ORIGINAL ARTICLE

Tacrolimus and mycophenolate regimen and subclinical tubulo-interstitial inflammation in low immunological risk renal transplants

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SUMMARY

The aim was to evaluate the relationship between maintenance immunosuppression, subclinical tubulo-interstitial inflammation and interstitial fibrosis/tubular atrophy (IF/TA) in surveillance biopsies performed in low immunological risk renal transplants at two transplant centers. The Barcelona cohort consisted of 109 early and 66 late biopsies in patients receiving high tacrolimus (TAC-C_0 target at 1-year 6–10 ng/ml) and reduced MMF dose (500 mg bid at 1-year). The Oslo cohort consisted of 262 early and 237 late biopsies performed in patients treated with low TAC-C_0 (target 3–7 ng/ml) and standard MMF dose (750 mg bid). Subclinical inflammation, adjusted for confounders, was associated with low TAC-C_0 in the early (OR: 0.75, 95% CI: 0.61–0.92; $P = 0.006$) and late biopsies (OR: 0.69, 95% CI: 0.50–0.95; $P = 0.023$) from Barcelona. In the Oslo cohort, it was associated with low MMF in early biopsies (OR: 0.90, 95% CI: 0.83–0.98; $P = 0.0101$) and with low TAC-C_0 in late biopsies (OR: 0.77, 95% CI: 0.61–0.97; $P = 0.0286$). MMF dose was significantly reduced in Oslo between early and late biopsies. IF/TA was not associated with TAC-C_0 or MMF dose in the multivariate analysis. Our data suggest that in TAC- and MMF-based regimens, TAC-C_0 levels are associated with subclinical inflammation in patients receiving reduced MMF dose.

Transplant International 2017; 30: 1119–1131

Key words
calcineurin antagonists, immunosuppression clinical, kidney clinical, mycophenolate mofetil, subclinical rejection, tacrolimus

Received: 9 April 2017; Revision requested: 8 May 2017; Accepted: 23 June 2017; Published online: 27 July 2017

Introduction

Early surveillance biopsy studies showed that subclinical tubulo-interstitial inflammation is present in more than 50% of biopsies and is associated with an accelerated progression of chronic tubulo-interstitial damage [1] and glomerular sclerosis [2]. This observation raised the question whether treatment of subclinical tubulo-interstitial inflammation could prevent progression of chronic lesions and improve outcome. This point was addressed in a clinical trial performed in cyclosporine- and azathioprine-treated patients, in which the study group was biopsied early after transplantation at defined time points and treated with steroid pulses if subclinical

doi:10.1111/tri.13002

tubulo-interstitial inflammation was present, while the control group was not biopsied and accordingly not treated. The study group had a lower degree of fibrosis at 6 months and a better renal function at 24 months [3]. Years later, this trial was repeated in patients treated with tacrolimus (TAC) and mycophenolate mofetil (MMF) and no benefit of treatment of subclinical tubulo-interstitial inflammation could be demonstrated [4]. The main difference between the first and second study was that the prevalence of subclinical inflammation was over 50% in the first and less than 10% in the second study, suggesting that TAC and MMF regimens prevent subclinical inflammation as confirmed in other observational studies [5–7]. However, it has recently been shown that even in TAC- and MMF-treated patients, the presence of subclinical tubulo-interstitial inflammation is not only associated with an accelerated progression of interstitial fibrosis and tubular atrophy but also with an increased risk for the appearance of *de novo* donor-specific antibodies and antibody-mediated rejection [8].

During the last decade, the combination of TAC and MMF has become the standard of care in the majority of renal transplant units. In some centers, TAC minimization has been favoured, especially after the publication of the Elite-Symphony trial that showed that reduced TAC (target TAC-C_0 of 3–7 ng/ml) associated with daclizumab, full-dose MMF (2 g/day) and prednisone were superior to a cyclosporine- or sirolimus-based regimen [9]. However, tacrolimus and cyclosporine have different effects on exposure to concomitantly administered MMF and for this reason, it has been recommended to use a 50% lower dose of MMF in combination with TAC compared to cyclosporin [10]. Thus, there exists significant variability in TAC and MMF dosing between centers and the consequence of these different schedules on subclinical tubulo-interstitial inflammation has not been evaluated. Accordingly, the aim was to evaluate whether TAC-C_0 and/or MMF dose at the time of surveillance biopsy are associated with subclinical tubulo-interstitial inflammation and IF/TA in low immunological risk transplants. Our hypothesis is that low TAC-C_0 and/or MMF dose at the time of biopsy are associated with subclinical tubulo-interstitial inflammation and/or IF/TA progression. To test this hypothesis, we evaluated two independent cohorts of patients (Barcelona and Oslo) treated with TAC and MMF but using different target TAC-C_0 levels (lower in Oslo than in Barcelona) and MMF dose (higher in Oslo than in Barcelona).

Patients and methods

Patients

For this study, two cohorts of adult ($\geq$ 18 years), low immunological risk, single kidney recipients of an ABO-compatible and non-HLA identical renal transplant, treated with tacrolimus and MMF with a stable well-functioning graft (eGFR $\geq$ 40 ml/min) at the time of the early surveillance biopsy were evaluated. Low immunological risk was defined as the absence of anti-HLA donor-specific antibodies (DSA) at the time of transplant, last PRA $\leq$ 20% and negative donor/recipient complement-dependent cytotoxicity cross-match.

The first cohort consisted of renal transplants with an early (3–4 months) and a late (12–18 months) surveillance biopsies performed between February 2012 and December 2015 at Vall Hebron University Hospital from Barcelona. The second cohort consisted of renal transplant recipients performed at the Oslo University Hospital Rikshospitalet between January 2009 and December 2012 with an early (6 weeks) and a late (12 months) biopsy. Written informed consent was obtained for all patients. In both cohorts, the protocol was approved by the Ethics Committee of each centre and was performed in accordance with the Declaration of Helsinki and is consistent with the Principles of the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.

