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ORIGINAL ARTICLE

Evolution of allograft fibrosis and function in kidney transplant recipients: a retrospective analysis of stable patients under CNI and mTORi

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Summary

Histological evaluations of renal allograft biopsies are essential for diagnosis, but still show a low predictive value for long-term allograft function. One limitation relies on the fact that the analysis is usually based on a single biopsy sample, and therefore, no dynamic changes are considered. Using two distinct approaches, we evaluated the evolution of fibrosis and related markers in 36 stable kidney transplant patients under calcineurin inhibitor therapy with two indication biopsies each, prior and at least 6 months after substitution by mTORi ($N = 18$), or maintenance on CNI ($N = 18$). In the method comparison, both Banff chronicity score and the digitally assessed fibrosis were correlated with allograft function at biopsy ($r = -0.36$ and $r = -0.72$, $P = 0.002$ and $P < 0.0001$, respectively). However, only the progression of fibrosis digitally assessed was correlated with allograft function loss, not only within the time between biopsies ($r = -0.47$, $P = 0.004$) but also in the 60-month follow-up ($r = -0.47$, $P = 0.006$). In the group analysis, despite of a higher incidence of C4d positivity ($P = 0.05$), progression of fibrosis, TGF-β1 expression, and allograft function decline were significantly lower after conversion to mTORi compared with maintenance on CNI ($P = 0.05$, $P = 0.02$ and $P = 0.01$, respectively). PDGF, VEGF, b-FGF, and HIF1A expressions remained stable over time regardless of therapy.

Introduction

In kidney transplantation, clinical studies show that early reduction or elimination of CNIs results in a significant improvement of renal allograft function [1–3], but a histological or immunohistological correlation between CNI withdrawal and prevention of any feature of CNI nephropathy has not been proven [4]. Histological abnormalities due to CNI therapy may occur early in the transplantation course, usually preceding the decline in the creatinine clearance, but only more advanced changes seem to be associated with graft loss [5–7]. One possible explanation is that these analyses are usually based on a semi-quantitative grading in the context of the Banff classification [8,9], which lacks standardization among different observers [10].

Digital analysis of transplant biopsies has been used to precisely quantify chronic alterations since the early 2000s, especially interstitial fibrosis, enhancing the potential of predicting graft function loss and/or failure through the histology [11,12]. Even with the development of better reproducible methods using automated devices [4,13,14], when the analysis is based on a single biopsy result, the predictive value of these techniques for functional outcomes of patients without severe interstitial fibrosis remains controversial [15,16]. Apart from the lack of representation of the

tissue biopsy sample [17], the absence of a second biopsy control to assess the evolution of the morphological changes may represent one of the key factors responsible for this lack of correlativity. The analysis of sequential protocol biopsies enabled not only a more precise description of the natural history of chronic CNI toxicity [7,11,18–20] but also established a correlation between evolution of fibrosis and function decline [21]. Reports evaluating these findings in indication biopsies, however, are still lacking.

To address these questions, we assessed the evolution of allograft fibrosis and related markers of selected patients, comparing maintenance therapy with CNI or conversion to mTORi. We also compared a digitally assisted method based on a proof and counterproof system and the pathologist's score (MBChS) for the assessment of these changes.

Patients and methods

Patient and clinical data

Sixty adult kidney-only allograft recipients with a low immunological risk (PRA <20%,) transplanted between 2003 and 2009 were considered eligible for analysis. Participants were selected from our database according to the following criteria: (i) two or more indication biopsies with adequate material according to Banff criteria [9] (at least 7 glomeruli, an artery sample and two separated cores); (ii) sufficient remaining material in the paraffin blocks for further slides; (iii) immunosuppression at the first biopsy based on methylprednisolone, mycophenolate, and a CNI; (iv) a period of at least 6 months between biopsies and, if the patient had the immunosuppression converted from CNI to mTORi, a period of at least 6 months after switch to the following biopsy; (v) no severe loss of allograft function during the time between biopsies, defined as a baseline function loss of <20 ml/min*1.73 m^2/year between both analyzed biopsies; (vi) adequate immunosuppressive therapy, confirmed by stable blood levels of immunosuppressives within target range; and (vi) absence of *de novo* glomerulonephritis, recurrence of underlying disease, malignant infiltration, virus infection, or urinary obstruction. Clinical information was obtained from patients' medical records. The baseline allograft function by the time of biopsy was defined as the optimal MDRD [22] value within ± 30 days from biopsy day, as the MDRD calculated at biopsy day would not necessarily reflect the allograft's potential.

The study was approved by the local institutional ethics committee (Ethical board/committee of the Medical Faculty, Ruprecht-Karls-University, Heidelberg, Germany; Internal Protocol Number S-530/2010) and conducted according to the Declaration of Helsinki. All patients provided written informed consent.

Immunosuppression

All patients were on immunosuppressive triple therapy consisting of low-dose methylprednisolone, mycophenolate acid, and a CNI at the time of the first renal allograft biopsy. Eighteen of these patients had the CNI therapy switched to mTORi therapy according to a clinical indication or a study protocol [23]; by the time of the second biopsy, 12 patients were under everolimus and six patients were under sirolimus therapy. From the remaining 42 patients who continued on CNI therapy, 18 matched patients were selected as controls according to either participation in the same study protocol ($N = 12$) or the next transplanted patient who fulfilled the selection criteria ($N = 6$). Patients who participated in the study protocol of the Herakles study were randomized after 4.5 months of transplantation to maintain cyclosporine treatment [target level 100–150 μg/l (C0)] or convert to Everolimus, [target level 6–10 μg/l (C0)].

