TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Progression of pulse pressure in kidney recipients durably exposed to CsA is a risk factor for epithelial phenotypic changes: an ancillary study of the CONCEPT trial

Yi-Chun Xu-Dubois,[1,2] Alexandre Hertig,[2,3,4] Yvon Lebranchu,[5] Bruno Hurault de Ligny,[6] Eric Thervet,[7] Maïté Jauréguy,[8] Guy Touchard,[9] Patrick Le Pogamp,[10] Yann Le Meur,[11] Olivier Toupance,[12] Anne-Elisabeth Heng,[13] Francis Bayle,[14] Sandrine Girardot-Seguin,[15] Aude Servais,[7] Vannary Meas-Yedid[16] and Eric Rondeau[2,3,4]

1 Département de Santé Publique, APHP, Hôpital Tenon, Paris, France
2 Sorbonne Universités, UPMC Univ Paris 06, UMR_S1155, Paris, France
3 Urgences Néphrologiques et Transplantation Rénale, APHP, Hopital Tenon, Paris, France
4 Université Pierre et Marie Curie, Paris, France
5 CHU Bretonneau, Tours, France
6 CHG Clémenceau, Caen, France
7 Hôpital Necker, Paris, France
8 Hôpital Sud, Amiens, France
9 CHU La Milétrie, Poitiers, France
10 Hôpital Pontchaillou, Rennes, France
11 Hôpital La Cavale Blanche, Brest, France
12 Hôpital Maison Blanche, Reims, France
13 Hôpital Saint Jacques, Clermont Ferrand, France
14 CHU Grenoble, La Tronche, France
15 Laboratoires Roche, Neuilly/Seine, France
16 Institut Pasteur, Paris, France

Keywords
cyclosporin, epithelial to mesenchymal transition, interstitial fibrosis and tubular atrophy.

Correspondence
Pofessor Alexandre Hertig, Urgences Néphrologiques et Transplantation Rénale, Hôpital Tenon, 4 rue de la Chine, 75020 Paris, France. Tel.: 33156016510;
fax: 33156016922659;
e-mail: alexandre.hertig@tnn.aphp.fr

Conflict of Interest
None (there are no financial connections that might directly or indirectly raise the question of bias in the work reported here).

Received: 23 April 2013
Revision requested: 23 June 2013
Accepted: 24 November 2013
Published online: 28 January 2014

doi:10.1111/tri.12253

Summary

In this ancillary study of the CONCEPT trial, we studied the role of CsA withdrawal at 3 months (3M) post-transplant on the intensity of epithelial phenotypic changes (EPC, an early marker for kidney fibrogenesis) on the 12M surveillance biopsy. Although conversion from CsA to sirolimus (SRL) at 3M was reported to have improved mean graft function at 12M, it did not reduce the score of EPC (1.73 ± 1.15 in the SRL group vs. 1.87 ± 1 in the CsA group, $P = 0.61$). Acute rejection, which had occurred twice more frequently in SRL-converted patients included here, was associated with 12M EPC. Interestingly, we observed that the patients durably exposed to CsA and who developed 12M EPC had a significant progression of blood pulse pressure (pp) from 1 to 6M post-transplantation ($\Delta pp = +12.3$ mmHg, $P = 0.0035$). Pulse pressure at 4, 6, and 9M and pp progression from 1 to 6M were significantly associated with the development of EPC at 12M in renal grafts. Logistic regression analysis revealed that a high 6M pp (≥60 mmHg) was an independent risk factor for 12M EPC with an odds ratio of 2.25 per additional 10 mmHg pp (95%CI: 1.14–4.4, $P = 0.02$) after adjustment with recipient's and donor's age, acute rejection incidence and immunosuppressive regimen. A *post hoc* analysis of the data collected in the whole population CONCEPT study revealed that pp was significantly higher at 6 months in patients maintained on CsA and that at this time point pp correlated negatively with GFR at 1 year.

Introduction

Calcineurin inhibitors (CNIs) are universally used in organ transplantation. They are effective in preventing acute rejection and have considerably improved 1-year survival postgraft. However, it is suspected that their chronic use promotes serious complications, such as cancer, hypertension, and chronic nephrotoxicity. The recognition of these side effects sparked interest in sparing strategies including the minimization, avoidance, and withdrawal of CNIs. In the context of kidney transplantation, the early replacement of cyclosporin (CsA) by sirolimus (SRL), an inhibitor of mTOR, has been shown to improve kidney perfusion [1] and function [2–7], but whether CsA withdrawal alleviates renal fibrogenesis is still a matter of debate [4,5,8–10]. The increased risk of graft rejection and of developing proteinuria [11] after the CSA-SRL switch could conversely promote interstitial fibrosis in some patients, thus counteracting the benefit resulting from a CNI-free regimen. Early markers of CsA-induced chronic nephrotoxicity would be helpful, but at present none is available.