Biopsies

Surveillance renal biopsies were performed as an outpatient procedure [11] under ultrasound guidance using spring-loaded 16- and 18-G needles and two cores of tissue were evaluated. One core was used for optical microscopy, and the other for immunofluorescence studies.

Biopsies were processed for routine light microscopy and stained with hematoxylin–eosin, periodic acid Schiff (PAS) and Masson's trichrome. Sample adequacy and histological lesions were evaluated according to the last update of the Banff criteria [12] at each center by local pathologists. In the Barcelona cohort, inflammation in areas of interstitial fibrosis (i-IFTA) and tubulitis in areas of tubular atrophy (t-IFTA) were also evaluated according to Mannon *et al.* [13] All biopsies were stained with an anti-SV40 antibody to discard BK polyomavirus nephropathy in Barcelona and only in patients with BK nephropathy suspicion in Oslo.

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The second core of tissue was embedded in OCT, frozen in liquid nitrogen and stored at −70 °C. Immunofluorescence studies were performed in 3-μm cryostat sections stained with FITC-conjugated anti-human IgG, IgA, IgM, C3, κ and λ light chain. C4d was stained with indirect immunofluorescence with a monoclonal antibody (Quidel, San Diego, CA, USA), and its deposition in peritubular capillaries was graded according to the Banff criteria [12]. Conventional histology and immunofluorescence were evaluated in Barcelona and Oslo by local pathologists (M.V. and M.S in Barcelona and F.P.R. and H.S. in Oslo).

In the Barcelona cohort, surveillance biopsies were not used for the clinical management of patients and, therefore, subclinical rejection episodes were not treated. In the Oslo cohort, subclinical rejection episodes were treated with steroid pulses according to the attending physician criteria.

Immunosuppression

Standard immunosuppression in the Barcelona cohort included the use of induction therapy for all renal transplants. Recipients of a first renal transplant without HLA antibodies received 20 mg of Basiliximab (Simulect®; Novartis, Basel, Switzerland) at days 0 and 4. Patients with previous transplants and/or patients with positive non-DSA anti-HLA antibodies were treated with three to five doses of rabbit antithymocyte globulin (Thymoglobulin®; Sanofi-Aventis, Paris, France) on alternate days to reach a total dose of 4–6 mg/kg. For this study, we considered patients receiving maintenance immunosuppression based on the combination of modified-release TAC (Advagraf; Astellas Pharma, Meppel, The Netherlands) and MMF (Cellcept; Roche Pharmaceuticals, Basel, Switzerland) at the time of surveillance biopsies. Target TAC-C_0 was 8–12 ng/ml during the first 3 months after transplant and 6–10 ng/ml thereafter. All patients received MMF 1 g bid during the first month and 500 mg bid thereafter. Daily dose of MMF was further reduced according to attending physician criteria in cases of suspected toxicity (mainly gastrointestinal or hematologic). The day of transplant patients received 500 mg of methylprednisolone, 125 mg at day 1 and 20 mg of prednisone at day 2. Thereafter, prednisone dose was progressively reduced to reach a daily dose of 0.1 mg/kg at 3 months and maintained during follow-up.

In the Oslo cohort, all patients received induction with Basiliximab and maintenance immunosuppression with reduced tacrolimus (TAC-C_0 3–7 ng/ml), full-dose MMF (1.5 g/day) that was reduced according to attending physician criteria in cases of suspected toxicity and prednisolone. The starting dose of prednisolone was 80 mg/day, tapered to 20 mg/day by day 8, 15 mg/day from day 30, 10 mg/day from day 60 aiming for 5 mg/day from day 90 [14].

Clinical variables

Demographic characteristics of donors and recipients as well as transplant-related variables were recorded in both cohorts. Anti-HLA antibodies at the time of transplant and at the time of each biopsy were determined by Luminex methodology in each center as previously described [15,16]. Briefly, in Barcelona, anti-HLA antibodies were determined by Luminex methodology using the product Lifecodes LifeScreen Deluxe (Gen-Probe, CA, USA) and IgG specificities were examined by single antigen beads testing with Lifecodes Luminex single antigen class I and class II kits. In Oslo, HLA antibodies were determined by Luminex platform LX200, using the LSM12- screening kit (One Lambda, Canoga Park, CA, USA). IgG antibody specificities were examined using single antigen-coated flow beads provided by One Lambda. A mean fluorescence intensity of 1000 as the cut-off value was employed. As a negative control, serum (LS-NC) delivered by the kit Producer (One Lambda) was used.

At the time of biopsy, serum creatinine, TAC-C_0 and MMF dose were recorded. Tacrolimus trough levels were measured by CMIA immunoassay (Abbott Laboratories, Abbott Park, IL, USA), and the intra-assay and interassay coefficient of variation at Barcelona and Oslo laboratories were lower than 6%. MMF dose at the time of each biopsy was recorded and expressed as mg/kg/day [17]. In patients receiving enteric-coated mycophenolic acid (EC-MPA), equimolar doses to MMF were used (720 mg of EC-MPA is equivalent to 1000 mg of MMF).

CMV prophylaxis and polyoma virus surveillance were performed according to local practice following the international criteria [18].

Statistics

Results are expressed as absolute frequencies for categorical variables and as the mean ± standard deviation for continuous variables. Comparison between groups for categorical variables was performed by Fisher's exact test. Comparison between groups for ordinal and continuous not normally distributed variables was

performed by Mann–Whitney's *U*-test. Comparison between groups for continuous normally distributed variables was performed by Student's *t*-test or by the analysis of variance and post hoc comparisons between individual groups by the Scheffé test. Similarly, Student's paired *t*-test and Wilcoxon signed-rank test were used to compare paired data.