Analysis of renal allograft biopsies

The estimation of fibrosis in the tissue fragments was performed using Masson's trichrome and Sirius red stains for the analysis of connective tissue and collagen, respectively. The Masson's trichrome-stained fragment was analyzed by the pathologist for the ascertainment of the modified Banff's chronicity score (MBChS) [8,18] and for the digitally assisted analysis of the cortical fibrosis. The MBChS was calculated after the sum of gs + ci + ct + cv from the biopsy report, whereas gs corresponds to the degree of glomerulosclerosis (0, no global gs; 1, up to 25% gs; 2, 26–50% gs; and 3, >50% gs) and ci, ct, and cv correspond to interstitial fibrosis, tubular atrophy, and chronic vascular changes as specified in the Banff classification [9].

The digitally assisted analysis was performed in a blinded manner at the same subsequently coded fragments used by the pathologist, from which five pictures of the cortical area were obtained using a digital camera (Polaroid DMC 1, Cambridge, MA, USA) attached to the optical microscope (Olympus BX45, Tokyo, Japan) under 200× magnification. These were analyzed with the assistance of image software (Image Pro Plus 6.0; Media Cybernetics, Bethesda, MD, USA), manually calibrated for the distinction of the different tissue compartments after a color cube-based method. The cortical tubulointerstitium without the subcapsular area was selected as the area of interest; the tubular lumina (white areas) were removed from the calculations to minimize the effect of the tubular atrophy on the percent count. During the method establishment phase, it became clear that technical differences in the trichrome stain were evident among the routine biopsies, so no single color

algorithm would be suitable for the analysis of all routine slides. To achieve an optimal result, we decided to analyze each slide with a different color selection and counterbalance with a counterproof system. A positive and a negative selection of the positive stain for connective tissue were performed, and the result was considered valid if the difference in the results from both kinds of analyses was less than 10%. If not, the procedure was repeated indefinitely.

All other stains were performed on extra slides prepared from the remaining tissue block and were analyzed digitally. A color cube-based selection tool was developed for each marker. Immunohistochemical stainings were performed using the following primary antibodies: IgG rabbit polyclonal anti-TGF-β1 (Santa Cruz, sc-146, Santa Cruz, CA, USA), IgG mouse monoclonal anti-HIF-1-alpha (Millipore, MAB 5382, Billerica, MA, USA), IgG goat polyclonal anti-PDGF (Upstate, 06-127, Lake Placid, NY, USA), IgG rabbit polyclonal anti-b-FGF (Abcam, ab16828, Cambridge, UK) IgG rabbit polyclonal anti-VEGF (Santa Cruz, sc-152).

The digital analyses of both histological and immunohistochemical stains of the fragments generated results given as a percentage of area positively stained/area of interest. The variation in the expression of the different markers was calculated for each patient, and the result of the first biopsy was used as the reference. The final patient's result is given as a variation of the expression in a percentage. Assuming that the development of fibrosis occurred in a linear way, this variation was corrected by the time between biopsies in the case of the Masson's trichrome and Sirius red stains.

Statistical analysis

Results of continuous variables are expressed as mean ± SEM; dichotomous variables are expressed as percentages (number of patients). Continuous variables were analyzed using a nonparametric *t*-test with Well's correction or the Mann–Whitney *U*-test when appropriate. Correlation analyses between clinical and histological variables were performed using Spearman or Pearson's correlation when appropriate. Linear regression was used to evaluate the progress of allograft function, serum creatinine, and proteinuria. Evolution of the different tissue markers was analyzed using two-way ANOVA with repeated measurements. For dichotomous variables, chi-square or Fischer's tests were performed when appropriate. The null hypothesis was rejected by $P < 0.05$.

Results

Baseline data, immunosuppression

Patient baseline characteristics were similar between the groups (Table 1). The mean patient follow-up was 86 ± 9 months; one patient from the CNI group lost follow-up at month 12. The mean time between biopsies under target immunosuppression was 18.5 ± 2.3 months and tended to be higher in the mTORi group compared with the CNI group ($P = 0.18$, Table 2). At the second biopsy, one patient of the CNI group and two patients of the mTORi group were solely on double immunosuppression with steroids and the target drug because of immunosuppression side effects. Six patients from the CNI group had their immunosuppression changed from cyclosporine to tacrolimus; for three of those patients, the change was performed prior to the second biopsy. Two patients from the mTORi group had their mTORi therapy paused at 2 and 143 months after the second biopsy because of persistent leucopenia. For another five patients in the mTORi group, the mTORi therapy was substituted by CNI. Reasons for the substitution were as follows: antibody-mediated

Table 1. Baseline characteristics of the studied groups.

	CNI ($N = 18$)	mTORi ($N = 18$)	*P*
Age (years) – mean ± SEM	48 ± 3.4	44 ± 3.4	ns
Female gender – *n* (percent)	6 (33%)	5 (29%)	ns
Baseline kidney disease – *n* (percent)			
Glomerulonephritis	11 (61%)	9 (50%)	ns
Vasculitis	0	0	ns
Diabetes	1 (6%)	0	ns
Nephrosclerosis	0	1 (6%)	ns
Obstructive	3 (17%)	1 (6%)	ns
Genetic (ADPKD, Alport's disease)	2 (11%)	4 (22%)	ns
Other/Unknown	1 (6%)	3 (17%)	ns
Hypertension – *n* (percent)	17 (94%)	17 (94%)	ns
Insulin-dependent diabetes – *n* (percent)	2 (11%)	2 (11%)	ns
Living donor – *n* (percent)	7 (39%)	6 (33%)	ns
Donor age (years) – mean ± SEM	51 ± 2.1	54 ± 3.7	ns
Patients with prior transplants – *n* (percent)	2 (11%)	1 (6%)	ns

Table 2. Biopsy and biopsy-related data.