We and others [12–18] have previously demonstrated that the phenotype of renal tubular epithelial cells in the graft may be altered, in a way that is reminiscent of the epithelial to mesenchymal transition (EMT), an instrumental process in embryogenesis and tumorigenesis. Thus, a number of the "epithelial phenotypic changes" (EPC), observed inside the tubular structures, are easily detected using immunohistochemistry. These changes include the translocation of β-catenin into the cytoplasm and the *de novo* tubular expression of vimentin, which are useful predictors of the progression of interstitial fibrosis and tubular atrophy (IF/TA) in sequential surveillance biopsies [16]. In addition, we have recently shown that patients exhibiting intense EPC when exposed to CsA will experience a rapid loss of graft function, up to 4 years post-Tx, if CsA is not withdrawn [19]. This suggests that chronic exposure to CsA accelerates fibrogenesis in some patients. However, the discontinuation of CsA was also deleterious, as a result of an increased risk of graft rejection. In the modern era, this risk can partially be circumvented by adding a non-CNI agent, typically an mTOR inhibitor.

The CONCEPT trial was a randomized and multicenter study that included 237 recipients of a first renal transplant who were transplanted between 2004 and 2006 in France, and was intended to evaluate the benefit afforded by delaying the replacement of CSA by SRL, to 12 weeks post-Tx [6]. Although the SRL group displayed an increase in biopsy-proven acute rejection that was borderline significant (16.8% vs. 8.2% in the CSA group), the estimated glomerular filtration rate [18] was better as early as 4 weeks after the switch and was still better at 1 and 4 years post-Tx. The subsequent observation that interstitial fibrosis—measured by morphometry—was similar in the two groups at 1 year post-Tx [9] is a conundrum and in apparent contradiction with the findings of previous studies that had also addressed this issue 1 [20] and 2 years [21] post-Tx.

In this ancillary study of the CONCEPT trial, we asked whether a CsA-SRL switch at 3 months would favorably influence the score for EPC at 1 year. Our hypothesis was that unlike eGFR, which rapidly improves after the withdrawal of CSA because of relief of vasoconstrictive properties of the drug, interstitial fibrosis is a slow process that needs to be evaluated in the long term. If long-term surveillance biopsies are not practicable, a marker of fibrogenesis such as EPC, which is validated in human kidney recipients [16,22] and has been shown to revert rapidly after CSA withdrawal in rodents [23], could reveal the antifibrotic effect of replacing CSA by SRL.

Patients and methods

Patients and protocol

Clinical data of patients were obtained from the centers that participated in CONCEPT trial. The short- and medium-term results of this open-label, randomized, multicenter French study have already been reported before [6,7]. The study was conducted in French hospitals in full compliance with the amended Declaration of Helsinki (ICH) and European Community Good Clinical Practice (CPMP/ICH/135/95), and approved by an Independent Ethics Committee and by the relevant authorities (Eudract Number 2004-002987-62).

All patients were given daclizumab (Zenapax, Roche Pharmaceuticals, Basel, Switzerland) as induction treatment. Maintenance immunosuppression comprised mycophenolate mofetil (MMF) (Cellcept®, Roche Pharmaceuticals, Basel, Switzerland), CsA (Neoral®, Novartis Pharma AG, Basel, Switzerland) and steroids. CsA concentrations were measured two hours postdose (C2). Unless patients displayed a noninclusion criterion (episode of acute rejection ≥grade I, an estimated glomerular filtration rate (eGFR, using the Gault and Cockcroft formula) <40 ml/min, serum creatinine variation >30% during the 15 days prior to randomization, or proteinuria >1 g/24 h), patients were randomized at 3 months (3M) either to continue CsA treatment or switch to SRL (see references on the CONCEPT trial for further details).

Arterial pressure was measured using an automatic apparatus, on the arm of patients in a seated position in a quiet room after resting for at least ten minutes. Pulse pressure was calculated at the 1,2,3,4,6, and 9 M visits as the difference between the systolic and diastolic blood pressures. Renal graft function was estimated by serum creatinine level and glomerular filtration rate (eGFR) calculated by the Gault and Cockcrft formula.

Immunohistochemistry and semi-quantitative analysis of tubular staining

Immunohistochemistry was performed on paraffin-embedded tissue as previously described [15]. Target retrieval was carried out by heating the tissue in citrate buffer (pH = 6) (DakoCytomation). Endogenous peroxidase was inactivated by incubation for 10 min at room temperature in 0.03% H_2O_2. The sections were incubated overnight at 4 °C with phosphate-buffered saline containing 1:4000 antivimentin [mAb V9 (Zymed)] or 1 μg/ml anti-β-catenin [rabbit polyclonal antibody (Santa Cruz Biotechnology)]. The immunoreactive proteins were visualized with envision + system HRP (AEC) (DakoCytomation). Finally, the tissue sections were counterstained with hematoxylin and mounted with aqueous mounting medium (Dako). For negative controls, the primary antibodies were replaced by an equal concentration of rabbit or mouse IgG (Dako).

The expression of the mesenchymal cell marker vimentin was semi-quantified according to the proportion of tubules positively stained: 0, none; 1, <10%; 2, 10% to 24%; 3, 25% to 50%; 4, >50%. Less than or equal to 10% of the tubules positively stained for vimentin were defined as EPC-positive grafts. Staining was scored three times in a blind fashion by an observer without any knowledge of the patient's clinical, biological, and morphological data. When the readings were discordant, not classifying the graft as either EPC+ or EPC−, the semi-quantification of the translocation of beta-catenin (also carried out three times) was used, because this marker is highly sensitive to tubular epithelial cells undergoing phenotypic changes. The reason why we exclusively studied vimentin as a cytoskeleton protein typical for mesenchymal cells, and not other classical markers such as alpha smooth muscle actin, for example, has been discussed in a previous publication from our group.