As biopsies were graded by different pathologists, the best cut-off value for tubulo-interstitial inflammation to explore a potential association between TAC-C_0 and/or MMF daily dose was evaluated in each cohort. IF/TA at one year was defined as ci+ct score ≥ 2. Logistic regression analysis was employed to analyze variables associated with subclinical tubulo-interstitial inflammation and IF/TA at one year. Those variables with a *P*-value < 0.20 in the univariate analysis were considered for the multivariate analysis. All tests are two-tailed, and a *P*-value < 0.05 was considered significant. We used STATA software package version 13.0 (Stata Corp LP, College Station, TX , USA) for statistical analysis.

Results

Patients

During the study period, 210 kidney transplants in Barcelona and 478 in Oslo accomplishing inclusion criteria were performed. The flow chart of included patients is shown in Fig. 1.

Demographic and transplant-related variables in both cohorts are summarized in Table 1. Donors and recipients were older, the proportion of living renal transplants was lower and prevalence of delayed graft function was higher in the Barcelona cohort. The prevalence of acute rejection at the time of both biopsies was not different between centers. Serum creatinine was higher in the Barcelona cohort at the time of both biopsies. Donor-specific antibodies (DSA) were negative in the Barcelona cohort at the time of both biopsies. In the Oslo cohort, five patients displayed *de novo* DSA at the time of early biopsy (1.9%) and 17 at the time of late biopsy (7.2%). According to the immunosuppressive protocol at each centre, TAC-C_0 levels were higher in Barcelona while daily MMF dose was higher in Oslo at the time of both biopsies. Prednisone dose was similar between cohorts. Between the early and late biopsies, MMF dose was not modified in the Barcelona cohort and significantly reduced in the Oslo cohort (1.5 ± 0.2 vs. 1.3 ± 0.3 g/day; $P < 0.001$). In the Barcelona cohort, there were only four patients (6.1%) in whom MMF dose between biopsies was reduced ≥ 500 mg/day due to increasing polyoma BK viruria ($n = 1$), gastrointestinal symptoms ($n = 2$) and hematologic toxicity ($n = 1$). In Oslo, there were 57 patients (24.1%) in whom MMF dose between biopsies was reduced ≥ 500 mg/day due to trough mycophenolic acid plasma (MPA) levels ≥ 3.5 μg/ml ($n = 21$), polyoma virus replication ($n = 12$), gastrointestinal symptoms ($n = 15$), hematologic toxicity ($n = 8$) and a wart on scalp ($n = 1$).

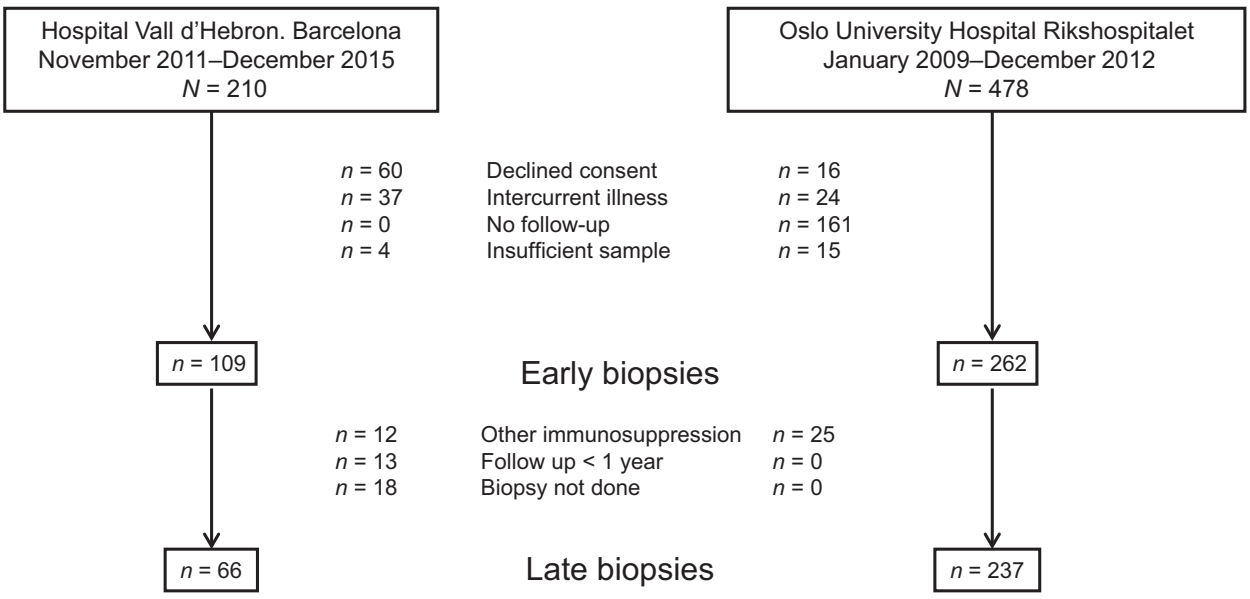

Figure 1 Flow chart of renal transplants performed in both cohorts of patients (Barcelona and Oslo) and the number of early and late surveillance biopsies obtained. DSA; HLA donor-specific antibodies; PRA, panel-reactive antibodies.

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Table 1. Demographic and transplant-related variables from the Barcelona and Oslo cohorts.