	CNI (N = 18)	mTORi (N = 18)	P
First Biopsy			
Serum creatinine at biopsy day (mg/dl) – mean ± SEM	2.2 ± 0.25	2.3 ± 0.25	ns
MDRD at biopsy day (ml/min/1.73 m^2) – mean ± SEM	33 ± 4.5	35 ± 2.9	ns
Patients on triple immunosuppression – *n* (percent)	18 (100%)	18 (100%)	ns
Primary pathological diagnosis – *n* (percent)			
No significant immunological changes	3 (16%)	1 (6%)	ns
Borderline changes	7 (39%)	2 (11%)	0.18
Acute T-cell-mediated interstitial rejection	0	2 (11%)	ns
Acute T-cell-mediated vascular rejection	1 (6%)	1 (6%)	ns
C4d deposition without active rejection	1 (6%)	1 (6%)	ns
Interstitial fibrosis/tubular atrophy alone	2 (11%)	7 (39%)	0.2
Other	4 (22%)	4 (22%)	ns
MBChS (gs + ci + ct + cv) – mean ± SEM	2.9 ± 0.4	3.3 ± 0.5	ns
Following biopsy			
Time between biopsies (on target immunosuppression) (months) – mean ± SEM	14 ± 1.9	23 ± 4.0	0.18
Serum creatinine at biopsy day (mg/dl) – mean ± SEM	2.3 ± 0.4	2.1 ± 0.2	ns
MDRD at biopsy day (ml/min/1.73 m^2) – mean ± SEM	38 ± 4.6	41 ± 4.1	ns
Patients on triple immunosuppression – *n* (percent)	17 (94%)	17 (94%)	ns
Primary pathological diagnosis – *n* (percent)			
Borderline changes	12 (66%)	4 (22%)	0.05
Acute T-cell-mediated Interstitial rejection	5 (28%)	2 (11%)	ns
C4d deposition without active rejection	0	1 (6%)	ns
Acute or chronic active antibody-mediated rejection	0	4 (22%)	0.1
Interstitial fibrosis/tubular atrophy alone	1 (6%)	7 (39%)	0.05
Overall C4d deposition	0	5 (28)	0.05
MBChS (gs + ci + ct + cv) – mean ± SEM	4.2 ± 0.4	4.1 ± 0.4	ns

rejection in two patients (3.3 and 29.8 months after second biopsy); acute cellular rejection, with and without presence of DSA in one patient each (0.7 and 37.8 months after second biopsy, respectively); and recurrence of focal segmental glomerulosclerosis in one patient (2.9 months after second biopsy).

Allograft function and survival

During an observation period of 60 months after transplantation, the serum creatinine increase tended to be higher in the CNI group compared with mTORi (slope 0.008 ± 0.002 vs. 0.004 ± 0.001, $P = 0.13$), which was confirmed by the analysis of the MDRD, whereas allograft function loss was significantly higher in the CNI group compared with mTORi (slope −0.25 ± 0.16 vs. −0.18 ± 0.02, respectively, $P = 0.01$). Evolution of proteinuria was not significantly different between the groups; only two patients developed proteinuria of over 1.0 g/24 h at month 60, both from the CNI group. Overall allograft survival was not significantly different between the groups; only two patients from the CNI group lost their allografts in the first 60 months of transplantation (months 12 and 56). The data are summarized on Fig. 1.

Biopsy and biopsy-related data

In line with the fact that all biopsies performed were indication ones, MDRD at first biopsy day was significantly lower than the baseline (33.9 ± 12.5 vs. 47.4 ± 15 ml/min/1.73 m^2, $P = 0.01$). At second biopsy, a similar tendency was seen (39.6 ± 18.3 vs. 43.6 ± 18.3, $P = 0.08$).

At the first biopsy, while all patients were under CNI treatment, no significant difference in the diagnostic profile was seen between the groups; C4d positivity was evidenced in one patient from each group, none of them with other signs of antibody-mediated rejection. Assessment of the MBChS also showed no significant difference between the groups. At the second biopsy, none of the patients in the CNI group presented a C4d positivity, whereas five in the mTORi group did ($P = 0.05$). Two of these biopsies had significant microvascular inflammation and another two had evidence of chronic tissue injury, confirming the presence of antibody-mediated rejection in four patients from the mTORi group ($P = 0.1$ vs. CNI).

On the other hand, 17 (94%) patients from the CNI group presented at least a sign of rejection at the second biopsy (12 of them had borderline changes), compared with 11 (61%) in the mTORi group ($P = 0.04$). MBChS at

