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ORIGINAL ARTICLE

Lower tacrolimus trough levels are associated with subsequently higher acute rejection risk during the first 12 months after kidney transplantation

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biopsy-proven acute rejection, kidney transplant recipient, tacrolimus trough level.

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Summary

The premise that lower TAC trough levels are associated with subsequently higher first BPAR risk during the first 12 mo post-transplant was recently questioned. Using our prospectively followed cohort of 528 adult, primary kidney transplant recipients (pooled across four randomized trials) who received reduced TAC dosing plus an IMPDH inhibitor, TAC trough levels measured at seven time points, 7, 14 days, 1, 2, 3, 6 and 9 months post-transplant, were utilized along with Cox's model to determine the multivariable significance of TAC level(t) (a continuous time-dependent covariate equaling the most recently measured TAC level prior to time t) on the hazard rate of developing first BPAR during the first 12 months post-transplant. The percentage developing BPAR during the first 12 months post-transplant was 10.2% (54/528). In univariable analysis, lower TAC level(t) was associated with a significantly higher BPAR rate ($P = 0.00006$), and its significance was maintained even after controlling for 2 significant baseline predictors (African-American/Hispanic Recipient and Developed DGF) in Cox's model (multivariable $P = 0.0003$). Use of a cutpoint, TAC level(t) <4.0 vs. ≥4.0 ng/ml, yielded an even greater association with BPAR rate (univariable and multivariable $P < 0.000001$), with an estimated hazard ratio of 6.33. These results suggest that TAC levels <4.0 ng/ml should be avoided during the first 12 months post-transplant when TAC is used in combination with fixed-dose mycophenolate with or without corticosteroids and induction therapy.

Introduction

The premise that a certain tacrolimus (TAC) trough level threshold (or range) exists below which the risk of developing a biopsy-proven acute rejection (BPAR) is significantly increased forms part of the rationale for the universally accepted approach of therapeutic drug monitoring of TAC —maintaining TAC levels within a target range so as to avoid CNI (calcineurin inhibitor) toxicity forms the other part. However, statistical validation of the first part of this hypothesis with actual kidney transplant data is rather sparse. In fact, a recent study [1] found no such association between TAC whole-blood trough level and acute rejection risk during the first 12 months post-transplant among 1304 kidney transplant recipients pooled across 3 large multicenter randomized trials [2–4]. We were surprised by this finding, having believed that there must be some threshold for the TAC trough level below which the risk of developing BPAR is increased. Other studies have shown that lower TAC trough (or area under the curve) levels during the first week [5–7], at discharge [8], during the first month [9], and during the first 6 weeks [10,11] post-transplant were significantly associated with subsequently higher BPAR rates. However, the current strategy of reduced TAC

dosing combined with an IMPDH (inosine monophosphate dehydrogenase) inhibitor was not utilized in most of these studies, and the sample size was fewer than 100 patients in 5 of 7 such studies [5,6,9–11]. One recent multivariable analysis of 216 moderately sensitized recipients [8] controlling for recipient race, age, and degree of sensitization found a significantly higher first BPAR rate during the first 12 months post-transplant among patients having a TAC trough level <8 ng/ml at discharge (i.e. below the initial target TAC trough level of 8–11 ng/ml). Another recent study of 1930 patients [12] found a significant association between lower TAC trough levels (on a continuous scale) and a significantly higher AR rate during the first 6 months post-transplant in a Cox multivariable model, with an even stronger relationship found during 3–6 months vs. 0–3 months post-transplant (i.e. interaction effect of TAC trough level with time post-transplant).

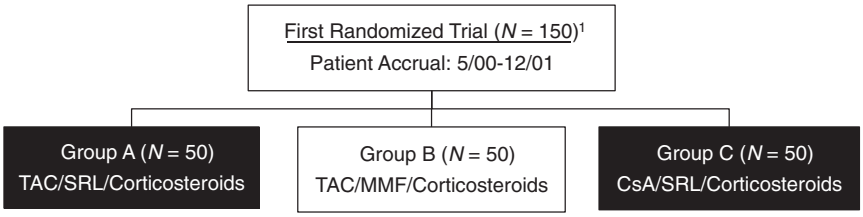
Given these major differences reported in the relationship between TAC trough level and subsequent rejection risk during the first 12 months post-transplant, we decided to perform a similar analysis using a prospectively followed cohort of 528 adult, primary kidney-alone transplant recipients. This cohort consists of all study participants assigned to receive reduced TAC dosing combined with an IMPDH inhibitor as part of maintenance therapy in four randomized immunosuppression trials performed at our center since May 2000 [13–23]; participants were combined across these randomized trials to maximize statistical power. Our goal was to determine whether (i) a significant association between TAC trough level and subsequent first BPAR risk does, in fact, exist during the first 12 months post-transplant, (ii) the relationship between TAC trough level and subsequent first BPAR risk is more accurately described using a continuous or dichotomous scale for TAC trough level, and (iii) the relationship between TAC trough level and subsequent first BPAR risk remains constant or changes over time. The results of this observational study are presented here.

