonephritis/interstitial nephritis, other/unknown)	24/8/19/6/22
DONOR CHARACTERISTICS	DONOR SOURCE (decased/living)	55/24
	AGE (years)	43.89 ± 15.55
	GENDER (f/m)	36/43
TRANSPLANTATION CHARACTERISTICS	TRANSPLANTED ORGAN (KIDNEY/SPKT)	58/21
	INITIAL IMMUNOSUPRESSION (anti-IL2,TAC,MMF/anti-IL2, CyA,MMF)	53/26
	DELAYED GRAFT FUNCTION (no/yes)	53/26
	STEROID FREE (yes/no)	36/43
	HLA MM	3.33 ± 1.51

Table 2. eCr_{cl}, MMF dose and CNI concentration during first year post transplant

	Month posttransplant			
	1	3	6	12
eCr_{cl} (ml/min)		56.98 ± 15.79	58.94 ± 16.94	61.47 ± 16.75
MMF dose (mg)	2500 (750 – 4000) 2427 ± 643.17	2000 (750 – 4000) 2167.72 ± 733.49	2000 (1000-4000) 2188.29 ± 716.91	2000 (1000-4000) 2193.04 ± 642.95
Tacrolimus conc. (µg/L) (n=53)	10.79 ± 4.16	9.69 ± 3.00	9.03 ± 5.52	7.83 ± 2.45
Cyclosporin conc. (µg/L) (n=26)	335.07 (274 – 413)	231.05 (181-265)	206 (170 – 257)	131 (125 – 171)

Table 3. Association of variables with eCr_{cl} on 1 year

	Estimated creatinine clearance (ml/min)	p value
Kidney *vs.* SPKT	57.88 ± 15.47 *vs*. 71.38 ± 13.45	0.001
DGF (yes *vs.* no)	56.15 ± 17.55 *vs*. 64.08 ± 15.87	0.05
Recipient gender (m *vs.* f)	59.83 ± 16.02 *vs*. 65 ± 18.07	0.2
Donor gender (m *vs*. f)	63.87 ± 16.71 *vs*. 58.60 ± 16.58	0.17
Donor source (D *vs*. L)	62.36 ± 17.85 *vs*. 59.43 ± 14.05	0.47
Steroid-free (yes *vs*. no)	63.94 ± 17.73 *vs*. 59.39 ± 15.81	0.23
Acute rejection (yes *vs*. no)	61.64 ± 16.59 *vs*. 61.39 ± 16.97	0.95
	R	p value
Recipient age	-0.45	<0.001
Donor age	-0.46	<0.001
HLA MM	0.07	0.52
Average tacrolimus concentration	-0.02	0.9
Average MMF dose	0.18	0.1
ci at 1 year post Tx	-0.34	0.002
ct at 1 year post Tx	-0.35	0.002
cv at 1 year post Tx	-0.20	0.07

Table 4. Multiple regression analysis of factors associated with kidney function

	Beta (β)	St.Err. β	p value
Tx (kidney)	-0.17	0.13	0.19
DGF (no)	0.04	0.1	0.71
Recipient age	-0.41	0.1	<0.001
Donor age	-0.1	0.14	0.45
ci at 12 months	-0.18	0.11	0.09
Average MMF dose	0.21	0.1	0.04

Table 5. One-year progression of chronic allograft scores

Banff score	N	At transplantation	N	12 month	p
Interstitial fibrosis (ci)	79	0.16 ± 0.44	79	0.94 ± 0.85	<0.001
Tubular atrophy (ct)	79	0.24 ± 0.46	79	1.05 ± 0.77	<0.001
Chronic glomerulopathy (cg)	79	0	79	0	
Mesangial matrix (mm)	79	0.01 ± 0.11	79	0.09 ± 0.36	0.09
Fibrointimal thickening (cv)	76	0.37 ± 0.83	78	0.29 ± 0.70	0.47
Arteriolar hyalinosis (ah)	78	0.68 ± 1,04	79	0.79 ± 1.04	0.26