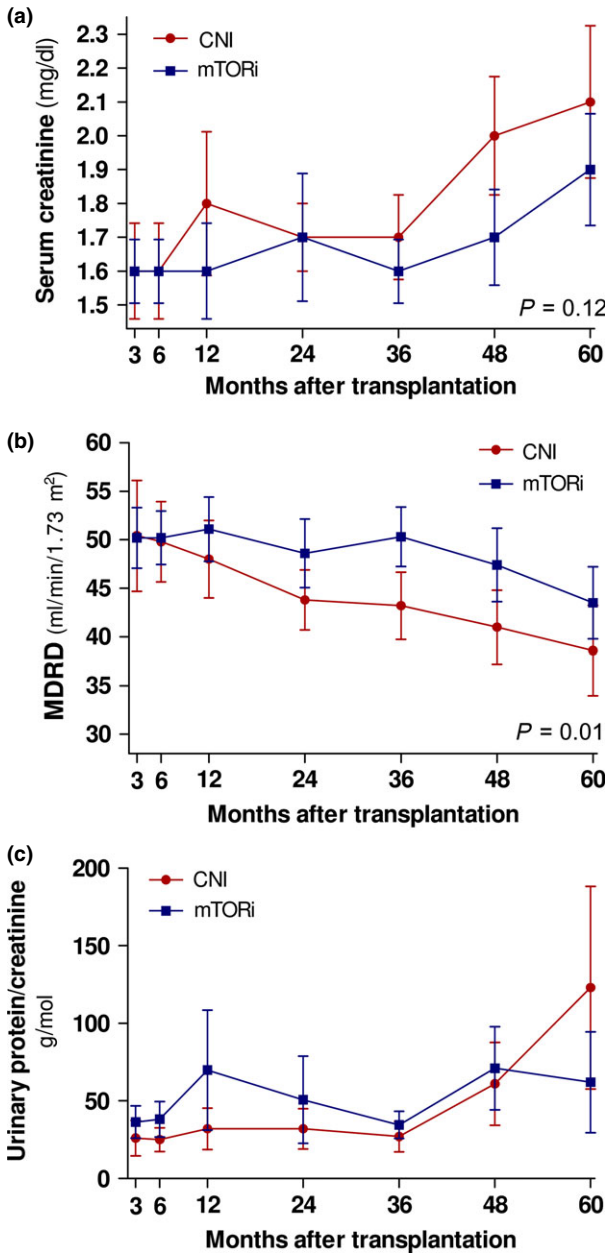

Figure 1 Evolution of allograft function, proteinuria, and survival after transplantation in the studied groups. After randomization, the increase in serum creatinine (a) tended to be higher in the CNI group compared with mTORi, by MDRD (b) a significant difference between both groups was seen. Urine protein/creatinine ratio (c) did not change significantly; the sudden rise observed at month 60 on the CNI group is due to the occurrence of significant proteinuria (>1.0 g/24 h) in two patients. The 60-month allograft survival was also not significantly different. Data are plotted as mean ± SEM.

second biopsy were practically equal in both groups (4.2 ± 0.4 vs. 4.1 ± 0.4 in the CNI and mTORi groups, respectively). All data are summarized in Table 2.

Evolution of kidney fibrosis

The increase in the connective tissues' and collagens' positively stained area on the kidney biopsies was significantly lower in patients under mTORi therapy compared with CNI.

Even when no significant differences in the individual MBChS in the first and second biopsies between both groups were identified, a significantly higher increase in the score corrected by the time between biopsies in the CNI group compared with mTORi could be observed (+1.5 ± 0.4% vs. +0.4 ± 0.2% change/year, $P = 0.04$). The digitally assisted analysis of the biopsy specimen's cortex confirmed this result, showing a + 2.7 ± 0.8% compared with a −0.25 ± 0.9% change/year in the CNI and in the mTORi group, respectively ($P = 0.02$).

In accordance with the results of the analyses in the Masson's trichrome-stained slides, the digitally assisted analysis of the Sirius red stain showed a significantly higher yearly increase in the collagens' positively stained area in the CNI group (+6.1 ± 1.4%) compared with mTORi (+0.8 ± 0.6% change/year, $P = 0.002$). A correlation between pathological diagnosis and evolution of fibrosis could not be established. Representative pictures of Masson's trichrome- and Sirius red-stained slides are shown in Fig. 2.

Immunohistochemical expression of fibrosis-related markers

Tubular expression of TGF-β significantly decreased in the mTORi-treated patients. Kidney allograft biopsies from patients of the mTORi group showed a significant decrease in the tubular TGF-β1 expression (10.1 ± 0.9% vs. 7.9 ± 0.7% of area positively stained, first to last biopsy, $P = 0.05$). Additionally, the variation in the expression between the first and second biopsy was significantly different between the CNI and mTORi groups (0.6 ± 1.0% vs. −2.2 ± 0.9% change, $P = 0.05$). The expression in the tubulointerstitium was concentrated in the tubular compartment, primarily in the proximal and distal tubuli and more prominent when compared with the expression in the glomeruli. Glomerular expression was observed primarily in the mesangium, but also variable within the kidney biopsy. No significant differences in the TGF-β1 expression in the glomerular compartment were seen. Representative sections of TGF-β1 expression are shown in Fig. 3.

To investigate a possible chronic effect contribution of chronic calcineurin-induced ischemia in the allograft tissue of CNI-treated patients, we assessed the expression of HIF1A and VEGF (as a response to the HIF2A signaling) in the different kidney compartments: HIF1A stain in the