Materials and methods

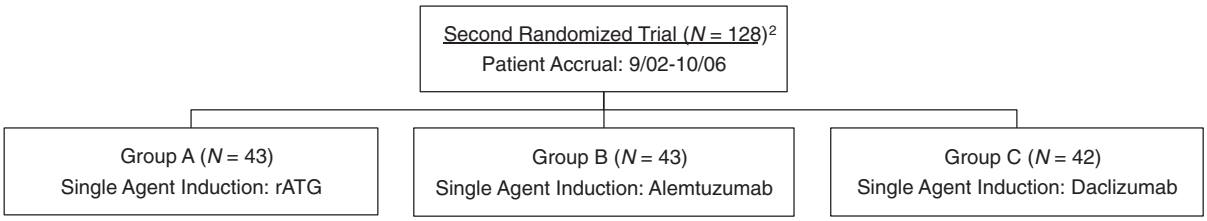
Data description

A flow diagram of the treatment arms for each of the four randomized trials appears in Fig. 1. Briefly, between May 2000 and December 2001 a randomized trial of 150 living donor (LD) and deceased donor (DD) recipients was performed comparing TAC/sirolimus (SRL) versus TAC/mycophenolate mofetil (MMF) versus cyclosporine microemulsion (CsA)/SRL (50 per arm) [13–16]. Patients assigned to the TAC/SRL and CsA/SRL arms were not included here. All patients received daclizumab induction and maintenance corticosteroids; last follow-up date was May 1, 2009. Between November 2002 and September 2004, a randomized trial of 90 DD recipients was performed comparing single-agent induction with rabbit antithymocyte globulin (rATG) (thymoglobulin) versus alemtuzumab versus daclizumab (30 patients per arm) [17,18]. TAC, MMF, and maintenance corticosteroids were given in the rATG and daclizumab arms, whereas TAC, one-half of standard MMF dose, and early corticosteroid withdrawal (by 7–10 days post-transplant) were scheduled in the alemtuzumab arm. A concurrently run randomized trial of 38 LD recipients was performed using exactly the same three treatment arms between September 2002 and October 2006 [19]. Last follow-up date for this combined study of 128 patients was August 31, 2011 [20]. Between December 2004 and February 2006, a randomized trial of 150 (LD and DD) recipients was performed comparing TAC/MMF versus TAC/enteric-coated mycophenolate sodium (EC-MPS) as maintenance, with both arms receiving rATG/daclizumab as dual induction and planned early corticosteroid withdrawal (75 patients per arm) [21,22]; last follow-up date was March 1, 2010. Lastly, between February 2006 and April 2009, a randomized trial of 200 (LD and DD) recipients was performed comparing two dual induction strategies, rATG/daclizumab versus rATG/alemtuzumab (100 patients per arm), with maintenance in both arms consisting of TAC, EC-MPS (using one-half of standard dose in the rATG/alemtuzumab arm), and planned early corticosteroid withdrawal [23]; last follow-up date was May 1, 2010. Of note, patient eligibility and exclusionary criteria were essentially the same in each trial. The center institutional review board approved each protocol; all patients gave written informed consent prior to enrollment (Clinical Trials.gov ID: NCT00681213, NCT00685061, NCT00681343, NCT00533624, and NCT01172418 – note: two distinct registration numbers exist in the second trial, because separate randomizations of DD and LD recipients were performed). Results of these randomized trials have been previously reported (details omitted here) [13–23]; again, these 528 randomized trial participants were combined to maximize statistical power.

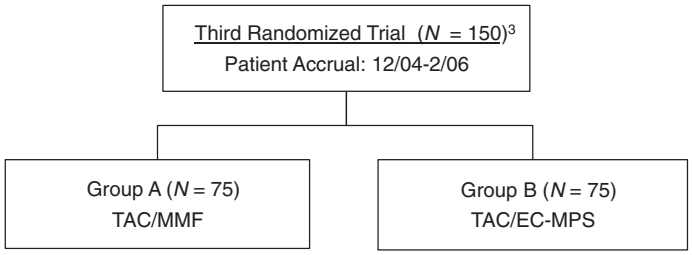
In each trial, TAC was initiated at a dose of 0.1 mg/kg twice daily once renal function had improved (i.e. serum creatinine <4 mg/dl absent dialysis). TAC trough levels were measured in whole blood by immunoassay. Target TAC trough levels ranged from 4 to 10 ng/ml (i.e. reduced tacrolimus dosing) throughout these trials, being higher in the first randomized trial (target: 6–10 ng/ml) and lower in subsequent trials (target: 4–8 ng/ml). TAC trough levels measured at seven distinct times post-transplant, that is, at 7, 14 days, 1, 2, 3, 6 and 9 months post-transplant, were utilized in the statistical analysis. If TAC had not yet been initiated at one or more of these times, then a TAC level of 0.0 was assumed. In addition, TAC was discontinued in three patients during the first 12 months post-transplant



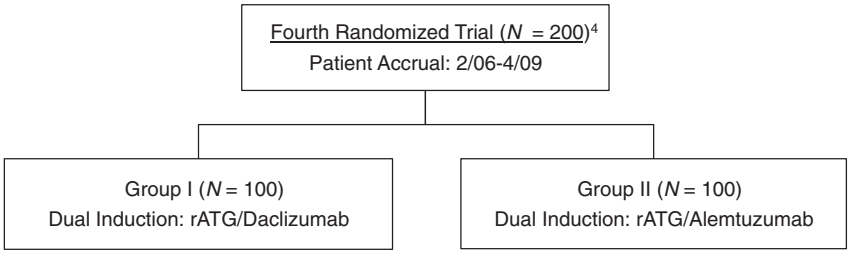
[1]All patients in the first randomized trial received single agent induction with daclizumab. Patients in Groups A and C were excluded from this analysis.



[2]Maintenance therapy consisted of TAC/MMF/Corticosteroids in Groups A and C and TAC/MMF with planned early corticosteroid withdrawal (by7-10 days post-transplant) in Group B. Of note, 94.5% (121/128) of patients accrued between 9/02-11/04.



[3]All patients in the third randomized trial received dual induction with rATG/daclizumab and planned early corticosteroid withdrawal (by 7-10 days post-transplant).