Table 6. Correlation of factors associated with progression of ci and ct scores

	Δci		Δct	
	mean ± SD	p	mean ± SD	p
Kidney *vs.* SPKT	0.86 ± 0.91 *vs*. 0.67 ± 0.73	0.51	0.85 ± 0.87 vs. 0.86 ± 0.65	0.74
DGF (yes *vs.* no)	1.19 ± 0.98 *vs*. 0.62 ± 0.74	0.02	1.15 ± 0.92 vs. 0.69 ± 0.72	0.05
Recipient gender (m *vs.* f)	0.83 ± 0.88 *vs*. 0.76 ± 0.83	0.78	0.91 ± 0.83 vs. 0.72 ± 0.79	0.35
Donor gender (m *vs*. f)	0.91 ± 0.95 *vs*. 0.69 ± 0.75	0.43	0.88 ± 0.93 vs. 0.81 ± 0.67	0.96
Donor source (D *vs*. L)	0.84 ± 0.88 *vs*. 0.75 ± 0.85	0.73	0.87 ± 0.82 vs. 0.79 ± 0.83	0.71
Steroid free (no *vs*. yes)	1.09 ± 0.87 *vs*. 0.47 ± 0.74	0.002	1.07 ± 0.83 vs. 0.58 ± 0.73	0.01
Acute rejection (yes *vs*. no)	0.8 ± 0.89 *vs*. 0.83 ± 0.82	0.78	0.93 ± 0.84 vs. 0.67 ± 0.76	0.23
	R	p	R	p
Recipient age	-0.11	0.33	-0.11	0.32
Donor age	0.17	0.13	0.04	0.73
HLA MM	-0.09	0.43	-0.002	0.99
Average tacrolimus conc.	-0.009	0.95	0.003	0.98
Average MMF dose	-0.37	<0.001	-0.38	<0.001
ci at implantation	-0.32	0.003		
ct at implantation			-0.45	<0.001

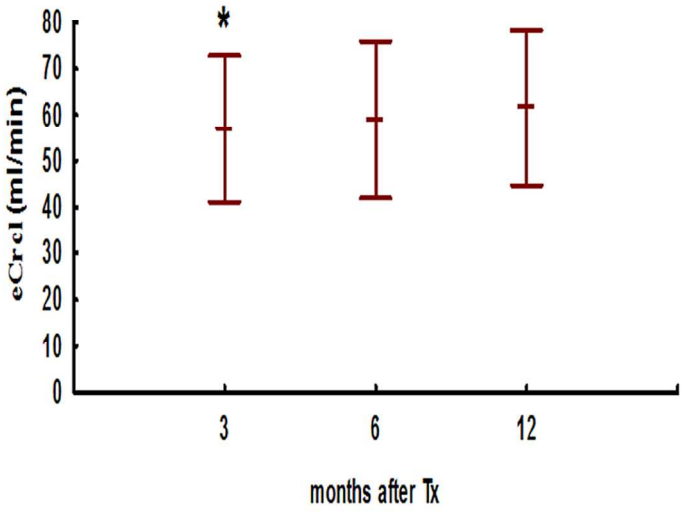
Table 7. Multivariate general regression analysis for factors related to progression of ci and ct score

Δci			
	Beta (β)	Std. Err. β	p
ci0	-0.43	0.09	<0.001
DGF (no)	-0.22	0.11	<0.05
Average MMF dose	-0.20	0.09	<0.05
Donor age	0.32	0.09	<0.05
Steroid free (yes)	-0.25	0.11	0.02
Δct			
	Beta (β)	Std. Err. β	p
ct0	-0.44	0.09	<0.001
Average MMF dose	-0.29	0.1	<0.05
DGF (no)	-0.29	0.1	<0.05
Steroid free (yes)	-0.09	0.11	0.39

Table 8. Adverse events with respect to 1 year average median MMF dose.

