Different renal toxicity profiles in the association of cyclosporine and tacrolimus with sirolimus in rats

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Abstract

Background. The association of calcineurin inhibitors (CNIs) with mTOR inhibitors (mTORi) is still a problem in clinical practice and there is substantial interest in better understanding the impact of these associations on kidney toxicity. We aimed to analyse the functional and histological profiles of damage and to define the contribution of inflammatory and pro-fibrotic mediators in the association of cyclosporine (CsA) and/or tacrolimus (Tac) with sirolimus (SRL).

Methods. A well-defined model of nephrotoxicity in salt-depleted male rats was used. Monotherapy groups were distributed as a non-treated control group with saline solution (n = 12), the Tac group (n = 16) (tacrolimus 6 mg/kg/day) and the CsA group (n = 13) (CsA 15 mg/kg/day). The groups with different associations were scattered as the Tac + SRL group (n = 14) (tacrolimus 6 mg/kg/day and rapamycin 3 mg/kg/day) and the CsA + SRL group (n = 7) (CsA 15 mg/kg/day and rapamycin 3 mg/kg/day). Groups were divided into 30 and 70 days of follow-up, but the CsA + SRL group was only studied for 30 days because animals became sick.

Results. Rats with the CsA + SRL association were the only ones which showed a significant reduction in body weight, impairment of renal function and severe and diffuse tubular vacuolization and tubular atrophy following a striped distribution, and scarce areas of the kidney were still preserved. The Tac + SRL association did not produce renal function impairment, and mild histological damage including enhanced periglomerular tubular atrophy was observed. This local damage affected the distal convoluted tubule involving macula densa and juxtaglomerular apparatus. Pro-inflammatory mediators paralleled functional and structural data. ED-1 and TNF-α were noticeably higher in the CsA + SRL than in the Tac + SRL association. Only in the CsA + SRL association an important increase in α-SMA+ cells was seen, mainly found in the areas with tubular atrophy.

Conclusion. We conclude that the association of SRL with high doses of CsA or Tac produces a different functional, histological, inflammatory and pro-fibrogenic pattern. Thus, the addition of SRL to high doses of CsA leads to severe renal injury. Combination with high doses of Tac is clearly less deleterious in the short term. However, there is a low grade of pro-fibrogenic inflammatory expression when this association is prolonged.

Keywords: fibrosis; immunosuppression; inflammation; renal toxicity

Introduction

The nephrotoxicity of calcineurin inhibitors (CNIs) contributes to the development and progression of chronic allograft nephropathy in renal transplantation [1–4]. Unfortunately, this undesirable effect of CNIs is also described in other solid organs as in cardiac transplantation [5]. In the last decade, Tac has progressively displaced CsA in clinical practice [4]. Although Tac is reported to have a nephrotoxic effect, it seems to induce fewer haemodynamic and fibrogenic alterations at equivalent doses of CsA [6–8]. Accordingly, it has been said that tacrolimus has less fibrogenic potential than cyclosporin A in a model of renal ischaemia-reperfusion injury [9].
Sirolimus is a macrolide lactone with a potent immunosuppressive effect. It inhibits the mammalian target of rapamycin, m-TOR [10]. SRL was initially used in immunosuppressive regimens, thus allowing the minimization of avoidance or occurrence of CNIs. Although SRL has little or no nephrotoxicity by itself [7], it potentiates CsA nephrotoxicity [6]. Podder et al. suggested that this impairment of renal function is due to a pharmacokinetic interaction of SRL that greatly increases the CsA concentration in whole blood and, particularly, in kidney tissue [11]. The Tac–SRL combination offers excellent acute rejection prophylaxis [12]; however, its deleterious effect on renal function is less constant than that of the CsA–SRL association at least in the short term [13]. However, in the clinical setting, both associations seem to be related to worse renal allograft survival than CsA + mycophenolic acid [14] and Tac + mycophenolic acid [15], respectively.