Morphometric analysis of interstitial fibrosis

Cortical sections from 1-year biopsies were centrally imaged in by a blind observer using a color video camera (Nikon DXM1200) mounted on a light microscope (Nikon Eclips E1000M). Images were acquired using the 40× objective and quantified automatically as recently described [9,24]. The detailed results of this analysis have been published previously.

Statistical analysis

The association of the patient's clinical and biological data (recipient's age, donor's age, pulse pressure, CsA dose, plasma creatinine, and eGFR) with EPC markers and renal interstitial fibrosis was assessed by correlation analysis. Results are presented with Pearson's correlation coefficient R and the *P* value. Clinical, biological, 1-year EPC marker score, and interstitial fibrosis were compared by an unpaired *t*-test in the patients still being treated by CsA or who had been switched to SRL at 3M or according to their 1 year EPC status or 1 year graft interstitial fibrosis score. Results are presented as mean ± standard deviation (SD) together with *P* values generated by two-sided tests. The cut-off points for EPC [15] or high pulsed pressure (pp) [25] was defined before statistical analysis according to the previously published data. The increase in pp from 1 to 6 months and the improvement of renal graft function (eGFR) from three to twelve months were assessed by paired *t*-test and shown by mean ± SD along with *P* value. Logistic regression was used to estimate the adjusted odds ratios (OR) and 95% confidence intervals (CI) of the potential risk factors for 12-month graft EPC. Statistical analysis was carried out using STATA 8 (Stata Corp. College Station, Texas, USA). The test was defined as being significant when $P < 0.05$.

Results

Demographical characteristics

A total of 60 patients, from 11 centers that had participated in the CONCEPT trial, had blank slides available for the assessment of EPC (Table 1). Thirty–three patients had been randomized to switch to SRL at 3M post-transplantation, and the other 27 continued on CsA. No significant difference was observed between the two groups of patients in terms of age, gender, donor age, donor gender, cold ischemia time, blood pulse pressure, and renal graft function 1 month after transplantation.

Acute rejection rate, and evolution of eGFR and pulse pressure after switching to SRL

The percentage of patients with acute rejection (AR) during the first year post-transplantation was twice as high in the group of patients who had switched to SRL than in the patients with CsA, but this difference was not statistically significant (30.3% vs. 14.8%, $P = 0.16$) (Table 1).

Although the difference was only statistically borderline significant, we observed that 6M pulse pressure (pp) was 6 mmHg higher in patients who were still being treated by CsA than that in patients switched to SRL (61.3 ± 13 vs. 55.1 ± 11 mmHg, $P = 0.0565$). A *post hoc* analysis of the whole population study strengthened this result, showing that mean pp at 6M was higher in patients maintained on CsA (60 ± 17 mmHg in the CsA group, vs. 55 ± 14 mmHg in the SRL group, $P = 0.03$). Interestingly, 6M pp correlated negatively with eGFR at 12M in the whole

Table 1. Comparison of clinical data, 12M vimentin score and graft fibrosis between the patients continually treated by CsA (n = 27) or switched at 3M by SRL (n = 33).

	CsA group (n = 27)	SRL group (n = 33)	P
Patient's age (years)	49 ± 11	47 ± 13	0.6*
Patient's gender (m/f) (%)	18/9 (67%)	20/13 (61%)	0.6†
Donor's age (year)	44 ± 14	40 ± 14	0.2*
Donor's gender (m/f) (%)	22/5 (81.5%)	21/12 (63.6%)	0.13†
Cold ischemia time (hour)	19 h ± 8	18 h ± 6	0.58*
Renal graft function (eGFR) (ml/min/1.73 m^2)			
At 1 month	57 ± 27	54.3 ± 16	0.65*
At 3 month	60 ± 16	63 ± 16	0.55*
At 6 month	64 ± 17	68.5 ± 19	0.35*
At 12 month	62 ± 16	70.7 ± 21	0.08*
Improvement in eGFR from 3 to 12 month	Δ = +1.3, P = 0.7‡	Δ = +7.8, P = 0.006‡	
Pulse pressure at 1 month (mmHg)	56.8 ± 13	55.3 ± 10	0.64*
Pulse pressure at 6 month (mmHg)	61.3 ± 13	55.1 ± 11	0.057*
Progression of pulse pressure from 1 to 6 months (mmHg)	Δ = +4.5, P = 0.12‡	Δ = −0.2, P = 0.83‡	
Number of acute rejection during first year	4 (14.8%)	10 (30.3%)	0.16†
EPC score at 12 month	1.87 ± 1	1.73 ± 1.15	0.61*
Graft fibrosis (%) at 12 month	30 ± 19%	25.5 ± 14%	0.36*

*Student t-test.
†Chi-square test.
‡Paired t-test.

population study (r = −0.334, P < 0.0001, n = 170), as well as in each group taken individually [r = −0.363, P = 0.0005, in the CsA group (n = 89) vs. r = −0.253, P = 0.023 in the SRL group (n = 81)].