Variable	Barcelona cohort	Oslo cohort	*P*-value
N	109	262	
Donor age (years)	54 ± 15	47 ± 14	<0.001
Donor sex (m/f)	56/53	139/123	0.8197
Donor type (deceased/living)	95/14	158/104	<0.001
Patient age (years)	55 ± 13	46 ± 14	<0.001
Patient sex (m/f)	86/23	177/85	0.0328
First Tx/Re-Tx	102/7	231/31	0.1350
HLA-DR mismatches	1.06 ± 0.64	0.71 ± 0.60	<0.001
DGF (no/yes)	91/18	249/13	<0.001
AR before early Bx (no/yes)	101/8	235/27	0.3232
Time of early biopsy (months)	4.3 ± 1.5	1.5 ± 0.5	<0.001
SCr at early Bx (mg/dl)	1.39 ± 0.31	1.22 ± 0.26	<0.001
TAC-C_0 at early Bx (ng/ml)	9.6 ± 2.5	6.9 ± 2.0	<0.001
MMF dose at early Bx (g)	0.9 ± 0.2	1.5 ± 0.2	<0.001
MMF dose at early Bx (mg/kg)	13 ± 3	21 ± 5	<0.001
DSA at early Bx	0	5	0.3272
N	66	237	
Time of late biopsy (months)	17.3 ± 1.5	12.0 ± 0.5	<0.001
SCr at late Bx (mg/dl)	1.31 ± 0.32	1.16 ± 0.28	<0.001
TAC-C_0 at late Bx (ng/ml)	8.5 ± 2.2	6.3 ± 2.0	<0.001
MMF dose at late Bx (g)	0.9 ± 0.2	1.3 ± 0.3	<0.001
MMF dose at late Bx (mg/kg)	13 ± 3	18 ± 4	<0.001
Prednisone dose (mg/day)	5.4 ± 1.8	5.3 ± 1.6	0.6626
AR between early and late Bx	0	12	0.0756
DSA at late Bx	0	17	0.0289

Tx, transplantation; Bx, biopsy; DGF, delayed graft function; AR, acute rejection; SCr, serum creatinine; TAC-C_0, tacrolimus trough levels; MMF, daily MMF dose; DSA, HLA donor-specific antibodies.

Biopsies

There were significant differences in the histological findings in early and late biopsies between study cohorts (Table 2). The degree of tubulitis (t-score) was higher in the Oslo cohort in early biopsies, while the degree of interstitial inflammation (i-score) was similar. The prevalence of subclinical tubulo-interstitial rejection (i-score ≥ 1 and *t*-score ≥ 1) was not different in the early (11.9% in Barcelona vs. 18.3% in Oslo, *P*-value = 0.1660) and late biopsies (9.1% in Barcelona vs. 15.2% in Oslo, *P*-value = 0.2332) between cohorts. Despite the severity of interstitial fibrosis (ci-score) being higher in the early and late biopsies from the Barcelona cohort, the proportion of biopsies with IF/TA (ci+ct score ≥ 2) was not different in early (50.5% in the Barcelona cohort vs. 53.4% in the Oslo cohort, *P*-value = 0.6485) and late biopsies (65.1% in the Barcelona cohort and 56.5% in the Oslo cohort, *P*-value = 0.2587).

Additionally, the presence of intimal arteritis was very low in both cohorts, but it was present in 2.7% (7 of 262) early biopsies from the Oslo cohort and it was not observed in the Barcelona cohort (*P*-value = 0.1106). In late biopsies, intimal arteritis was not observed in any cohort. Similarly, the presence of glomerulitis and peritubular capillaritis was low in both cohorts but the presence of microcirculation inflammation (g-score plus ptc-score ≥ 2) was higher in the early biopsies from the Barcelona cohort (3.7% vs. 0.4% in early biopsies, *P*-value = 0.0276) but not in late biopsies (6.1% vs. 2.5%, *P*-value = 0.2329). Staining for C4d was positive in one early biopsy from both cohorts (*P*-value = 0.5018), and it was negative in all late biopsies from Barcelona and positive in five late biopsies from Oslo (*P*-value = 0.5892).

Maintenance immunosuppression and tubulo-interstitial inflammation

Barcelona cohort

There was an association between the severity of tubulo-interstitial inflammation and TAC-C_0 levels at the time of early ($P = 0.0083$) and late biopsies ($P = 0.0483$) while there was no association with MMF

Table 2. Number of glomeruli, percentage of global glomerular sclerosis and acute and chronic Banff scores in early and late surveillance biopsies from the Barcelona and Oslo cohorts.

Variable	Barcelona cohort	Oslo cohort	*P*-value
Scores early biopsies			
N	109	262	
Glomerular sections	12 ± 7	16 ± 7	<0.001
Glomerular sclerosis (%)	8.6 ± 10.4	5.1 ± 7.6	0.0004
g-score	0.08 ± 0.28	0.01 ± 0.09	0.0003
i-score	0.31 ± 0.54	0.29 ± 0.66	0.7798
t-score	0.21 ± 0.43	0.38 ± 0.70	0.0190
v-score	0	0.04 ± 0.22	0.0586
ah-score	0.42 ± 0.66	0.46 ± 0.68	0.6030
cg-score	0.01 ± 0.09	0	0.0724
ci-score	0.72 ± 0.73	0.58 ± 0.55	0.0420
ct-score	0.80 ± 0.77	0.73 ± 0.52	0.3099
cv-score	0.61 ± 0.78	0.75 ± 1.07	0.2173
mm-score	0.02 ± 0.13	0.02 ± 0.16	1.000
ptc-score	0.11 ± 0.31	0.02 ± 0.17	0.0004
Scores late biopsies			
N	66	237	
Glomerular sections	13 ± 8	14 ± 7	0.3210
Glomerular sclerosis (%)	8.0 ± 8.9	4.1 ± 7.2	0.0003
g-score	0.14 ± 0.39	0.05 ± 0.29	0.0405
i-score	0.18 ± 0.42	0.22 ± 0.57	0.5957
t-score	0.23 ± 0.42	0.35 ± 0.67	0.1685
v-score	0	0	0.999
ah-score	0.68 ± 0.75	0.64 ± 0.71	0.8310
cg-score	0.02 ± 0.12	0.01 ± 0.13	0.5747
ci-score	0.97 ± 0.84	0.66 ± 0.65	0.0015
ct-score	1.05 ± 0.69	0.84 ± 0.63	0.0197
cv-score	0.70 ± 0.91	0.59 ± 0.95	0.4019
mm-score	0.06 ± 0.39	0.04 ± 0.27	0.6323
ptc-score	0.15 ± 0.40	0.02 ± 0.17	0.0001

g-score, glomerulitis; i-score, interstitial inflammation; t-score, tubulitis; v-score, intimal arteritis; ah-score, arteriolar hyalinosis; cg-score, transplant glomerulopathy; ci-score, interstitial fibrosis; ct-score, tubular atrophy; cv-score, vascular intimal thickening; mm-score, mesangial matrix expansion; ptc-score, peritubular capillaritis.

daily dose (Table 3). As the number of cases in the category i+t ≥ 2 were too low to perform a multivariate analysis (13 in early biopsies and 6 in late biopsies) and TAC-C_0 levels were similar in patients with i+t = 1 and i+t ≥ 2, biopsies were categorized as i+t = 0 and i+t ≥ 1. TAC-C_0 was the only independent predictor of i+t ≥ 1 in the early (odds ratio [OR]: 0.75 and 95% confidence interval [CI]: 0.61–0.92; *P*-value = 0.006) (Table 4A) and late biopsies (OR: 0.69 and 95% CI: 0.50–0.95; *P*-value = 0.023) (Table 5A).