CNIs' associated toxicities are related to their blood and tissue concentrations. However, drug levels are unpredictable, due to intravascular and interindividual differences in drug pharmacokinetics, including hepatic drug metabolizing activity and drug absorption in the small intestine [16]. The high affinity of CsA for P-glycoprotein (Pgp) [17] suggests that this efflux protein is one of the factors involved in CsA-induced nephrotoxicity. Given the role of Pgp in protection from toxic drugs and the knowledge that CsA, Tac and SRL are actively transported by Pgp [18–20], their interaction with the same transporter may influence their therapeutic effectiveness and the incidence of toxic side effects in target cells [21–23]. The increase in graft nephrotoxicity of the CsA+SRL association has been well described, clinically and experimentally, but Tac + SRL has scarcely been studied. As these associations efficiently prevent allograft rejection, there is significant interest in better understanding the different impact of those associations on kidney toxicity. In this experimental study, we examined the impact of the association of SRL with both CNIs on functional and histological parameters in a rat toxicity model. Furthermore, we examined the underlying molecular inflammatory and pro-fibrogenic mechanisms, as well as the involvement of Pgp in this immunosuppressor-related renal toxicity.

Subjects and methods

Animals

All the procedures and housing conditions were in accordance with current European Union legislation on animal experiments and approved by our Institution’s Ethics Committee for Investigation with Animals. Young (4- to 8-week-old) male Sprague Dawley rats (200–250 g body weight) were purchased from Harlam Iberica (Spain).

Study groups

A well-defined model of nephrotoxicity in salt-depleted male rats was used. The study follow-up was of 30 or 70 days. A non-treated control group (n = 12) was treated with a saline solution. The group Tac (n = 16) was given tacrolimus (6 mg/kg/day). The group CsA (n = 13) received CsA (15 mg/kg/day). The group Tac + SRL (n = 14) was given tacrolimus (6 mg/kg/day) and rapamycin (3 mg/kg/day). The group CsA + SRL (n = 7, only studies 30 days) received CsA (15 mg/kg/day) and rapamycin (3 mg/kg/day).

Serum and urine chemistry

Rats were placed in metabolic cages in order to collect 24-h urine specimens on Day 0 (before therapeutic intervention) and at 30 and 70 days. Blood was obtained from the tail vein. Serum and urine creatinine (sCr, mg/dl) levels were determined, and creatinine clearance (CrCl, μl/min/100 g body weight) was calculated.

Histological studies

Tissue sections (3–4 μm) were stained with haematoxylin–eosin, periodic acid–Schiff (PAS) and silver–methenamine (PAM). A pathologist blinded to the treatment groups evaluated focal and diffuse tubular atrophy, cytoplasmic vacuolization and vascular changes, with a semi-quantitative scale graded from 0 to 4+.

Immunohistochemical analyses

Representative tissue sections were immunostained using the immunoperoxidase technique for α-SMA and ED-1 as described previously [24,25] and were blindly evaluated as 0 (absent), 1 (mild), 2 (moderate) and 3 (severe). For apoptotic cells as described previously [26], positive cells in kidneys were counted and expressed as mean ± standard error of the mean (SEM) of cells per field of view (+cells/FV; ×40, ≥20 counted fields/kidney).

Apoptotic cells were stained by an Apotag peroxidase kit (Labcinetics, Barcelona, Spain) with a 50% diluted primary antibody. They were then revealed with DAB (Sigma, Spain). Positive apoptotic cells in kidneys were counted and expressed as mean ± SEM of cells per field of view (+cells/FV; ×40, ≥20 counted fields/kidney).

Western blot of Pgp

Renal tissue (0.1 μg) was sonicated in a lysis buffer as described previously [24]. 50 μg of protein lysates were separated on 7% SDS–polyacrylamide gels. The membranes were blocked in 5% (w/v) non-fat dry milk in Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 (TBST) at room temperature for 1 h. The blocked membranes were incubated with primary rabbit antibodies specific to Pgp, C219 (1:100) (Calbiochem-Novabiochem, San Diego, CA, USA) in TBST containing 5% BSA at 4°C overnight. After incubation, immunoblots were washed with TBST and incubated at room temperature for 1 h with the horseradish peroxidase conjugated antimouse secondary antibody (1:5000, Dako, Denmark) in TBST containing 5% milk. Pgp levels were expressed as a percentage of respective control groups. β-Actin was used as an internal loading control.
Different renal toxicity profiles

Fig. 1. Functional effects of the calcineurin-inhibitor and sirolimus association: (A) body weight, (B) serum creatinine and (C) urea values, at 30 and 70 days. (a) \( P < 0.01 \) CsA + SRL (\( n = 7 \) at 30 days) versus Tac + SRL (\( n = 8 \) at 30 days, \( n = 8 \) at 70 days) and monotherapy groups (\( n = 8 \) at 30 days, \( n = 8 \) at 70 days) in all the functional parameters.