	MMF dose < median	**MMF dose > median**	**p**
Average number of infection episodes per patient	1.16 ± 0.97	1.23 ± 1.22	0.88
Mean time to first infection (days)	157±138	175±143	0.76
Proportion of patients with leucopenia	6 /31	7 /48	0.58

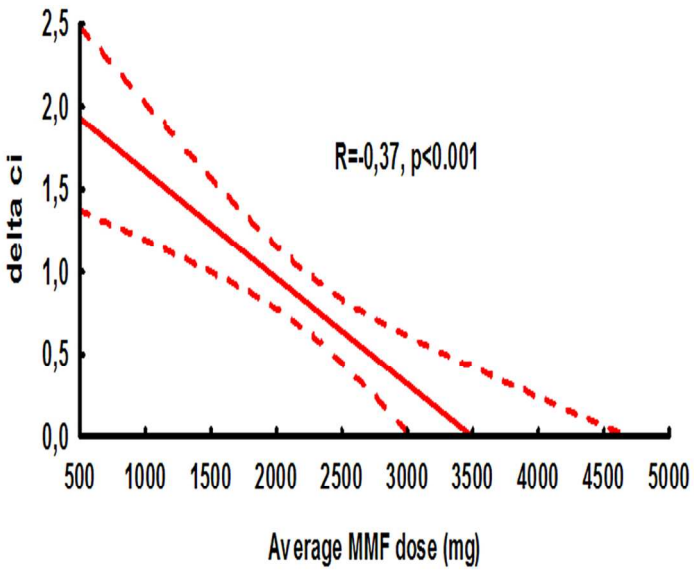
Figure 1: Estimated creatinine clearance during first year posttransplant



*, p<0.001; 3 month *vs.* 12 months eCr_{cl}. Values are shown as mean ± SD

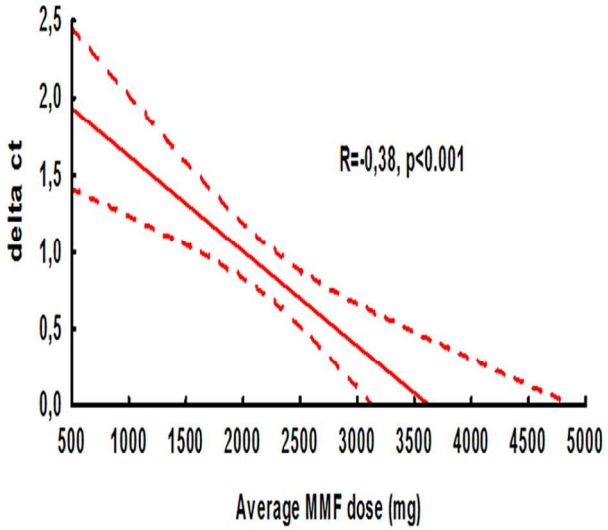
90x105mm (300 x 300 DPI)

Figure 2A: Correlation between average MMF dose and progression of ci score



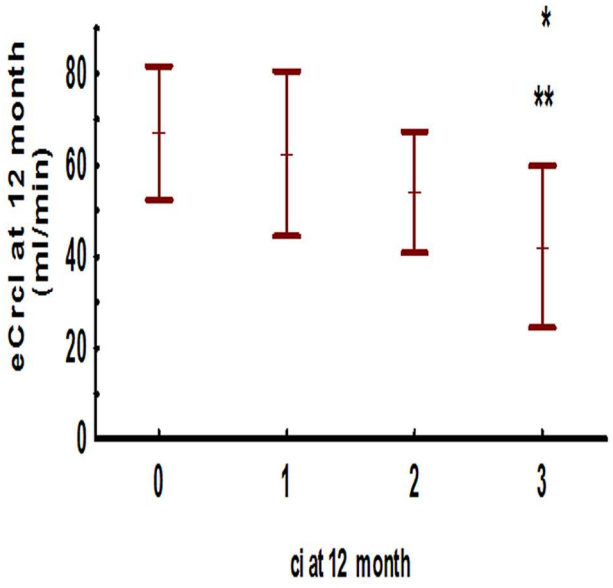
90x121mm (300 x 300 DPI)