While patients treated with CsA had actually increased their pp over time (56.8 ± 13 at M1 vs. 61.3 ± 13 mmHg at M6, P = 0.12), patients switched to SRL had a stable pp over time (55.3 ± 10 vs. 55.1 ± 11 mmHg, P = 0.83).

A significant improvement in eGFR between 3 and 12M post-Tx (from 63 ± 16 to 71 ± 21 ml/min, P = 0.006) was observed in the group of patients who were converted to SRL. No significant change was found (60 ± 16 vs. 62 ± 16 ml/min, P = 0.7) in those still taking CsA.

Detection of EPC at the 12M protocol biopsy

As previously reported [15], we observed that in the normal kidneys, vimentin was not detected in the tubules (Fig. 1a), whereas a thin line of expression of β-catenin was found in proximal tubular epithelial cells (basal staining), in distal tubules and in collecting ducts (basolateral staining), as shown in Fig. 1b. In contrast, strong *de novo* expression of vimentin was observed in some tubular epithelial cells in 1-year protocol biopsies (Fig. 1c). In serial sections of these tubules, we also observed upregulated expression of β-catenin translocated from the basal surface to cytoplasm (Fig. 1d). Importantly, EPCs were detected in both atrophic and nonatrophic tubules. After semi-quantification of the tubular expression of vimentin, we found a significant correlation between 1-year EPC scores and the intensity of renal graft interstitial fibrosis as assessed by morphometry (r = 0.454, P = 0.0005).

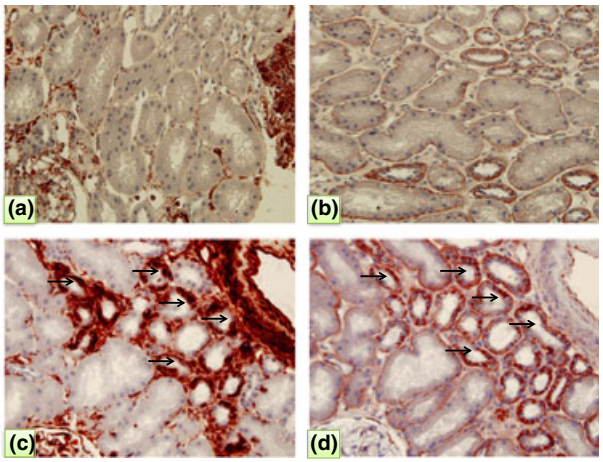

Figure 1 Epithelial phenotype changes in renal grafts. In normal kidneys, the expression of vimentin (a) was undetectable, and beta-catenin staining (b) displayed a thin, linear pattern. In contrast, serial sections from grafts revealed *de novo* expression of vimentin (c) in tubules concomitantly showing changes in the expression and localization of beta-catenin (d).

12M EPC score in patients exposed to SRL or CsA

At 1 year, the EPC score was similar in patients who had been switched to SRL and in those who continued on CsA: 1.73 ± 1.15 vs. 1.87 ± 1 (P = 0.61), respectively (Table 1).

It is noteworthy that CsA C2 was unrelated to 12M renal graft tubular vimentin score at any time point (data not shown). In keeping with what had been previously reported in the general population study, interstitial fibrosis (assessed by morphometry) was also equivalent in the two groups of patients included in this ancillary study (25.5 ± 14% vs. 30 ± 19%, $P = 0.36$). As (i) the EPC score in patients who had experienced acute rejection (AR) during the first year was significantly higher than in AR-free patients (2.29 ± 1.2 vs. 1.64 ± 1.4, $P = 0.0498$) and (ii) AR was numerically more frequent in the group switched to SRL, we reasoned that AR episodes could be masking the benefits of the switch. We therefore carried out a subgroup analysis in AR-free patients. Here too, vimentin score (1.57 ± 1.1 vs. 1.72 ± 0.9, $P = 0.61$) and graft fibrosis (22.2 ± 14% vs. 27.4 ± 18%, $P = 0.293$) were similar in the two groups of patients.

Risk factors for 12M EPC

According to our previous definition, when ≥10% of tubules displayed EPCs, the graft was classified as EPC+ (Table 2). As shown in Table 2a, there was no significant difference between patients with EPC+ and EPC− grafts at 12 months for recipient age, cold ischemia time, or immunosuppressive regimen. Donor age was numerically higher, and the acute rejection rate was twice as high in the EPC+ group. Six-month pp was higher in patients with EPC+ grafts, which was because of a significant increase in pp between 1 and 6 months post-transplantation (from 55.6 mmHg ± 13 to 63 mmHg ± 20, $P = 0.0066$). This was in contrast to patients with an EPC− graft, in whom pp had decreased slightly from 1 to 6M (from 56 mmHg ± 10 to 54 mmHg ± 10, $P = 0.2$). The increase in pp was even more obvious in CsA-treated patients (Table 2b) with an EPC+ graft ($\Delta pp = +12$ mmHg, $P = 0.0035$). In EPC−, group of patients was continuously treated by CsA, but pp had not changed between 1 and 6M ($\Delta pp = -4$ mmHg, $P = 0.22$). Moreover, we observed that in EPC+ patients and treated by CsA, patient's and donor's age were relatively more elevated than those in EPC− group; in patients converted to SRL, pp progression was similar between EPC+ and EPC− grafts of Table 2c.