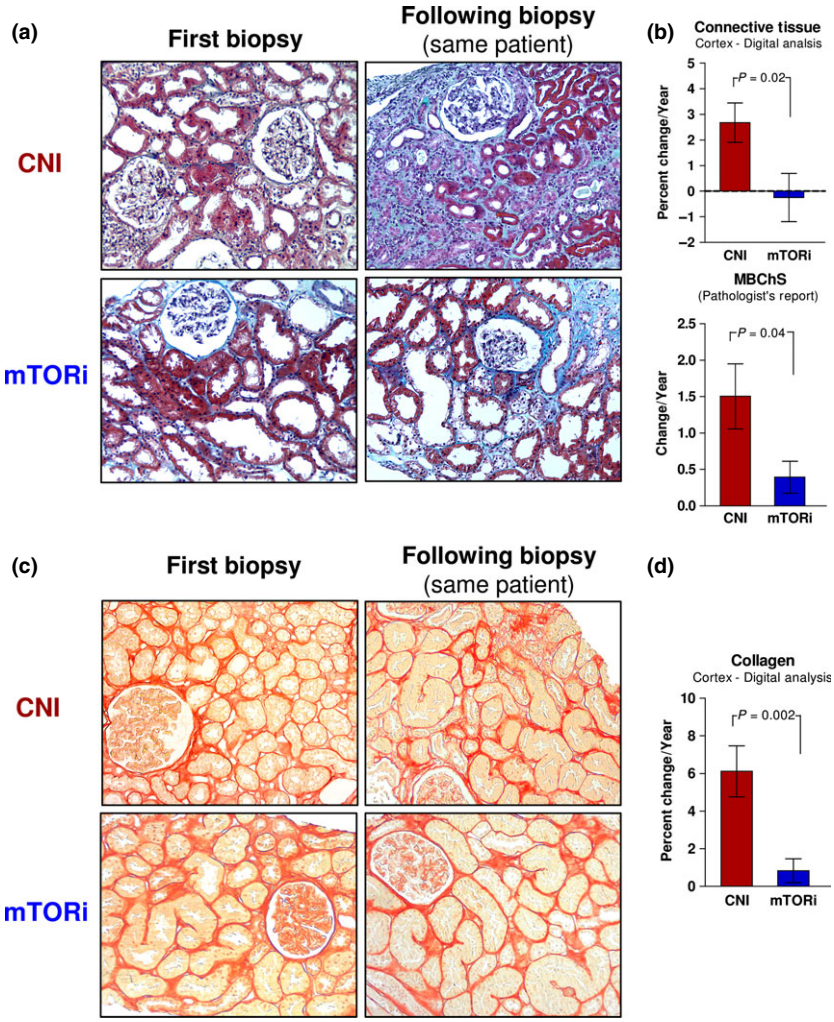

Figure 2 Evolution of allograft fibrosis in the studied groups accessed through measurements of connective tissue (Masson's trichrome stain) and collagen (Sirius red) in paraffin sections. Representative sections of allograft biopsies were obtained before and 1.5 years after randomization. Note the diffuse increase of the connective tissue area in blue (a), which was significantly less pronounced in the mTORi group compared with CNI, as accessed by both the digitally assisted method and the pathologist's MBChS, corrected by the time between biopsies (b). In the Sirius red stain for collagen (c, lower pictures), a similar pattern before and after the change in immunosuppression was observed. Although cortical collagen expression suffered a slight increase in the CNI group, it remained practically stable in the mTORi group as confirmed by the digitally assisted analysis (d). T bars indicate standard errors; bars indicate mean values. Photographs performed under a 200× magnification.

kidney was highly specific, but only observed in tubular cells. The expression was overall low and quite variable within the kidney. Neither the expression in the second biopsy (after the change in the immunosuppression) nor the variation between the two biopsies was different between the groups.

Expression of VEGF in the glomeruli and in vessels remained stable and was not different between the groups. Tubulointerstitial VEGF expression tended to decrease after switching to mTORi ($5.5 \pm 0.9\%$ vs. $3.7 \pm 0.6\%$ of area positively stained, first to last biopsy, $P = 0.1$).

Additionally, the investigation of fibrogenic factors in the kidney allografts was complemented by the analysis of b-FGF and PDGF. Expression of b-FGF was primarily identified in the tubulointerstitium and tended to increase in the CNI group compared with the first biopsy ($3.6 \pm 0.8\%$ vs. $5.3 \pm 0.7\%$ of area positively stained, first to last biopsy, $P = 0.09$), but was not significantly different from the mTORi group. PDGF expression in the different compartments (glomeruli, tubulointerstitium and vessels) remained practically stable and was not significantly different between the groups.