[4]All patients in the fourth randomized trial received maintenance therapy consisting of TAC/EC-MPS and planned early corticosteroid withdrawal (by 7-10 days post-transplant).

Figure 1 Flow diagram of the treatment arms for each randomized trial.

(uncontrolled diabetes in one case; elevated serum Cr/suspected CNI toxicity in two cases). For these three patients, the TAC level was set to 0.0 at those times at which it was not taken (none of these three patients developed BPAR).

An IMPDH inhibitor was initiated immediately post-transplant in all patients, with a target dose of 1000 mg BID for MMF and 720 mg BID for EC-MPS, except among alemtuzumab-treated patients, who received one-half of standard dose to avoid severe leukopenia. Mycophenolate acid (MPA) levels were measured in only a small subset of patients, and therapeutic drug monitoring of the IMPDH inhibitor was not performed in any of the trials. Of note, all transplanted DD kidneys received hypothermic machine perfusion preservation with the RM3 Renal Preservation

Machine using Belzer-MPS Machine Perfusion Solution as perfusate [24].

Delayed graft function (DGF) was defined as requirement for dialysis during the first week post-transplant. BPAR was defined as a rise of ≥0.3 mg/dl from the nadir serum creatinine accompanied by a kidney transplant biopsy to confirm the diagnosis within 24 h of initiation of antirejection therapy. Grading of BPAR was performed according to the Banff classification [25,26]. Initial rejection episodes were treated with intravenous corticosteroids, with antilymphocyte antibody treatment being added for histologically proven Banff grade II/III or steroid-resistant rejection. Graft loss was determined as the time of re-establishment of long-term dialysis therapy or death. All causes of graft failure and death were determined prospectively from the attending physician's ongoing clinical evaluation of each patient's post-transplant follow-up.

Baseline variables that were considered for their prognostic value included date of transplant, recipient age, sex, race/ethnicity, and body mass index, pretransplant diabetes status (no/yes), pretransplant coronary artery disease (CAD) status, %PRA for ABC, %PRA for DR, # of HLA mismatches, # of DR mismatches, donor and recipient CMV status, preemptive transplant status, pretransplant time spent on dialysis, donor type (LD or DD), donor age, donor race/ethnicity, expanded criteria donor (ECD) status, donation after cardiac death (DCD) status, deceased donor cold ischemia time (CIT), type of induction (daclizumab alone versus use of lymphocyte-depleting agent), planned maintenance with corticosteroids, and developed DGF.

Statistical analysis

Similar to the approach taken by Israni *et al.* [12], we used the most powerful statistical approach, that being the use of time-dependent covariates to test the prognostic impact of TAC trough level on the hazard rate of developing a first BPAR during the first 12 months post-transplant (patients having graft loss were censored at their times of graft loss). Specifically, the continuous time-dependent covariate, TAC level (t), was defined as the most recent TAC trough level prior or equal to time t months post-transplant, that is, equaled the TAC level at 7 days for $t < 14$ days, TAC level at 14 days for 14 days $\leq t < 1$ month, TAC level at month 1 for $1 \leq t < 2$ months, TAC level at month 2 for $2 \leq t < 3$ months, TAC level at month 3 for $3 \leq t < 6$ months, TAC level at month 6 for $6 \leq t < 9$ months, and TAC level at month 9 for $9 \leq t < 12$ months. Various threshold cut points for TAC level (<3.0 vs. ≥3.0 ng/ml, <3.5 vs. ≥3.5 ng/ml, <4.0 vs. ≥4.0 ng/ml, <4.5 vs. ≥4.5 ng/ml, <5.0 vs. ≥5.0 ng/ml, and <8.0 vs. ≥8.0 ng/ml) were also considered in testing the prognostic impact of TAC level (t), for example, TAC level 4.0 (t) = {1 if TAC level (t) <4.0 ng/ml, 0 otherwise}. Using the various categorizations for TAC level (t), nonparametric graphical display of its prognostic impact on the hazard rate of first BPAR during the first 12 months post-transplant was performed using Nelson–Aalen cumulative hazard plots, whereby the hazard rate is visualized by the slopes of the curves. Univariable analysis of the impact of TAC level (t) (defined as both continuous and categorical) was performed using Cox model score and log-rank tests, each correctly calculated based on the time-dependent nature of these covariates. Cox stepwise regression was used to determine a multivariable model of significant baseline predictors of the hazard rate of developing a first BPAR during the first 12 months post-transplant. The multivariable influence of TAC level (t) on this hazard rate was then tested after controlling for the significant baseline predictors. P-values ≤ 0.05 were considered to be statistically significant. A separate Cox model of baseline predictors using a less stringent criterion of $P \leq 0.15$ was also performed to allow for a more conservative test of the multivariable influence of TAC level (t).

Results

Patient demographics, early outcomes, and TAC trough levels

Distributions of selected baseline variables appear in Table 1. Mean age at transplant was 49.3 years; Caucasians, African-Americans, and Hispanics represented 30.1% (159/528), 27.5% (145/528), and 37.5% (198/528), respectively. Percentages of recipients having pretransplant diabetes, a pretransplant history of CAD, donor age ≥50 years, and receiving a DD kidney were 25.8% (136/528), 9.5% (50/528), 20.5% (108/528), and 78.4% (414/528), respectively. All patients were primary transplant cases, and the percentage having a pretransplant PRA ≥20% was only 3.4% (18/528). Thus, the great majority were nonhighly sensitized.

The percentage of patients developing DGF was 4.7% (25/528). The percentage of patients developing a first BPAR during the first 12 months post-transplant was 10.2% (54/528); its actuarial estimate was 10.5 ± 1.4% (figure not shown). Lastly, the observed percentage of patients who experienced graft loss during the first 12 months post-transplant was 5.9% (32/528); 4/32, 12/32, and 16/32 were due to a never functioning graft, graft failure, and death with a functioning graft, respectively.