Correlation analysis showed also that the EPC score was significantly correlated with patient's age ($r = 0.41$, $P = 0.03$, $n = 27$), with pp at 4, 6, 9M, and with pp progression from 1 to 6M ($r = 0.51$, $P = 0.007$; $r = 0.7$, $P = 0.0001$; $r = 0.46$, $P = 0.03$; $r = 0.365$, $P = 0.0048$) in patients still receiving CsA. The level of pp at 6M was significantly correlated with patient's 6, 9, and 12M eGFR in both CsA ($r = -0.53$, $P = 0.006$; $r = -0.71$, $P = 0.0001$; $r = -0.59$, $P = 0.0013$) and SRL ($r = -0.38$, $P = 0.03$; $r = -0.49$, $P = 0.0044$; $r = -0.52$, $P = 0.0023$) groups. Pulse pressure at 1M was significantly correlated with 6, 9, and 12M eGFR only in SRL-converted patients ($r = -0.53$, $P = 0.002$; $r = -0.59$, $P = 0.0003$; $r = -0.577$, $P = 0.0004$), not in CsA-treated patients. By logistic regression analysis, carried out after adjustment for patient's age, donor's age, incidence of acute rejection and immunosuppressive treatment, an admittedly high (≥60 mmHg) [25] 6M pp was the only independent risk factor for 12M EPC with an odds ratio of 2.25 [95% CI = (1.14–4.4), $P = 0.02$] per additional 10 mmHg pp (Table 3). This risk was 5.67 [95% CI = (1.2–26.5), $P = 0.03$] in patients continuously exposed to CsA.

Discussion

Studying the phenotype of tubular epithelial cells in renal grafts is justified by the replicated observation that the intensity of the expression of mesenchymal markers, such as *de novo* expression of vimentin and translocation of beta-catenin into the cytoplasm, predicts the development of interstitial fibrosis and the decrease in renal graft function and hence the long-term progression toward graft loss [16,18,19,26]. In contrast, when EPC was measured at an earlier time point (1 month), where the graft is also repairing from tubular injuries induced by the death of the donor and cold ischemia, EPC has no predictive value [27].

A major finding of the CONCEPT trial, in which patients were randomly assigned at 3 months post-transplant either to continue on CsA or to stop it and start on an mTOR inhibitor (in this case, sirolimus), was that CsA withdrawal resulted in a significant improvement in eGFR at 1 year [6], which was maintained up to 4 years post-transplantation [7]. Similar long-term results were recently reported from the Spiesser study [28]. This makes it unlikely that the benefit of switching treatment was merely due to discontinuing a drug that has a potent vasoconstrictive effect on afferent arterioles. However, with respect to the progression of interstitial fibrosis, Servais *et al.* [24] did not detect any benefit 1-year post-transplantation in patients who had been switched from CsA to SRL. Here, we consolidate this latter observation and show that the EPC score, which is supposed to be predictive of future fibrosis, is also similar in both treatment groups. In our opinion, there are three possible interpretations of this observation.

First, we could argue that CsA is less fibrogenic, or at least not more fibrogenic than other immunosuppressants (here, sirolimus). This goes against an abundant literature, but the fact is that at present, there is no histological lesion known to be specific for chronic CsA toxicity in the kidney [29]. However, a proportion of these patients admittedly free from kidney disease would go on to develop interstitial fibrosis and progressive renal failure when exposed to CsA, which seems to show that the received dogma is relatively safe.

Table 2. (a) Comparison of clinical data between the patients with 12 month's EPC-positive or EPC-negative renal grafts (n = 60). (b) Comparison of clinical data between the patients with 12 month's EPC-positive or EPC-negative renal grafts under CsA ongoing treatment (n = 27). (c) Comparison of clinical data between the patients with 12 month's EPC-positive or EPC-negative renal grafts converted to SRL treatment at 3 months post-transplantation (n = 33).