Figure 2B: Correlation between average MMF dose and progression of ct score



90x121mm (300 x 300 DPI)

Figure 3A: Estimated creatinine clearance by ci score



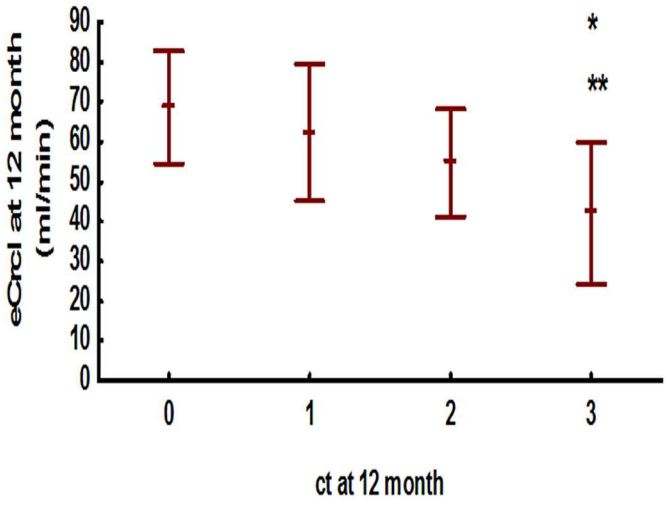
* p<0.05; score ci 0 *vs.* score ci 3 (*post hoc* Newman-Keuls test)

** p=0.03; score ci 1 *vs.* score ci 3

Values are shown as mean ± SD

90x121mm (300 x 300 DPI)

Figure 3B: Estimated creatinine clearance by ct score



* p<0.05; score ct 0 *vs.* score ct 3 (post hoc Newman-Keuls test)

** p=0.03; score ct 1 *vs.* score ct 3

Values are shown as mean ± SD

90x121mm (300 x 300 DPI)

STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No/page number	Recommendation
Title and abstract	1/1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2/5	Explain the scientific background and rationale for the investigation being reported
Objectives	3/5,6	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4/6	Present key elements of study design early in the paper
Setting	5/6,7, 8,9	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6/6	(*a*) *Cohort study*—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up *Case-control study*—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls *Cross-sectional study*—Give the eligibility criteria, and the sources and methods of selection of participants
		(*b*) *Cohort study*—For matched studies, give matching criteria and number of exposed and unexposed *Case-control study*—For matched studies, give matching criteria and the number of controls per case
Variables	7/6,7, 8	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*/8	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group
Bias	9	Describe any efforts to address potential sources of bias
Study size	10/6	Explain how the study size was arrived at
Quantitative variables	11/9	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12/9	(*a*) Describe all statistical methods, including those used to control for confounding
		(*b*) Describe any methods used to examine subgroups and interactions
		(*c*) Explain how missing data were addressed
		(*d*) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy
		(*e*) Describe any sensitivity analyses

Continued on next page

Results		
Participants	13*/9, 10	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*/9, 10	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(b) Indicate number of participants with missing data for each variable of interest
		(c) *Cohort study*—Summarise follow-up time (eg, average and total amount)
Outcome data	15*/11	*Cohort study*—Report numbers of outcome events or summary measures over time
		Case-control study—Report numbers in each exposure category, or summary measures of exposure
		Cross-sectional study—Report numbers of outcome events or summary measures
Main results	16/10, 11	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included
		(*b*) Report category boundaries when continuous variables were categorized
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
Key results	18/12	Summarise key results with reference to study objectives
Limitations	19/16	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20/12-16	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21/12-16	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22/2	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.