TAC trough level distributions at the seven distinct times post-transplant are shown in Table 2. Mean TAC trough level ± SD at 1, 3, 6 and 9 months post-transplant was 7.3 ± 2.8, 6.9 ± 2.2, 6.4 ± 2.5, and 6.6 ± 2.7 ng/ml, respectively. The percentage of patients having a TAC level

Table 1. Distributions of selected baseline variables and early outcomes.

Baseline variable	Mean ± SD if continuous; % with characteristic if categorical
Recipient age at transplant (year)	49.3 ± 13.3 (*N* = 528)
Recipient age at transplant	
<50 year	47.2% (249/528)
≥50 year	52.8% (279/528)
Recipient sex	
Male	69.7% (368/528)
Female	30.3% (160/528)
Recipient race/ethnicity	
Caucasian	30.1% (159/528)
African-American	27.5% (145/528)
Hispanic	37.5% (198/528)
Other†	4.9% (26/528)
Pretransplant diabetes	
No	74.2% (392/528)
Yes	25.8% (136/528)
Pretransplant history of CAD‡	
No	90.5% (478/528)
Yes	9.5% (50/528)
Pretransplant PRA ≥20%§	
No	96.7% (510/528)
Yes	3.4% (18/528)
Total # HLA mismatches	4.0 ± 1.3 (N = 528)
# HLA DR mismatches	
0	18.6% (98/528)
1	69.1% (365/528)
2	12.3% (65/528)
Donor age (year)	37.0 ± 14.4 (*N* = 528)
Donor age	
<50 year	79.5% (420/528)
≥50 year	20.5% (108/528)
Received a DD kidney	
No	21.6% (114/528)
Yes	78.4% (414/528)
Induction with a lymphocyte-depleting agent*	
No	17.4% (92/528)
Yes	82.6% (436/528)
Assigned to receive maintenance corticosteroids	
No	74.4% (393/528)
Yes	25.6% (135/528)
Developed DGF	
No	95.3% (503/528)
Yes	4.7% (25/528)
Developed BPAR during the 1st 12 months post-transplant	
No	89.8% (474/528)
Yes	10.2% (54/528)

*Lymphocyte-depleting agent includes rATG or alemtuzumab (or both).
†"Other" includes 26 patients of Asian, Indian-Pakistani, and Middle Eastern descent.
‡Pretransplant History of CAD includes nonfatal myocardial infarction (*N* = 19), coronary artery bypass surgery (*N* = 11), and angioplasty for blocked coronary arteries (*N* = 20).
§PRA ≥20% implies that either PRA ABC ≥20% or PRA DR ≥20% (or both).

Table 2. TAC trough level distributions at the seven distinct times post-transplant.

		TAC trough level (ng/ml)		
Time	*N*	Mean ± SD	Median [interquartile range]	TAC level <4.0 ng/ml
Day 7	525	5.5 ± 3.9	5.2 [2.9–7.9]	34.9% (183/525)
Day 14	522	8.2 ± 4.2	8.0 [5.8–10.3]	12.6% (66/522)
Month 1	521	7.3 ± 2.8	7.1 [5.6–8.7]	5.4% (28/521)
Month 2	517	7.0 ± 2.3	6.9 [5.6–8.2]	6.2% (32/517)
Month 3	515	6.9 ± 2.2	6.6 [5.5–8.2]	6.0% (31/515)
Month 6	507	6.4 ± 2.5	6.2 [5.0–7.5]	9.3% (47/507)
Month 9	489	6.6 ± 2.7	6.2 [5.0–7.7]	10.2% (50/489)

<4.0 ng/ml at these times was 5.4% (28/521), 6.0% (31/515), 9.3% (47/507), and 10.2% (50/489), respectively.

Cox multivariable model of baseline predictors for the hazard rate of first BPAR

Two baseline variables were selected into the Cox multivariable model predicting a greater hazard rate of developing a first BPAR during the first 12 months post-transplant (listed by the order of selection) (Table 3): African-American or Hispanic Recipient ($P = 0.002$) and Developed DGF ($P = 0.02$). Tests to include other baseline variables were not significant. If the selection criterion was relaxed to $P \leq 0.15$, then four baseline variables were selected into the Cox multivariable model (listed by the order of selection): African-American or Hispanic recipient ($P = 0.002$), Developed DGF ($P = 0.06$), Had Pretransplant CAD ($P = 0.10$), and # HLA Mismatches ($P = 0.14$).

Kaplan–Meier freedom-from-1st BPAR curves by race/ethnicity and Developed DGF in Fig. 2 show that the actuarial percentage of patients developing a 1st BPAR during the first 12mo post-transplant ± SE was 3.9 ± 1.5%, 13.0 ± 1.9%, and 28.9 ± 10.0% for non-African-American and non-Hispanic (i.e. Caucasian/Other) recipients without DGF (*N* = 182, seven events), African-American and Hispanic recipients without DGF (*N* = 321, 41 events), and those having DGF (*N* = 25, six events), respectively.

Testing the association of TAC trough level with the hazard rate of first BPAR

Lower values for the continuous time-dependent covariate TAC trough level (t) were associated with a significantly higher first BPAR rate in both univariable ($P = 0.00006$) and multivariable ($P = 0.0003$) analysis (i.e. after controlling for the effects of the two selected baseline variables,

Table 3. Testing the effect of the continuous time-dependent covariate TAC level (t) in a Cox multivariable model for the hazard rate of developing a first BPAR during the first 12 months post-transplant (54 events).