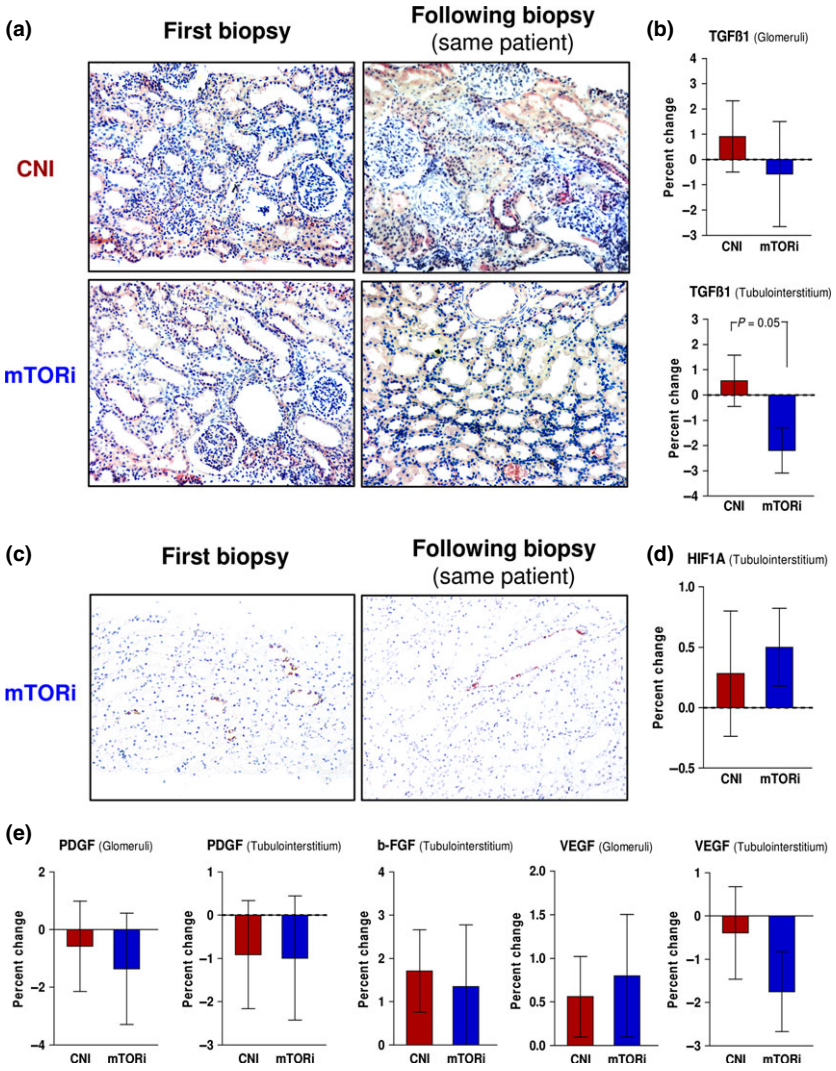

Figure 3 Evolution of the immunohistochemical expression of fibrosis-related markers in glomerular and tubulointerstitial compartments. After the switch to mTORi, a significant reduction in the TGF-β1 tubulointerstitial expression compared with both baseline and the CNI group was observed (a and b). HIF1A stain was very specific and restricted to the tubuli (c), but also variable and not significantly different between the two groups (d). Expressions of PDGF, b-FGF, and VEGF in the different allograft compartments remained practically stable in the two groups and were also similar. T bars indicate standard errors; bars indicate mean values. All photographs were performed under a 200× magnification.

Representative sections of the immunohistochemical stains and analyses are shown in Fig. 3.

Correlation between markers of fibrosis and allograft function

As the results regarding allograft function loss and increase in fibrosis were congruent, we decided to investigate the correlation between allograft function and fibrosis in the routine Masson's trichrome-stained slides, comparing the pathologist's MBChS and the digitally assisted analysis. First, we verified the correlation between the baseline allograft function ± 30 days from the time of biopsy and the MBChS and digitally assisted analysis fibrosis values (Fig. 4a and b). Although the MBChS value showed a strong (negative) correlation with the MDRD ($r = -0.72$, $P < 0.0001$), only a weak correlation between the digitally assisted analysis value and MDRD ($r = -0.36$, $P = 0.002$) could be observed. The two fibrosis-measurement techniques were also weakly correlated with each other ($r = 0.30$, $P = 0.01$) (Fig. 4c).

When we assessed the evolution of allograft function and fibrosis, however, a different pattern was observed. Although no significant correlation between the difference in the MDRD and MBChS was seen (Fig. 4d), the change