Oslo cohort

Patients with an early biopsy displaying i+t score ≥ 2 received lower MMF dose than patients with i+t = 0. Similarly, at the time of late biopsy, TAC-C_0 was significantly lower in patients with i+t ≥ 2 than in patients with i+t = 0 (Table 3). As MMF and TAC-C_0 levels were similar in patients with i+t = 0 and i+t = 1, patients were classified into two groups as i+t ≤ 1 and i+t ≥ 2. Logistic regression analysis showed that i+t ≥ 2 in the early biopsy was associated with MMF dose (OR: 0.90 and 95% CI: 0.83–0.98; *P*-value = 0.0101) (Table 4B) while in late biopsies it was associated with TAC-C_0 (OR: 0.77 and 95% CI: 0.61–0.97; *P*-value = 0.0286) after adjusting for confounding variables (Table 5B).

Maintenance immunosuppression and tubulointerstitial inflammation in scarred areas

This analysis was only performed in the Barcelona cohort as scoring for inflammation in scarred areas

Table 3. Relationship between subclinical tubulo-interstitial inflammation (i-score plus t-score) and tacrolimus trough levels and mycophenolate mofetil dose at the time of early and late biopsy in both cohorts of patients.

	Barcelona cohort			
Early biopsy	i + t = 0 (n = 69)	i + t = 1 (n = 27)	i + t ≥ 2 (n = 13)	P-value
TAC-C_0 (ng/ml)	10.1 ± 2.5	8.6 ± 2.0a	8.6 ± 2.7	0.0083
MMF (mg/kg)	13 ± 3	12 ± 3	14 ± 4	0.4523
Late biopsy	i + t = 0 (n = 46)	i + t = 1 (n = 14)	i + t ≥ 2 (n = 6)	P-value
TAC-C_0 (ng/ml)	9.0 ± 2.2	7.8 ± 1.9a	7.1 ± 2.3	0.0483
MMF (mg/kg)	13 ± 3	13 ± 4	14 ± 2	0.7640
	Oslo cohort			
Early biopsy	i + t = 0 (n = 184)	i + t = 1 (n = 30)	i + t ≥ 2 (n = 48)	P-value
TAC-C_0 (ng/ml)	6.8 ± 1.7	6.8 ± 2.2	7.0 ± 2.5	0.7987
MMF (mg/kg)	21 ± 5	21 ± 6	19 ± 4a	0.0452
Late biopsy	i + t = 0 (n = 171)	i + t = 1 (n = 30)	i + t ≥ 2 (n = 36)	P-value
TAC-C_0 (ng/ml)	6.5 ± 1.8	6.5 ± 3.2	5.7 ± 1.5a	0.0957
MMF (mg/kg)	18 ± 4	19 ± 5	18 ± 6	0.6988

TAC-C_0, tacrolimus trough levels; MMF, mycophenolate mofetil daily dose. [a]$P < 0.05$ vs. i + t = 0 group by Scheffé test.

Table 4. (A) Logistic regression analysis of clinical variables associated with subclinical inflammation (i-score plus t-score ≥ 1) in the early surveillance biopsy in the Barcelona cohort. (B) Logistic regression analysis of clinical variables associated with subclinical inflammation (i-score plus t-score ≥ 2) in the early surveillance biopsy in the Oslo cohort.

	Univariate		Multivariate	
Variable	OR (95% CI)	P-value	OR (95% CI)	P-value
(A)				
Donor age	1.02 (0.99–1.04)	0.208		
Recipient age	1.02 (0.99–1.05)	0.152	1.01 (0.98–1.05)	0.426
HLA-DR mm	1.54 (0.83–2.89)	0.172	1.44 (0.75–2.75)	0.272
AR until early Bx	1.81 (0.43–7.65)	0.423		
TAC-C_0 at early Bx	0.74 (0.61–0.90)	0.003	0.75 (0.61–0.92)	0.006
MMF dose at early Bx	1.00 (0.89–1.13)	0.971		
	Univariate		Multivariate	
Variable	OR (95% CI)	P-value	OR (95% CI)	P-value
(B)				
Donor age	0.97 (0.95–0.99)	0.0151	0.98 (0.96–1.01)	0.1564
Recipient age	0.98 (0.96–1.00)	0.0792	0.98 (0.95–1.00)	0.0987
HLA-DR mm	1.87 (1.10–3.18)	0.0211	1.83 (1.02–3.27)	0.0425
AR before early Bx	1.66 (0.66–4.17)	0.2848		
TAC-C_0 at early Bx	1.05 (0.90–1.22)	0.5076		
MMF dose at early Bx	0.91 (0.85–0.98)	0.0143	0.90 (0.83–0.98)	0.0101
DSA at early Bx	19.4 (2.11–177.5)	0.0087	18.5 (1.66–205.8)	0.0176

HLA-DR mm, number of HLA mismatches at DR loci; AR, acute rejection; TAC-C_0, tacrolimus trough levels; MMF, mycophenolate mofetil.