		Cox multivariable models			
		Includes only		Includes	
		Baseline variables		TAC level (*t*)†	
Baseline variable*	Univariable *P*-value	*P*-value	Coeff ± SE	*P*-value	Coeff ± SE
Afr-Am/Hisp recipient	0.001	0.002	1.118 ± 0.386	0.006	1.067 ± 0.386
Developed DGF	0.003	0.02	0.980 ± 0.436	0.31	0.475 ± 0.464
Pre-Tx CAD	0.05				
# HLA mismatches	0.11				
DD recipient	0.19				
Recipient age	0.45				
PRA ≥20%	0.33				
Donor Age ≥50 year	0.63				
Induction w depleting agent	0.94				
TAC Level (*t*)	0.00006			0.0003	−0.199 ± 0.056

Coeff, model coefficient; Afr-Am/Hisp, African-American or hispanic; Pre-Tx, pretransplant; w, with.

*Listed baseline variables were defined as follows: Afr-Am/Hisp Recipient = {1 if Recipient Race/Ethnicity was African-American or Hispanic, 0 otherwise}; Developed DGF = {1 if patient developed DGF, 0 otherwise}; Pre-Tx CAD = {1 if Recipient had Pretransplant CAD, 0 otherwise}; # HLA Mismatches (ordinal variable); DD Recipient = {1 if DD Recipient, 0 otherwise}; Recipient Age (continuous variable); PRA ≥20% = {1 if Pretransplant PRA ≥20%, 0 otherwise}; Donor Age ≥50 year = {1 if Donor Age ≥50 year, 0 otherwise}; and Induction w Depleting Agent = {1 if Recipient Received Induction with a Lymphodepleting Agent, 0 otherwise}. The order of selection for the 2 baseline variables selected into the Cox model was as follows: Afr-Am/Hisp Recipient and Developed DGF.

†TAC evel (*t*) was defined as a continuous time-dependent covariate representing the most recently measured TAC trough level prior or equal to time t months post-transplant.

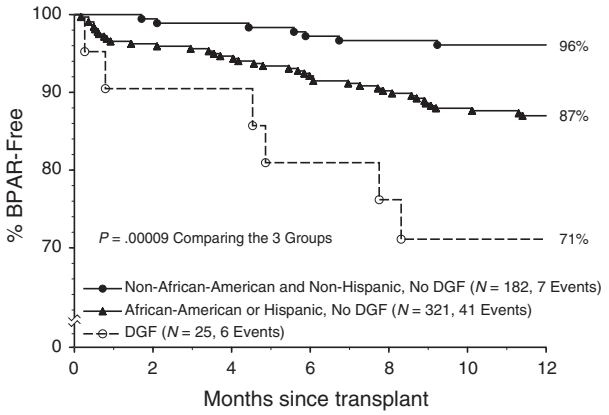


Figure 2 Kaplan–Meier freedom-from-first BPAR during the first 12mo post-transplant by race/ethnicity and DGF.

Table 3). The Cox model coefficient for this variable was −0.199, which translates into an estimated 18.0% lower BPAR rate for every 1 ng/ml increase in the TAC level. If the four baseline variables selected into the Cox model using $P \leq 0.15$ as the selection criterion (African-American/Hispanic recipient, Developed DGF, Pretransplant CAD, and # HLA mismatches) were controlled, the multivariable test of the TAC level (*t*) effect remained significant ($P = 0.0003$).

Consideration of various dichotomous cutpoints for TAC trough level yielded the following association that was even more statistically significant than the continuous variable: Patients with a TAC trough level (*t*) <4.0 ng/ml had a significantly higher first BPAR rate in comparison with the TAC level (*t*) being ≥4.0 ng/ml (i.e. the zero-one variable TAC Level 4.0 (*t*)), in both univariable and multivariable analysis ($P < 0.000001$ each, Table 4). The Cox model coefficient for this variable was 1.845, which translates into an estimated hazard ratio of 6.33, that is, a BPAR rate that is 6.33 times higher when the TAC trough level is below 4.0 ng/ml. Other threshold cutpoints, while also yielding highly significant differences, were not as strong as the <4.0 vs. ≥4.0 ng/ml comparison (see footnote #3 in Table 4). In addition, once the zero-one time-dependent covariate TAC trough level 4.0 (*t*) was controlled in the Cox model, no further categorization of TAC trough level was necessary (e.g. the comparison between TAC level 4.0–7.9 and ≥8.0 ng/ml was not significant—results not shown). Thus, it appears that the <4.0 vs. ≥4.0 ng/ml TAC trough level dichotomy provided the most accurate representation of the prognostic impact of TAC trough level on first BPAR rate during the first 12 months post-transplant, clearly more significant than using the continuous time-dependent covariate TAC level (t) and without needing any further categorization of TAC level.

Table 4. Testing the effect of the zero-one time-dependent covariate TAC level 4.0 (*t*) in a Cox multivariable model for the hazard rate of developing a first BPAR during the first 12 months post-transplant (54 events).