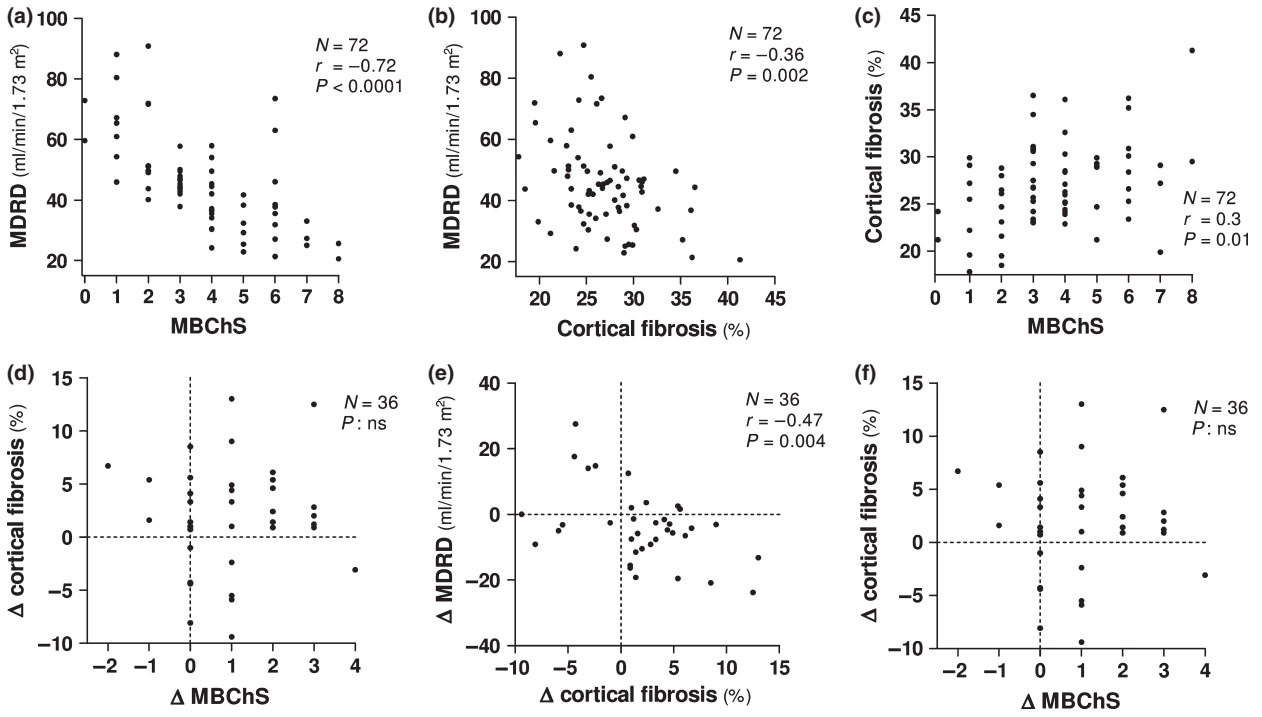

Figure 4 Relationship between the modified Banff chronicity score MBChS (gs + ci + ct + cv) obtained from the pathologist's report, cortical fibrosis electronically measured on the same Masson's trichrome fragment and estimated allograft function by MDRD. The MBChS showed a stronger correlation to the patient's baseline MDRD (a) than the electronic assessment of cortical fibrosis (b), and the absolute values of both kinds of analyses were intercorrelated (c). A distinct pattern was observed when the patient's evolution was analyzed: Although the change in the MBChS between the biopsies did not correlate with the change in the allograft function (d), a moderate correlation between the change in the cortical fibrosis and the change allograft function between the two biopsies was observed (e). The evolution of both parameters was also not intercorrelated (f).

in the MDRD between the biopsies was significantly correlated with the change in the allograft fibrosis ($r = -0.47$, $P = 0.004$) (Fig. 4e). The evolution of allograft fibrosis assessed by the two different methods was not significantly correlated (Fig. 4f). When both the CNI and mTORi groups were analyzed separately, all correlations were less significant because of the smaller group size, but no discrepancies were found.

To investigate whether the evolution of allograft fibrosis between the two biopsies could serve as a predictive value for the evolution of an allograft function in the long term, we performed the correlation analysis between the evolution of allograft fibrosis and the yearly loss of allograft function in an up-to-60-month observation period. Thirty-four patients were analyzed, as one patient suffered a graft loss and another was lost before the 60-month follow-up (mentioned previously). Although the digitally assisted evolution of allograft fibrosis was significantly correlated with the yearly loss of allograft function ($r = -0.47$, $P = 0.006$), no significant correlation between the change in MBChS and MDRD/year could be observed (Fig. 5a and b, respectively).

Discussion

Our study shows that, in stable kidney transplant patients, the evolution of the allograft fibrosis between two indication biopsies assessed by a digitally assisted method was at least modestly correlated with the fall in the MDRD allograft function not only by the time of biopsies, but also in a 60-month follow-up period. In the group analysis, we observed that the conversion from a CNI-based regimen to mTORi in a selected low immunological risk population was associated with a lower increase rate of allograft fibrosis when compared with patients under maintenance of CNI therapy, regardless of the method of tissue analysis or the pathological diagnosis. This was paralleled with a significant reduction in the TGF-β1 expression and a better allograft function in the mTORi group, compared with CNI. To our knowledge, all of the histological findings are novel.

The better results of allograft function in the mTORi group have to be interpreted with caution: 7 of the 18 patients converted to mTORi had their therapy either discontinued or substituted by CNI in the long term (all after the second biopsy) and four of them due to alloreactivity. Even with a better functional outcome after 5 years, we

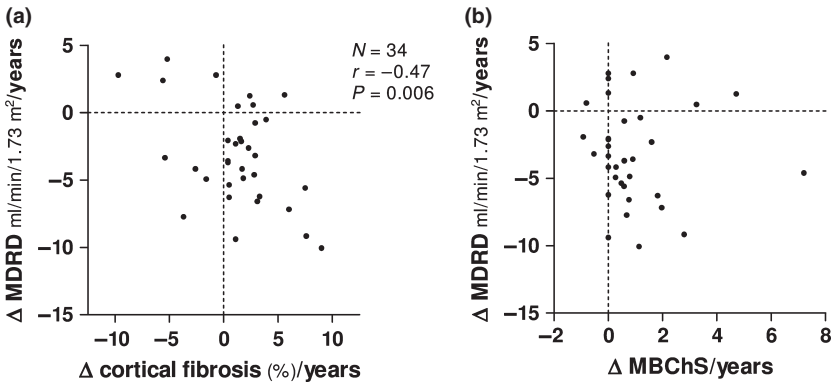

Figure 5 Correlation between the evolution of allograft fibrosis and the renal function loss. (a)The variation of cortical fibrosis corrected by the time between biopsies was moderately correlated with the yearly change of allograft function in the first 5 years after transplantation. This correlation was not observed in the analysis of the evolution of MBChS (b).

found a significantly higher incidence C4d positivity and a tendency for a higher incidence of antibody-mediated rejection in the mTORi group, which is in accordance with the findings in literature reporting not only higher incidence of *de novo* donor-specific antibodies [24,25] but also of antibody-mediated rejection in mTORi patients compared with CNI [26]. In the other hand, the incidence of biopsies without any sign of rejection was also significantly higher in the mTORi group compared with CNI, so that it was not possible to draw a uniform picture about the immunological processes in the graft after the conversion to mTORi.