	12 month EPC− (n = 32)	12 month EPC+ (n = 28)	P
(a)			
Patient's age (year)	46.4 ± 12	49.6 ± 11	0.3*
Donor's age (year)	38.4 ± 14	45.25 ± 13	0.0551*
Cold ischemia time (hour)	17.3 ± 5.7	19.8 ± 7.7	0.15*
Immunosuppression (CsA/SRL)	13/19	14/14	0.53†
Renal graft function at 1 month (eGFR) (ml/min/1.73 m^2)	59 ± 26	52 ± 14	0.21*
Renal graft function at 3 month (eGFR) (ml/min/1.73 m^2)	64 ± 16	60 ± 16	0.36*
Renal graft function at 6 month (eGFR) (ml/min/1.73 m^2)	70 ± 18	63 ± 17	0.114*
Renal graft function at 12 month (eGFR) (ml/min/1.73 m^2)	70 ± 19	63 ± 20	0.165*
Pulse pressure at 1 month (mmHg)	56 ± 10	55.6 ± 13	0.8*
Pulse pressure at 6 month (mmHg)	54 ± 10	63 ± 13	0.0042*
Progression of pulse pressure (1–6 months) (mmHg)	$\Delta = -2.5$, $P = 0.2$‡	$\Delta = +7.2$, $P = 0.0066$‡	
Number of acute rejection (%)	5 (15.6%)	9 (32%)	0.131†
Graft fibrosis (%) at 12 month	22 ± 14	35 ± 17	0.0022*
	12 month EPC− (n = 13)	12 month EPC+ (n = 14)	P
(b)			
Patient's age (year)	45 ± 13	52 ± 7.6	0.126§
Donor's age (year)	39 ± 15	48.5 ± 11.6	0.1§
Cold ischemia time (hour)	17.6 ± 7	20.2 ± 8	0.42§
Renal graft function at 1 month (eGFR) (ml/min/1.73 m^2)	63.7 ± 36	50.5 ± 14	0.41§
Renal graft function at 3 month (eGFR) (ml/min/1.73 m^2)	64 ± 19	56.6 ± 11	0.31§
Renal graft function at 6 month (eGFR) (ml/min/1.73 m^2)	70 ± 20	59 ± 13	0.11§
Renal graft function at 12 month (eGFR) (ml/min/1.73 m^2)	66.2 ± 16	57.5 ± 16	0.24§
1-month pulse pressure (mmHg)	58 ± 12.5	55.7 ± 14	0.79§
6-month pulse pressure (mmHg)	54 ± 10.5	68 ± 12	0.005§
Progression of pulse pressure (1–6 months) (mmHg)	$\Delta = -4$, $P = 0.22$¶	$\Delta = +12.3$, $P = 0.0035$¶	
Number of acute rejection (%)	1 (7.7%)	3 (21.4%)	0.32†
Graft fibrosis (%) at 12 month	21 ± 17%	38.4 ± 18%	0.011§
	12 month EPC− (n = 19)	12 month EPC+ (n = 14)	P
(c)			
Patient's age (year)	47 ± 12	47 ± 13.7	0.78§
Donor's age (year)	38 ± 13.5	42 ± 14	0.43§
Cold ischemia time (hour)	17 ± 5	19.3 ± 7	0.36§
Renal graft function at 1 month (eGFR) (ml/min/1.73 m^2)	55.4 ± 17	52.8 ± 15	0.61§
Renal graft function at 3 month (eGFR) (ml/min/1.73 m^2)	63 ± 15	63 ± 19	0.97§
Renal graft function at 6 month (eGFR) (ml/min/1.73 m^2)	70.4 ± 18	66.2 ± 20	0.27§
Renal graft function at 12 month (eGFR) (ml/min/1.73 m^2)	72.6 ± 21	68 ± 23	0.54§
1-month pp (mmHg)	55 ± 8.6	55 ± 12	0.97§
6-month pp (mmHg)	53.7 ± 11	57 ± 12	0.44§
Progression of pp (1–6 months) (mmHg)	$\Delta = -1.3$, $P = 0.55$¶	$\Delta = +1.2$, $P = 0.72$¶	
Number of acute rejection (%)	4 (21%)	6 (43%)	0.18†
Graft fibrosis (%) at 12 month	22 ± 13%	31 ± 15%	0.1§

*Student t-test.
†Chi-square test.
‡Paired t-test.
§Wilcoxon test.
¶Wilcoxon matched-pairs signed ranks test.

Table 3. Risk factors for EPC at 12 months (logistic regression analysis) in all patients (n = 60).

	OR [39]	*P*
6-month pp (per additional 10 mmHg pp)	2.25 [1.14–4.4]	0.02
Age of patients	0.96 [90.9–1.03]	0.216
Age of donors	1.04 [0.99–1.090]	0.138
Acute rejection	1.935 [0.45–8.37]	0.377
Immunosuppressive regimens (CsA = 1)	1.03 [0.3–3.6]	0.965

Second, exposure to CsA for 3 months could be sufficient to compromise matrix remodeling in the long term, reducing the likelihood of seeing any decrease in interstitial fibrosis in patients who stop taking CsA. This is also unlikely as we have recently reported that in rodents [23] as well as in human patients with a kidney transplant [19], withdrawing CsA does produce reduction or stabilization of the EPC.

Third, CsA withdrawal could alleviate fibrogenesis in some patients but promote it in others, resulting in the absence of any "overall benefit" when the strategy is randomly applied, as here in the CONCEPT trial. For example, CsA withdrawal always increases the risk of acute rejection in the following months, and acute rejection is an important risk factor for the occurrence of EPCs [13,15] and interstitial fibrosis, including in this ancillary study. Even though we failed to detect any significant difference in *post hoc* analyses after excluding patients with biopsy-proven acute rejection, we were dealing with a small number of patients (only 31% of the CONCEPT population study), in whom acute rejection is overrepresented (23% of patients, as opposed to 14% in the total population study), and the present study is not statistically powerful enough to demonstrate this difference. Subclinical inflammation could also be an issue, whether due to CsA withdrawal or switching to SRL [30], as inflammation is a well-known trigger of interstitial fibrosis.