	Cox multivariable model includes	
	TAC level 4.0 (*t*)†‡	
Baseline variable*	*P*-value	Coeff ± SE
Afr-Am/Hisp Recipient	0.004	1.107 ± 0.386
Developed DGF	0.57	0.260 ± 0.458
TAC Level 4.0 (*t*)	<0.000001	1.845 ± 0.305

Coeff, model coefficient; Afr-Am/Hisp, African-American or hispanic.
*Listed baseline variables were defined as follows: Afr-Am/Hisp Recipient = {1 if Recipient Race/Ethnicity was African-American or Hispanic, 0 otherwise}; and Developed DGF = {1 if patient developed DGF, 0 otherwise}.
†TAC Level 4.0 (*t*) was defined as a dichotomous (zero-one) time-dependent covariate representing whether or not the most recently measured TAC trough level prior or equal to time *t* months post-transplant was at or above vs. below 4.0 ng/ml (see Patients and Methods Section for further details). Other cutpoints considered for TAC Level (*t*) included 3.0, 3.5, 4.5, 5.0, and 8.0 ng/ml, respectively.
‡Univariable Score Chi-squared test statistics for the zero-one time-dependent covariates TAC Level 3.0 (*t*), TAC Level 3.5 (*t*), TAC Level 4.0 (*t*), TAC Level 4.5 (*t*), TAC Level 5.0 (*t*), and TAC Level 8.0 (*t*) were 36.5 ($P < 0.000001$), 45.2 ($P < 0.000001$), 55.4 ($P < 0.000001$), 26.3 ($P < 0.000001$), 13.3 ($P = 0.0003$), and 0.4 ($P = 0.54$), respectively; thus, the univariable score test of TAC Level 4.0 (*t*) yielded the most significant *P*-value.

Changes in the effect of the continuous time-dependent covariate TAC level (*t*) with time since transplant were considered by including a single covariate by time interaction effect into the Cox model; a nonsignficant result was obtained ($P = 0.35$ when testing a continuous change over time; $P = 0.97$ when testing a dichotomous change with time, < vs. ≥3 months). Similarly, changes in the effect of the zero-one time-dependent covariate TAC Level 4.0 (*t*) with time since transplant were considered by including a single covariate by time interaction effect into the Cox model; a nonsignficant result was also obtained ($P = 0.99$ when testing a continuous change over time; $P = 0.58$ when testing a dichotomous change with time, < vs. ≥3 months). Thus, the magnitude of prognostic effect of TAC trough level with the first BPAR hazard rate appeared to remain consistent during the first 12 months post-transplant.

The univariable prognostic impact of the zero-one time-dependent covariate TAC trough level 4.0 (*t*) is shown visually by the cumulative hazard plot in Fig. 3a; the hazard rate of first BPAR (slope of the curve) was consistently higher throughout the first 12 months post-transplant among patients having a TAC trough level <4.0 ng/ml at the most recently measured time point ($P < 0.000001$, with a nonparametric hazard ratio of 6.57, i.e. the cumulative hazard ratio at 12 months, 0.46/0.07). Among patients having a TAC trough level <4.0 ng/ml, the median risk set just prior to the occurrence of a 1st BPAR was 41 (range: 26–183), with 21 patients experiencing a 1st BPAR. Among patients having a TAC trough level ≥4.0 ng/ml, the median risk set just prior to the occurrence of a 1st BPAR was 456 (range: 399–482), with 33 patients experiencing a 1st BPAR. Also note the convex shape of the two cumulative hazard curves, which indicates that the hazard rate of first BPAR decreases with time since transplant.

Figure 3b shows no difference in the hazard rate of developing a first BPAR during the first 12 months post-transplant between patients having a TAC trough level 4.0–7.9 and ≥8.0 ng/ml ($P = 0.21$).

Excluding patients having DGF, lower and higher risk groups for first BPAR rate were simply defined (based on the Cox model results) according to race/ethnicity: non-African-American and non-Hispanic (i.e. Caucasian/Other) versus African-Americans and Hispanics combined. Using this simple stratification, Fig. 3c and d show a consistently higher first BPAR hazard rate for those having their most recent TAC trough level <4.0 ng/ml among lower risk ($P = 0.02$, nonparametric hazard ratio: 7.33) and higher risk ($P < 0.000001$, nonparametric hazard ratio: 5.80) patients, respectively. Of note, among the 25 patients having DGF, a higher (although nonsignificant) first BPAR hazard rate was observed among those having their most recent TAC trough level <4.0 ng/ml ($P = 0.24$, nonparametric hazard ratio: 3.25; figure not shown).

Finally, a cumulative hazard plot of the effect of TAC level 4.0 (*t*) was performed separately for the 92 patients who received daclizumab alone as induction therapy and for the 436 patients who received induction therapy with one or more lymphodepleting agents (Fig. 3e and f). In each figure, a significantly higher first BPAR hazard rate was observed for those having their most recent TAC trough level <4.0 ng/ml ($P < 0.000001$ each). In summary, a significant prognostic impact of TAC trough level <4.0 vs. ≥4.0 ng/ml on the first BPAR hazard rate was observed for all major subgroups of patients in this study.

Discussion

This single-center study of 528 prospectively followed adult, primary (nonhighly sensitized) kidney-alone transplant recipients investigated the association between TAC trough levels measured over the course of the first 9 months post-transplant and subsequent BPAR risk during the first 12 months post-transplant—in the era of reduced TAC dosing and concomitant use of an IMPDH inhibitor. The time-dependent covariate approach utilized here correlated the most recently measured TAC trough