Despite of the major concerns regarding the employment of mTORi-based protocols in kidney transplantation [27], more recent prospective evidence confirms that conversion from CNI to mTORi-based immunosuppression results in a significant increase in eGFR, especially after early conversion within 6 months after renal transplantation [2,3,28–30]. Late elimination of CNI therapy was less effective concerning renal allograft function and outcome [31–34]. For these reasons, it is generally accepted that an early conversion from CNI to mTORi therapy is advisable in an immunological low-risk population and offers the opportunity for an immediate [35,36] and sustained [29,37,38] improvement of renal allograft function. In the ZEUS study [38], an increased incidence of BPAR during the first 2–3 months after randomization has been shown; however, BPARs were classified by mild (Banff grade I) rejection, and only very few patients experienced BPAR in both treatment arms after month 12 after transplantation. In the SMART trial, patients on mTORi treatment with BPAR showed similar eGFR at 3 years compared with those without BPAR, suggesting that mild rejections are followed by recovery of allograft function, but no data on chronic allograft changes were shown in the study. Similarly to our observation, there was a high incidence of reconversion to CNI in the SMART study [39].

As the diagnosis of the following biopsies between CNI and mTORi groups were markedly different but did not seem to influence the evolution of fibrosis in our observation, we investigated the expression profile of fibrogenic factors which could contribute to the slower development of kidney fibrosis in the mTORi group [40]. Although the increase of TGF-β expression in the tissue allograft in CNI-treated patients was reported long ago [41–43], the evolution of the expression of TGF-β in the kidney tissue after mTORi treatment remains controversial [44–46]. In a recent experimental study investigating the amelioration of cyclosporine-induced nephropathy after conversion to sirolimus, the authors demonstrate a significant reduction of TGF-β expression in the kidney tissue after conversion, which may solely reflect the cessation of CNI therapy, at least in the short term [47]. However, an observation of 128 kidney transplant biopsies, the mRNA expression of TGF-β, was significantly influenced by the presence and the degree of interstitial fibrosis, rather than by any other pathological diagnosis including acute rejection [48]. We believe that both of these factors may have influenced the TGF-β expression in our biopsies, explaining, at least in part, the lower expression of TGF-β1 in the mTORi group.

Due to the chronic ischemia induced by CNI therapy [49] and the potential pathogenetic effect in the kidney of chronic HIF1A activation [50] associated with the upstream interaction between TGF-β, mTOR (and HIF1A) [51], we decided to investigate the expression of HIF1A in the different groups. In contrast to the findings of Rosenbergerger *et al.* [52] who found almost no HIF1A expression in stable patients and a higher expression of the marker in acute rejection 3 months after transplantation, the expression of HIF1A in our study was neither influenced by the pathological diagnosis nor by the conver-

sion to mTORi. A possible explanation for the similar results of HIF1A expression in both groups relies on the fact that HIF1A expression is directly influenced by calcineurin, so that the calcineurin inhibitor treatment could directly affect the production of HIF1A. As we were not able to establish a specific HIF2A stain in our kidneys samples, we decided to investigate the expression of VEGF as an additional maker for the hypoxic response [53], which also was not significantly different between the groups, the same for PDGF and b-FGF. Without the availability of frozen material, the analysis of pathomechanistic changes, especially in the chronic setting, is very difficult.

Another key issue was to evaluate the best method to correlate chronic histological changes with allograft function in indication biopsies. Morphometric analysis can better assess the different aspects of chronicity on a kidney biopsy [19,54], and the interpretation of an experienced renal pathologist is more helpful in ponderating these than a single analysis of fibrosis. Indeed, our results showed that only the MBChS assessed by the pathologist was strongly correlated with the optimal allograft function $\pm$ 1 month from biopsy. It has to be pointed out, however, that the pathologist had full access to the patients' clinical data, so no blinded comparison could be performed. Regardless, the Banff classification of the kidney fibrosis score "ci" in three gradings is not precise and may not be able to detect minor changes in fibrosis, compared with a digitally assisted method which is much more suitable to assessing the evolution in the same patient [21].

As previously mentioned in the recently published Banff fibrosis study [16], "there is no consensus regarding the best way to assess interstitial fibrosis (in a kidney biopsy)." Furthermore, tissue-processing techniques and stains are not standardized among different centers, generating discrepancies in the results. We encountered the same problem in our study with the trichrome analysis, which was performed using routine-stained slides, fixed, and stained using standardized techniques, but at different time points (see "Patients and methods" section). The use of a proof and counterproof system was proved to be effective to overcome this technical issue, so that even slight differences in fibrosis could be detected and these were congruent to clinical data, irrespective of the studied group.

The main advantages of the presented method rely on the possibility to integrate the interpretation of the researcher with the precision provided by the computer-based analysis. Additionally, the presented method has a better availability compared with the analysis of virtual slides, as it requires only a good quality microscope with an attached camera. The main disadvantage is that a congruent proof and counterproof analysis is relative laborious and time-consuming for the routine usage; nevertheless, the benefits of a more precise analysis of fibrosis (maybe in combination with other aspects of chronicity) are irrefutable.

In conclusion, conversion from CNI to mTORi with close clinical and histological monitoring may constitute a suitable alternative for stable transplant patients to reduce progression of chronic allograft changes including fibrosis and prolonged renal allograft survival. Digital analysis of fibrosis progression, even if performed on indication biopsies, may be indicative for the evolution of allograft function, a finding that still requires validation in a larger population.

Authorship

LEB: performed study conception and design, developed and performed the immunohistochemical and tissue analysis protocols, participated in patient data analysis, and wrote the manuscript. BW: assisted in patient data assessment, and in the histological and immunohistochemical analysis. XY: participated in the immunohistochemical tests and analysis. M-LG-W: performed histological evaluation of the biopsy specimens. RW: performed histological evaluation of the biopsy specimens and C4d staining. MZ: supervised study conception, performance of the study, and writing of the manuscript. CS: performed study conception and design, provided patient data, supervised the analysis of clinical data, collection of biopsies, and wrote the manuscript.

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