Table 5. (A) Logistic regression logistic analysis of clinical variables associated with subclinical inflammation (i-score plus *t*-score ≥ 1) in the late surveillance biopsy in the Barcelona cohort. (B) Regression logistic regression analysis of clinical variables associated with subclinical inflammation (i-score plus *t*-score ≥ 2) in the late surveillance biopsy in the Oslo cohort.

	Univariate		Multivariate	
Variable	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
(A)				
Donor age	0.99 (0.95–1.02)	0.463		
Recipient age	0.99 (0.96–1.03)	0.762		
HLA-DR mm	0.95 (0.44–2.08)	0.899		
AR until late Bx	0.40 (0.04–3.66)	0.417		
TAC-C_0 at late Bx	0.69 (0.50–0.95)	0.023	0.69 (0.50–0.95)	0.023
MMF dose late Bx	1.05 (0.90–1.24)	0.520		
	Univariate		Multivariate	
Variable	OR (95% CI)	*P*-value	OR (95% CI)	*P*-value
(B)				
Donor age	1.03 (1.00–1.06)	0.0360	1.03 (1.00–1.07)	0.0324
Recipient age	1.01 (0.98–1.03)	0.6253		
HLA-DR mm	1.00 (0.55–1.82)	0.9938		
AR until late Bx	5.90 (1.33–14.9)	0.0152	4.32 (1.19–15.7)	0.0260
TAC-C_0 at late Bx	0.77 (0.61–0.97)	0.0258	0.77 (0.61–0.97)	0.0286
MMF at late Bx	0.99 (0.91–1.09)	0.9421		
DSA at late Bx	2.79 (0.91–8.57)	0.0738	3.19 (0.94–10.8)	0.0622

HLA-DR mm, number of HLA mismatches at DR loci; AR, acute rejection; TAC-C_0, tacrolimus trough levels; MMF, mycophenolate mofetil dose.

Table 6. Relationship between subclinical tubulo-interstitial inflammation in scarred areas (i-IFTA score plus t-IFTA score) and tacrolimus through levels and mycophenolate mofetil dose at the time of early and late biopsy in the Barcelona cohort.

	Barcelona cohort			
Early biopsy	i-IFTA + t-IFTA = 0 (*n* = 40)	i-IFTA + t-IFTA = 1 (*n* = 16)	i-IFTA + t-IFTA ≥ 2 (*n* = 53)	*P*-value
TAC-C_0 (ng/ml)	10.0 ± 3.1	9.0 ± 1.9	9.4 ± 2.3	0.3877
MMF (mg/kg)	13 ± 4	13 ± 3	13 ± 3	0.9732
Late biopsy	i-IFTA + t-IFTA = 0 (*n* = 19)	i-IFTA + t-IFTA = 1 (*n* = 12)	i-IFTA + t-IFTA ≥ 2 (*n* = 35)	*P*-value
TAC-C_0 (ng/ml)	9.0 ± 1.9	9.4 ± 2.1	8.0 ± 2.3	0.1119
MMF (mg/kg)	13 ± 3	13 ± 3	13 ± 4	0.6750

TAC-C_0, tacrolimus through levels; MMF, mycophenolate mofetil daily dose.

was not evaluated in the Oslo cohort. There was no association between TAC-C_0 or MMF and i-IFTA+t-IFTA in early or late biopsies (Table 6). There was a correlation between the degree of tubulo-interstitial inflammation in scarred and nonscarred areas in early ($\rho = 0.26$, $P = 0.0077$) and late ($\rho = 0.38$, $P = 0.0004$) biopsies. Between early and late biopsies, the degree of i-IFTA+t-IFTA and ci+ct significantly increased while i+t remained stable.

Maintenance immunosuppression and IF/TA

Barcelona cohort

In early and late biopsies, IF/TA (ci+ct score ≥ 2) was observed in 55 of 109 biopsies (50.5%) and 43 of 66 cases (65.1%), respectively. There was no association between TAC-C_0 or MMF daily dose and IF/TA in the early or late biopsies (Table 7A). The

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Table 7. (A) Clinical and histological variables in the Barcelona cohort classified according to ci+ct score in the late biopsy. (B) Clinical and histological variables in the Oslo cohort classified according to ci+ct score in the late biopsy.

Variable	ci+ct < 2 (n = 23)	ci+ct ≥ 2 (n = 43)	P-value
(A)			
Donor age (years)	48 ± 16	56 ± 15	0.0378
Donor sex (m/f)	18/5	20/23	0.0020
LD/DD	4/19	7/36	0.5820
HLA-DR mm	1.0 ± 0.6	1.0 ± 0.7	0.6057
Patient age (years)	50 ± 13	56 ± 13	0.0568
Patient sex (m/f)	20/3	32/11	0.3460
AR before early Bx (n/y)	22/1	41/2	0.7240
TAC-C_0 early Bx (ng/ml)	10.2 ± 2.0	9.4 ± 2.3	0.1581
MMF dose early Bx (mg/kg)	13 ± 4	13 ± 3	0.5613
i+t early Bx	0.13 ± 0.34	0.63 ± 0.85	0.0090
ci+ct early Bx	0.61 ± 0.89	1.95 ± 1.11	<0.001
AR between Bx (n/y)	23/0	43/0	n.a.
TAC-C_0 late Bx (ng/ml)	9.1 ± 2.0	8.2 ± 2.2	0.1202
MMF dose late Bx (mg/kg)	13 ± 3	13 ± 4	0.5613
i+t late Bx	0.26 ± 0.45	0.49 ± 0.74	0.1813
ci+ct late Bx	0.52 ± 0.51	2.81 ± 1.10	n.a
Variable	**ci+ct < 2 (n = 103)**	**ci+ct ≥ 2 (n = 134)**	**P-value**
(B)			
Donor age (years)	42 ± 13	51 ± 12	<0.001
Donor sex (m/f)	48/55	75/59	0.1328
LD/DD	50/53	44/90	0.0170
HLA-DR mm	0.7 ± 0.6	0.7 ± 0.6	0.7800
Patient age (years)	45 ± 14	47 ± 14	0.2309
Patient sex (m/f)	73/30	87/47	0.3083
AR before early Bx (n/y)	95/8	116/18	0.1591
TAC-C_0 early Bx (ng/ml)	7.3 ± 2.4	6.5 ± 1.6	0.0092
MMF dose early Bx (mg/kg)	20 ± 4	21 ± 5	0.1024
i+t early Bx	0.58 ± 1.38	0.81 ± 1.29	0.1709
ci+ct early Bx	0.91 ± 0.89	1.61 ± 0.95	<0.001
AR between Bx (n/y)	100/3	125/9	0.1802
TAC-C_0 late Bx (ng/ml)	6.3 ± 1.6	6.3 ± 2.3	0.9782
MMF dose late Bx (mg/kg)	18 ± 4	18 ± 5	0.5645
i+t late Bx	0.17 ± 0.67	0.88 ± 1.35	<0.001
ci+ct late Bx	0.43 ± 0.50	2.34 ± 0.85	n.a.