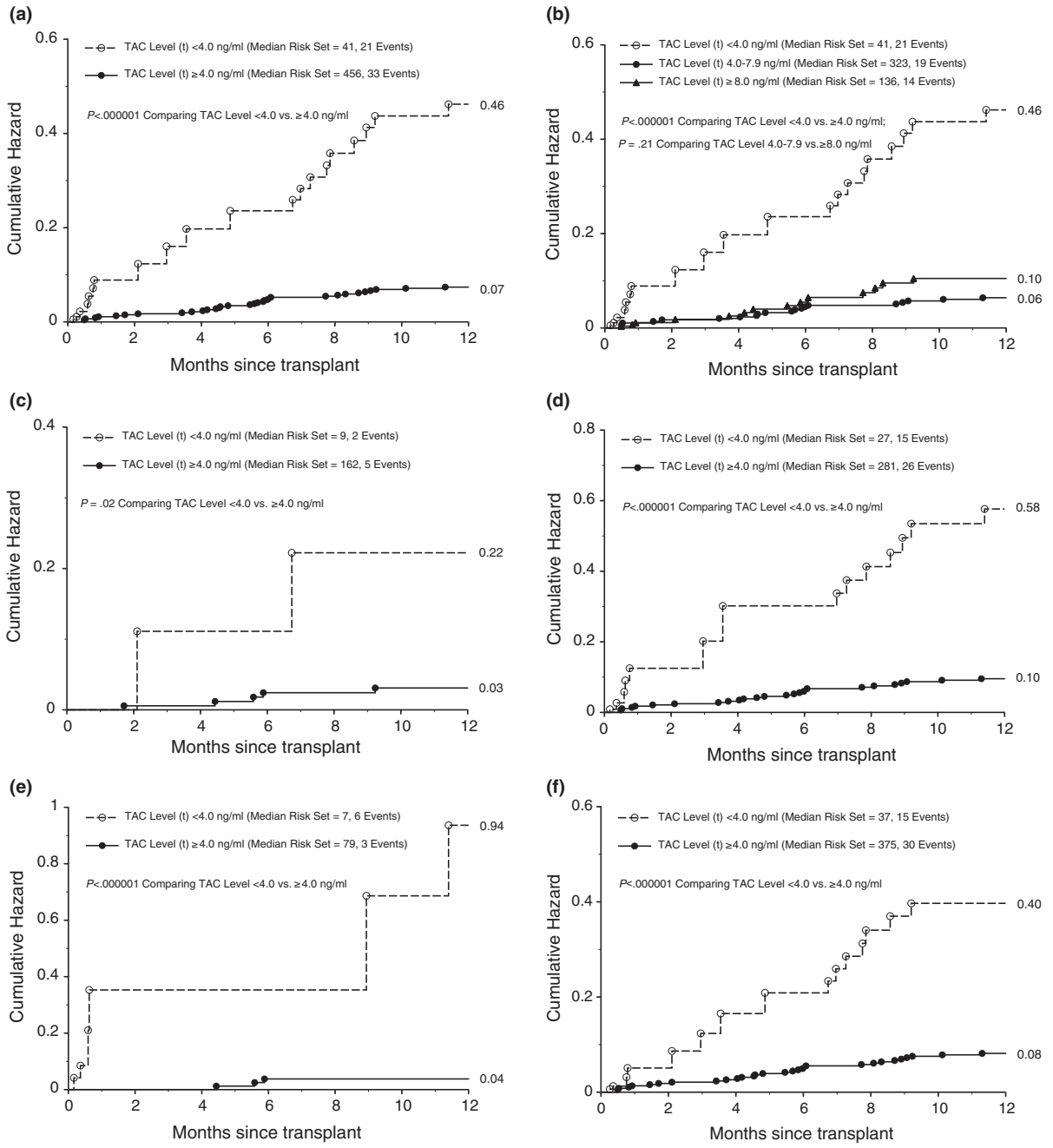


Figure 3 (a) Nelson–Aalen cumulative hazard plot showing the prognostic effect of TAC level 4.0 (*t*) (i.e. 2 TAC trough level categories: <4.0 vs. ≥4.0 ng/ml) on the hazard rate of developing a first BPAR during the first 12mo post-transplant. (b) Nelson–Aalen cumulative hazard plot showing the prognostic effects TAC level 4.0 (*t*) and TAC level 8.0 (*t*) (i.e. 3 TAC trough level categories: <4.0, 4.0–7.9 and ≥8.0 ng/ml) on the hazard rate of developing a first BPAR during the first 12mo post-transplant. (c) Nelson–Aalen cumulative hazard plot among 182 non-African-American and non-Hispanic (lower risk) patients without DGF showing the prognostic effect of TAC Level 4.0 (*t*) (i.e. 2 TAC trough level categories: <4.0 vs. ≥4.0 ng/ml) on the hazard rate of developing a first BPAR during the first 12mo post-transplant. (d) Nelson–Aalen cumulative hazard plot among 321 African-American and Hispanic (higher risk) patients without DGF showing the prognostic effect TAC level 4.0 (*t*) (i.e. 2 TAC trough level categories: <4.0 vs. ≥4.0 ng/ml) on the hazard rate of developing a first BPAR during the first 12 months post-transplant. (e) Nelson–Aalen cumulative hazard plot among 92 patients who received Daclizumab alone as induction therapy showing the prognostic effect of TAC level 4.0 (*t*) (i.e. 2 TAC trough level categories: <4.0 vs. ≥4.0 ng/ml) on the hazard rate of developing a first BPAR during the first 12 months post-transplant. (f) Nelson–Aalen cumulative hazard plot among 436 patients who received lymphocyte-depleting induction therapy showing the prognostic effect of TAC level 4.0 (*t*) (i.e. 2 TAC trough level categories: <4.0 vs. ≥4.0 ng/ml) on the hazard rate of developing a first BPAR during the first 12 months post-transplant.

level with subsequent BPAR risk, an approach which tends to maximize statistical power. We found that the hazard rate of developing a first BPAR during the first 12 months post-transplant was significantly higher among patients having a lower TAC trough level measured on a continuous scale prior to the time of BPAR occurrence. The significance of TAC trough level as a predictor of BPAR risk remained even after controlling for the effects of two significant (or four borderline significant) baseline variables in a Cox multivariable model. Use of the cutpoint <4.0 vs. ≥4.0 ng/ml for TAC level appeared to provide the most accurate description of the association of lower TAC trough levels with subsequently higher BPAR risk, and of note, 4.0 ng/ml represented the minimum of the target TAC trough level range specified in our protocols. Further categorization of TAC trough level provided no additional prognostic discrimination of first BPAR risk, and the association of TAC trough level with first BPAR risk, using either the continuous or zero-one (time dependent) covariate for TAC level, remained consistent throughout the first 12 months post-transplant (i.e. no significant interaction effect of TAC level with time since transplant was found).