To note, the main positive finding of this ancillary study concerns the predictive value of the evolution of pulse pressure (from 1 to 6M) for late (12M) EPC and graft dysfunction. First, mean pp was significantly higher at 6M in patients not converted to SRL, including in the whole population study (170 patients total). Second, 6-month pp was negatively correlated with 12M eGFR. Pulse pressure progression under CsA treatment was associated with EPC at 12M. In fact, 6M-high pp was a strong and independent risk factor for the presence of EPC at 1 year, after adjustment with patient's age, donor's age, incidence of acute rejection, and immunosuppressive treatment. The risk was particularly more important in patients not converted to SRL and thus continuously exposed to CsA. CsA, a potent vasoconstrictive drug, is known to increase blood pressure and pp [3,31]. Increased pp is a marker of vascular stiffness and may cause end-organ endothelial dysfunction, excessive collagen deposition in the vascular wall [32,33] and subsequently irreversible chronic organ ischemia due to reduced diameter of arterioles. In the kidneys, the blood flow is torrential but the vascular resistance to flow is low [34]: A rapid increase in pp after transplantation might thus cause small vascular damage. Although elevated pp is a well-known predictor of cardiovascular events [35–37], the relationship between pp and renal fibrogenesis has not yet been thoroughly explored [38]. Our study suggests that it should, especially in patients durably exposed to CNI.

In conclusion, our study demonstrates that CsA withdrawal at 3 months does not affect the expression of mesenchymal markers by tubular epithelial cells in kidney transplants at 1 year. Disappointing at first glance, these results mean that a random or blind CsA withdrawal is not the best way, as a one-size fits all approach, to prevent graft fibrogenesis in transplanted patients.

Authorship

Y-CX-D: involved in the study of epithelial phenotypic changes, the analysis of the data, and the writing of the manuscript. AH: involved in the analysis of the data and the writing of the manuscript. YL, BHL, ET, MJ, GT, PLP, YLM, OT, AEH, FB: all clinicians involved in the CONCEPT study and were all involved in the writing of the manuscript. SGS: an employee from Roche SAS Pharma, she was involved in the CONCEPT Study and commented on the manuscript. AS and VM-Y: involved in the quantification of interstitial fibrosis by morphometry. ER: supervised the work and was involved in writing the manuscript.

Funding

Roche funded the CONCEPT trial and provided financial support to realize immunohistochemistry on the leftover biopsy material for this ancillary study.

Acknowledgements

We would like to thank Roche SAS Pharma who provided the leftover biopsy material, patient's clinical and biological information and financial support to realize this ancillary study of CONCEPT trial.

References

1. Kihm LP, Hinkel UP, Michael K, *et al.* Contrast enhanced sonography shows superior microvascular renal allograft perfusion in patients switched from cyclosporine A to everolimus. *Transplantation* 2009; **88**: 261.