LD, living donor; DD, deceased donor; HLA-DR mm, number of donor/recipient mismatches in locus DR; AR, acute rejection; Bx, biopsy; TAC-C_0, tacrolimus trough levels; MMF, mycophenolate mofetil daily dose.

presence of IF/TA in the late biopsy was associated with donor age, donor gender, recipient age, i+t score and ci+ct score in the early biopsy (Table 7A). Multivariate logistic regression analysis showed that female donors (OR: 4.42, 95% CI: 1.01–19.3; P = 0.0480), i+t in the early biopsy (OR: 5.03, 95% CI: 0.89–29.5; P = 0.0740) and ci+ct in the early biopsy (OR: 4.01; 95% CI: 1.77–9.10; P < 0.001) were associated with IF/TA at 1 year.

Oslo cohort

In early and late biopsies, IF/TA (ci+ct score ≥ 2) was observed in 140 of 262 biopsies (53.4%) and 134 of 237 biopsies (56.5%), respectively. In the univariate analysis, lower TAC-C_0 level at the time of the early biopsy was associated with more severe ci+ct in the late biopsy (Table 7B). The presence of IF/TA in the late biopsy was also associated with donor age, donor type, ci+ct

score in the early biopsy and i+t in the late biopsy (Table 7B). Multivariate logistic regression analysis showed that donor age (OR: 1.05, 95% CI: 1.02–1.08; $P < 0.001$), deceased donors (OR: 4.20, 95% CI: 1.00–3.63; $P = 0.0403$), ci+ct in the early biopsy (OR: 1.92, 95% CI: 1.36–2.71; $P < 0.001$) and i+t in the late biopsy (OR: 2.27, 95% CI: 1.42–3.62; $P < 0.001$) were independently associated with IF/TA at one year. In this analysis, TAC-C_0 levels at the time of early biopsy were not included into the model (OR = 0.91, 95% CI: 0.78–1.06; $P = 0.2206$).

Discussion

In the present study, lower TAC-C_0 levels at the time of the early and late biopsies were associated with the severity of tubulo-interstitial inflammation in the Barcelona cohort that received full TAC and reduced MMF dose. In the Oslo cohort, treated with reduced TAC and full MMF dose, the severity of tubulo-interstitial inflammation was associated with lower MMF dose in the early biopsy and with lower TAC-C_0 levels in the late biopsy. Of note, MMF dose was significantly reduced in the Oslo cohort from the early to the late biopsy.

Until now, there is scarce information on the relationship between TAC and/or MMF regimens and subclinical tubulo-interstitial inflammation observed in surveillance biopsies. In a study comparing a historical cohort exposed to high TAC levels (target TAC-C_0 12–15 ng/ml during the first month, 10–12 ng/ml from months one to four and 8–10 ng/ml between months four and 12) with a more recent cohort exposed to lower TAC levels (target TAC-C_0 10–12 ng/ml during the first month, 8–10 ng/ml from months two to four and 6–8 ng/ml thereafter), lower TAC exposure was associated with a reduction in polyoma virus-associated nephropathy but not with subclinical inflammation. Importantly, in both cohorts, patients were treated with MMF at 1.5 g/day [19]. Another study evaluating the relationship between TAC exposure and subclinical histological findings at 3 and 12 month in patients treated with high TAC exposure (TAC-C_0 target of 12–15 ng/ml during the first 3 months after transplantation) also failed to show any association between TAC exposure and subclinical tubulo-interstitial inflammation. However, an association between lower TAC exposure and increased progression of tubulo-interstitial chronic damage was observed [20]. In the present study, we observed an association between TAC-C_0 levels and subclinical tubulo-interstitial inflammation in the early and late biopsies from the Barcelona cohort that was treated with full TAC and reduced MMF. In the Oslo cohort, treated with reduced TAC and full MMF, tubulo-interstitial inflammation in the early biopsy was associated with MMF dose but not TAC-C_0. However, at the time of late biopsy, MMF dose was reduced for clinical indications and tubulo-interstitial inflammation was associated with TAC-C_0 as in the Barcelona cohort. We interpret that tubulo-interstitial inflammation depends on TAC-C_0, in patients receiving an MMF dose lower than 1.5 g/day. These results are in agreement with a large epidemiological study showing that TAC-C_0 levels below 5 ng/ml at one year are associated with decreased renal allograft survival. This association was significant in patients receiving a MMF dose $\leq$ 1.5 g/day while it was not observed in patients receiving a MMF dose $>$ 1.5 g/day [21]. Recently, in a prospective, open-label, randomized trial conducted in low immunological risk steroid-free kidney transplants receiving MMF at approximately 1.2 g/day, it has been shown that TAC-C_0 $<$ 7 μg/l during the first year post-transplantation is associated with clinical and subclinical rejection [22]. Altogether, these studies suggest that patients treated with low TAC and reduced MMF dose are at risk of underimmunosuppression as it has been shown that subclinical tubulo-interstitial inflammation constitutes a risk factor for the progression of tubulo-interstitial fibrosis, the appearance of *de novo* DSA and late graft failure [8,16,22,23].