While our results do not match exactly with those reported by others, for example, Israni *et al.* [12] reported a significant interaction effect of TAC level with time since transplant, and Richards *et al.* [8]. reported above vs. below 8.0 ng/ml as the significant tacrolimus level cutpoint in moderately sensitized patients, these results clearly contradict the Bouamar *et al.* study [1], which reported no association of TAC level with subsequent first BPAR risk during the first 12 months post-transplant.

If one considers how patients were selected from the FDCC [2], Opticept [3], and Symphony [4] trials into the Bouamar *et al.* [1] study, a selection bias in favor of higher risk patients chosen from the FDCC study did exist. First, only patients who received TAC were utilized in the Bouamar *et al.* [1] study (i.e. patients who received CsA were excluded), and only 45.8% of FDCC trial patients received TAC (choice of CNI varied according to center-specific protocols). Second, in a separate analysis of 669 FDCC trial patients having a day 3 CNI trough level available [27], the observed percentage of high-risk patients (defined as having DGF, ≥2nd transplant, PRA >15%, ≥4 HLA mismatches, or black race) was significantly higher among those who received TAC vs. CsA, 64.9% (237/365) vs. 55.9% (170/304) ($P = 0.02$). Thus, by selecting only those patients who received TAC from the FDCC trial, a selection bias in favor of higher risk patients occurred. As reported by Ekberg *et al.* [28], among patients in the 3 trials who received TAC and were not prematurely withdrawn, the mean TAC level (averaged over months 5.5–12.5 post-transplant) was significantly higher for those in the FDCC vs. Opticept and Symphony trials (mean ± SD: 8.4 ± 2.4 vs. 6.9 ± 2.1 and 6.6 ± 1.8 ng/ml, respectively, $P = 0.0001$). In addition, the percentage who experienced a first BPAR during the first 12 months post-transplant was 11.6%, 6.3%, and 11.5% in the three trials, respectively [28]. Thus, even after controlling for the high-risk factors in a multivariable analysis as performed by Bouamar *et al.* [1], it may still not have been sufficient to overcome the selection bias existing in the FDCC trial. In addition, concentration-controlled MMF dosing was used in approximately two-thirds of Opticept [3] trial participants (versus only one-half of FDCC [2] and none of Symphony [1] trial participants, respectively), which may have further contributed to confounding of the results reported by Bouamar *et al.* [1].

In terms of study limitations, one must always be careful in generalizing the results from a single center to the whole transplant community. This is especially true for this study given that our cohort of 528 patients was pooled across four randomized trials utilizing somewhat different immunosuppressive regimens. Still, the statistical modeling was performed with the intent of overcoming this issue (i.e. use of Cox's multivariable model), and consistency in the unfavorable prognostic effect of lower TAC levels was seen across the four randomized trials. In addition, patient eligibility and exclusionary criteria remained unchanged during this time, and all of the outcomes data were collected prospectively. Second, even with the relatively large sample size, only 54 patients experienced a first BPAR during the first 12mo post-transplant, thus limiting statistical power to detect small differences. Third, as mycophenolate acid (MPA) levels were measured in only a small subset of this cohort, statistical analysis of the simultaneous impact of TAC and MPA trough levels on subsequent BPAR risk was not possible. A more ideal study would be able to simultaneously test the influence of TAC trough level while controlling for MPA trough level, and vice versa (with both variables being defined as time-dependent covariates).

In a recent report by Daher Abdi *et al.* [29], lower MPA AUC (area under the curve) values were associated with a significantly higher BPAR risk during the first 12 months post-transplant. In secondary analyses of the Opticept [3] and FDCC [2,27] trials, a lower MPA trough level and a lower MPA AUC at day 3 post-transplant were each associated with a significantly higher BPAR rate during the first 12mo post-transplant, respectively. However, in none of these analyses was the prognostic influence of CNI level either tested or controlled. A more recent study by Daher Abdi *et al.* [30]. did simultaneously test the prognostic impact of MPA AUC and CNI trough level as time-dependent covariates, with each variable measured at weeks 1 and 2 and months 1, 3, 6, and 12 post-transplant. While a significant association of lower MPA AUC but not lower TAC level with subsequently higher BPAR risk was reported,

only 96 patients had received TAC in that study, and very few of the TAC trough levels were below 5 ng/ml.

In summary, our main conclusion is that lower exposure to TAC is associated with increased rejection risk during the first 12 months post-transplant, and more specifically, a TAC predose concentration cutoff of 4 ng/ml is suggested as the lower limit of the therapeutic window. We believe that this study is very relevant as the therapeutic window of TAC, the cornerstone of modern immunosuppressive therapy, has still not been definitively defined after being in general use for more than 20 years.

Authorship

JJG: designed research/study, performed research/study, collected data, analyzed data, wrote the paper. GC: designed research/study, performed research/study, wrote the paper. GG: designed research/study, performed research/study, collected data. JS: designed research/study, performed research/study. DR: designed research/study, performed research/study. MJG: designed research/study, performed research/study. LC: designed research/study, performed research/study. WK: designed research/study, performed research/study. AM: designed research/study, performed research/study. LT: collected data. SF: collected data. LH: collected data. PR: performed research/study. RV: performed research/study. GWB: designed research/study, performed research/study, wrote the paper.

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