2. Flechner SM, Kobashigawa J, Klintmalm G. Calcineurin inhibitor-sparing regimens in solid organ transplantation: focus on improving renal function and nephrotoxicity. *Clin Transplant* 2008; **22**: 1.
3. Johnson RW, Kreis H, Oberbauer R, Brattstrom C, Claesson K, Eris J. Sirolimus allows early cyclosporine withdrawal in renal transplantation resulting in improved renal function and lower blood pressure. *Transplantation* 2001; **72**: 777.
4. Stallone G, Di Paolo S, Schena A, *et al.* Early withdrawal of cyclosporine A improves 1-year kidney graft structure and function in sirolimus-treated patients. *Transplantation* 2003; **75**: 998.
5. Mota A, Arias M, Taskinen EI, *et al.* Sirolimus-based therapy following early cyclosporine withdrawal provides significantly improved renal histology and function at 3 years. *Am J Transplant* 2004; **4**: 953.
6. Lebranchu Y, Thierry A, Toupance O, Westeel PF, Etienne I, Thervet E, *et al.* Efficacy on renal function of early conversion from cyclosporine to sirolimus 3 months after renal transplantation: concept study. *Am J Transplant* 2009; **9**: 1115.
7. Lebranchu Y, Thierry A, Thervet E, Buchler M, Etienne I, Westeel PF, *et al.* Efficacy and safety of early cyclosporine conversion to sirolimus with continued MMF-four-year results of the Postconcept study. *Am J Transplant* 2011; **11**: 1665.
8. Ruiz JC, Campistol JM, Grinyo JM, *et al.* Early cyclosporine a withdrawal in kidney-transplant recipients receiving sirolimus prevents progression of chronic pathologic allograft lesions. *Transplantation* 2004; **78**: 1312.
9. Servais A, Meas-Yedid V, Toupance O, *et al.* Interstitial fibrosis quantification in renal transplant recipients randomized to continue cyclosporine or convert to sirolimus. *Am J Transplant* 2009; **9**: 2552.
10. Hamdy AF, El-Agroudy AE, Bakr MA, *et al.* Comparison of sirolimus with low-dose tacrolimus versus sirolimus-based calcineurin inhibitor-free regimen in live donor renal transplantation. *Am J Transplant* 2005; **5**: 2531.
11. Ponticelli C, Graziani G. Proteinuria after kidney transplantation. *Transpl Int* 2012; **25**: 909.
12. Rastaldi MP, Ferrario F, Giardino L, *et al.* Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. *Kidney Int* 2002; **62**: 137.
13. Robertson H, Ali S, McDonnell BJ, Burt AD, Kirby JA. Chronic renal allograft dysfunction: the role of T cell-mediated tubular epithelial to mesenchymal cell transition. *J Am Soc Nephrol* 2004; **15**: 390.
14. Vongwiwatana A, Tasanarong A, Rayner DC, Melk A, Halloran PF. Epithelial to mesenchymal transition during late deterioration of human kidney transplants: the role of tubular cells in fibrogenesis. *Am J Transplant* 2005; **5**: 1367.
15. Hertig A, Verine J, Mougenot B, *et al.* Risk factors for early epithelial to mesenchymal transition in renal grafts. *Am J Transplant* 2006; **6**: 2937.
16. Hertig A, Anglicheau D, Verine J, *et al.* Early epithelial phenotypic changes predict graft fibrosis. *J Am Soc Nephrol* 2008; **19**: 1584.
17. Galichon P, Hertig A. Epithelial to mesenchymal transition as a biomarker in renal fibrosis: are we ready for the bedside? *Fibrogenesis Tissue Repair* 2011; **4**: 11.
18. Kers J, Xu-Dubois YC, Rondeau E, *et al.* Intragraft tubular vimentin and CD44 expression correlate with long-term renal allograft function and interstitial fibrosis and tubular atrophy. *Transplantation* 2010; **90**: 502.
19. Hazzan M, Hertig A, Buob D, *et al.* Epithelial-to-mesenchymal transition predicts cyclosporine nephrotoxicity in renal transplant recipients. *J Am Soc Nephrol* 2011; **22**: 1375.
20. Dean PG, Grande JP, Sethi S, *et al.* Kidney transplant histology after one year of continuous therapy with sirolimus compared with tacrolimus. *Transplantation* 2008; **85**: 1212.
21. Flechner SM, Kurian SM, Solez K, *et al.* De novo kidney transplantation without use of calcineurin inhibitors preserves renal structure and function at two years. *Am J Transplant* 2004; **4**: 1776.
22. Xu-Dubois YC, Baugey E, Peltier J, *et al.* Epithelial phenotypic changes are associated with a tubular active fibrogenic process in human renal grafts. *Hum Pathol* 2013; **44**: 1251.
23. Galichon P, Vittoz N, Xu-Dubois YC, *et al.* Epithelial phenotypic changes detect cyclosporine in vivo nephrotoxicity at a reversible stage. *Transplantation* 2011; **92**: 993.
24. Servais A, Meas-Yedid V, Buchler M, *et al.* Quantification of interstitial fibrosis by image analysis on routine renal biopsy in patients receiving cyclosporine. *Transplantation* 2007; **84**: 1595.
25. Safar ME. Peripheral pulse pressure, large arteries, and microvessels. *Hypertension* 2004; **44**: 121.
26. Xu-Dubois YC, Galichon P, Brocheriou I, *et al.* Expression of the transcriptional regulator snail 1 in kidney transplants displaying epithelial-to-mesenchymal transition features. *Nephrol Dial Transplant* 2013 [Epub ahead of print].
27. Vitalone MJ, O'Connell PJ, Jimenez-Vera E, *et al.* Epithelial-to-mesenchymal transition in early transplant tubulointerstitial damage. *J Am Soc Nephrol* 2008; **19**: 1571.
28. Lebranchu Y, Snanoudj R, Toupance O, *et al.* Five-year results of a randomized trial comparing de novo sirolimus and cyclosporine in renal transplantation: the SPIESSER study. *Am J Transplant* 2012; **12**: 1801.
29. Snanoudj R, Royal V, Elie C, *et al.* Specificity of histological markers of long-term CNI nephrotoxicity in kidney-transplant recipients under low-dose cyclosporine therapy. *Am J Transplant* 2011; **11**: 2635.
30. Thierry A, Thervet E, Vuiblet V, *et al.* Long-term impact of subclinical inflammation diagnosed by protocol biopsy one year after renal transplantation. *Am J Transplant* 2011; **11**: 2153.
31. Seckinger J, Sommerer C, Hinkel UP, Hoffmann O, Zeier M, Schwenger V. Switch of immunosuppression from cyclosporine A to everolimus: impact on pulse wave velocity in

stable de-novo renal allograft recipients. *J Hypertens* 2008; **26**: 2213.
32. Lehoux S. Redox signalling in vascular responses to shear and stretch. *Cardiovasc Res* 2006; **71**: 269.
33. Mitchell GF. Effects of central arterial aging on the structure and function of the peripheral vasculature: implications for end-organ damage. *J Appl Physiol* 2008; **105**: 1652.
34. O'Rourke MF, Safar ME. Relationship between aortic stiffening and microvascular disease in brain and kidney: cause and logic of therapy. *Hypertension* 2005; **46**: 200.
35. Franklin SS. Cardiovascular risks related to increased diastolic, systolic and pulse pressure. An epidemiologist's point of view. *Pathol Biol (Paris)* 1999; **47**: 594.
36. Safar ME, Levy BI, Struijker-Boudier H. Current perspectives on arterial stiffness and pulse pressure in hypertension and cardiovascular diseases. *Circulation* 2003; **107**: 2864.
37. Safar ME, Delahousse M, Bahous SA. Arterial stiffness and renal transplantation. *J Hypertens* 2008; **26**: 2101.
38. Vetromile F, Szwarc I, Garrigue V, *et al.* Early high pulse pressure is associated with graft dysfunction and predicts poor kidney allograft survival. *Transplantation* 2009; **88**: 1088.
39. Barenbrock M, Hausberg M, Kosch M, Kisters K, Hoeks AP, Rahn KH. A longitudinal study of vessel wall properties in normotensive and hypertensive renal transplant recipients. *J Hum Hypertens* 1998; **12**: 707.