Tubulitis (t-score) was higher in the early and late biopsies from the Oslo cohort. Histological evaluation was performed by local pathologists, and this difference may be the result of interobserver variability [24,25]. This interpretation is reinforced by the observation that other lesions such as microcirculation inflammation or endothelialitis were different between cohorts despite similar rejection rates. The different scoring of tubulitis between centers explains why the i+t threshold employed to classify biopsies according to the presence or absence of tubulo-interstitial inflammation was different between cohorts.

In the Barcelona cohort, interstitial inflammation and tubulitis were also evaluated in scarred areas. We did not observe any association between TAC or MMF exposure and inflammation in scarred tissue. This observation raises the question whether inflammation in scarred areas might be less responsive to immunosuppressive treatment. Unfortunately, this observation could not be tested in the Oslo cohort.

In both cohorts, IFTA in the late biopsy was mainly associated with donor characteristics and IF/TA degree in the early biopsy. In the multivariate analysis, TAC-C_0

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and MMF dose were not associated with IF/TA in the early or late biopsies in any cohort. In the Barcelona cohort, tubulo-interstitial inflammation in the early biopsy was associated with IF/TA at 1 year, and this association was on the verge of significance in the multivariate analysis. However, in the Oslo cohort, early inflammation was not associated with IF/TA at 1 year. This discrepancy may be related to the different timing of early biopsies between centers. In a study of early surveillance biopsies performed at 6 weeks, as in the Oslo cohort, the inflammatory molecular phenotype mostly reflected the injury repair response to implantation stress [26]. On the contrary, in a study of 6-month surveillance biopsies, as in the Barcelona cohort, interstitial inflammation correlated with enhanced donor-specific memory T-cell reactivity [27]. These studies suggest that in very early biopsies, tubulo-interstitial inflammation partly reflects the injury repair process, while tubulo-interstitial inflammation in biopsies performed later also reflects the donor-specific alloimmune response.

The appearance of *de novo* DSA one year after transplantation was higher in the Oslo than in the Barcelona cohort (7.2% vs. 0%). This result is in agreement with a recent prospective, randomized study showing that patients receiving a steroid-free regimen randomized to a target TAC-C_0 < 7 μg/l developed more frequently *de novo* DSA than patients randomized to a target TAC-C_0 > 7 μg/l (6.9% vs. 0%) [22]. However, our results should be interpreted with caution as the methodology to determine anti-HLA antibodies was different between centers. We understand that this finding deserves further evaluation in new prospective, randomized trials in patients receiving a maintenance immunosuppression containing steroids.

Our study was focused in low immunological risk patients with a well-functioning graft and, accordingly, they cannot be generalized to all kidney transplants. Studied cohorts were different in some transplant-related variables, reflecting different transplant policies between centers. Furthermore, TAC and MMF dosages were also different between centers. However, the association between immunosuppressive treatment and subclinical tubulo-interstitial inflammation in both cohorts supports that immunosuppressive regimen is a major determinant of subclinical inflammation. The present study has other limitations. We failed to show a significant association between early inflammation and late IF/TA as it has been previously described in other studies [16,20,28]. This might reflect insufficient statistical power, especially, if we take into consideration that subclinical tubulo-interstitial inflammation was rather low as it has been already described in TAC-treated patients in comparison with other immunosuppressive schedules [6,23]. Moreover, the progression of tubulo-interstitial chronic damage between biopsies was moderate in both cohorts as it has been described in serial biopsies obtained in TAC-treated patients [29]. The lack of centralized biopsy reading is a potential source of bias. However, systematic bias in the evaluation of tubulitis at any of both centers has been dealt using different thresholds to define the presence or absence of inflammation. Additionally, in none of the participating centers through serum mycophenolic acid levels at the time of biopsy were routinely obtained and this parameter may have contributed to better characterize the relationship between immunosuppression and subclinical inflammation. Finally, we were not able to explore whether minimization of both drugs, this means, MMF dose ≤ 1 g/day and TAC-C_0 levels < 5 ng/ml, is associated with a higher risk of subclinical inflammation as this schedule was not followed at any of both centers.

In summary, our data suggest that in low immunological risk renal transplants treated with TAC- and MMF-based regimens, TAC-C_0 levels are associated with subclinical inflammation in patients receiving reduced MMF.

Authorship

IBT: participated in the acquisition, analysis and interpretation of data, and drafts and approves the final version of the article. AVR: participated in the acquisition and interpretation of data, revising the manuscript and approves the final version of the article. FM: participated in the study design, acquisition, analysis and interpretation of data, drafts and approves the final version of the article. AA: participated in the acquisition and interpretation of data, revising the manuscript and approves the final version of the article. MV: participated in the acquisition of data, revising the manuscript and approves the final version of the article. CG-C: participated in the acquisition of data, revising the manuscript and approves the final version of the article. KM: participated in the acquisition and interpretation of the data, revising the manuscript and approves the final version of the article. FPR: participated in the acquisition of data, revising the manuscript and approves the final version of the article. HS: participated in the acquisition of data, revising the manuscript and approves the final version of the article. EC: participated in the acquisition of data, revising the manuscript and approves the final version of the article.

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Funding

This work was supported by a SET (Sociedad Española de Trasplante-Novartis) grant, Instituto Salud Carlos III (PI14/01383, PIE13/00027) and Red de Investigación renal (REDinREN 16/009/30) grants. IB Torres was supported by a VHIR (Vall d'Hebron Research Institute) grant and MA Azancot was supported by a PFIS 11/0246 (Fondo Investigación Sanitaria) grant.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

We thank Dr. Jaume Martorell and Dr. Eduard Palou from the Catalunya Histocompatibility Laboratory for anti-HLA antibody determination and Dr. Jaume Vima from the Hospital Vall d'Hebron Biochemistry Laboratory for tacrolimus trough-level determinations.

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