Quantification of renal TNF-α, IFN-γ, TGF-β1, VEGF and mdr1 by real-time PCR

Total RNA was isolated using trizol (Invitrogen, Barcelona, Spain). Results were expressed as ‘many fold of the unknown sample’ relative to non-treated kidneys. Results are expressed as mean ± SEM.

Statistical analysis

All data are presented as mean ± SEM. A Student’s \( t \)-test or analysis of variance (ANOVA) for parametric values and the Mann–Whitney \( U \)-test or the Kruskall–Wallis test for nonparametric values were used to compare group means. All \( P \) values were two-tailed. A \( P \) value of <0.05 was considered statistically significant.

Results

Functional effects of the association of sirolimus with anticalcineurinics

Animals in monotherapy with any of the three drugs or Tac + SRL association grew uniformly throughout the follow-up (Figure 1A). In contrast, the CsA + SRL association produced a significant reduction in BW and animals became progressively sick. To avoid animal suffering such rats were killed on Day 30, therefore there is no follow-up on Day 70.

At 30 days, plasma creatinine and urea were stable in both monotherapy and Tac + SRL groups (Figure 1B and C). The only group with renal impairment was CsA + SRL with a significant increase in both creatinine and urea. Presumably, this renal failure explains the illness of the animals. As the Tac + SRL association offered a better clinical evolution, we prolonged the follow-up. After 70 days, neither of the groups presented a decrease in BW nor renal impairment as the CsA + SRL association at 30 days.

Histological effects of the association of sirolimus with anticalcineurinics

The CsA + SRL and Tac + SRL groups presented different histological patterns (Table 1). At 30 days, CsA + SRL produced intense diffuse tubular atrophy and

Table 1. Histological data

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<th>Cytoplasmatic vacuolization</th>
<th>Focal tubular atrophy</th>
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<td>30 Days</td>
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<tr>
<td>Control</td>
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<td>1.0 ± 0.7</td>
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<tr>
<td>SRL</td>
<td>0.9 ± 0.3</td>
<td>1.1 ± 0.5</td>
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<td>Tac</td>
<td>0.7 ± 0.3</td>
<td>1.2 ± 0.4</td>
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<td>CsA</td>
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<td>1.0 ± 0.4</td>
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<td>CsA + SRL</td>
<td>2.7 ± 0.3</td>
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<td>70 Days</td>
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<tr>
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<tr>
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<tr>
<td>Tac</td>
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\( a P < 0.05 \) CsA versus control and monotherapy groups.

\( b P < 0.05 \) Tac + SRL versus control and monotherapy groups.

\( c P < 0.05 \) CsA + SRL versus Tac + SRL, control and monotherapy groups.

Spain). Results were expressed as ‘many fold of the unknown sample’ relative to non-treated kidneys. Results are expressed as mean ± SEM.
cytoplasmatic vacuolization following a striped distribution (Figure 2). Scarcely areas of the kidney were still preserved. In contrast, the association of Tac + SRL mainly produced an enhanced focal tubular atrophy around the glomeruli. This local damage affected the distal convoluted tubule involving macula densa and juxtaglomerular apparatus. Animals in monotherapy showed slightly diffuse tubular atrophy. The association of SRL with both CNIs led to tubular vacuolization, more pronounced in the CsA + SRL group. Also the CsA group showed significantly more vacuolization than all the other groups.

When CNI monotherapy was prolonged to 70 days, diffuse tubular atrophy increased. Tac + SRL also worsened tubular damage as monotherapy, but less severe than CsA + SRL at 30 days (Table 1). In the Tac or CsA monotherapy groups, tubular vacuolization was greater at 70 days, suggesting that damage increases with length of exposure. No arteriolopathy was seen in either group at any of the time points.

Toxic cellular injury showed a clear increase in the number of positive apoptotic cells in the CsA + SRL group significantly higher than in the other groups ($P < 0.05$). Interestingly, CsA or Tac + SRL administration did not increase apoptotic cells compared with non-treated animals (Figure 3).

The inflammatory pattern of the different immunosuppressor associations

At 30 days, ED1 quantification revealed a slight increase of glomerular and interstitial macrophages in CsA or Tac groups with respect to non-treated animals. Rats treated with the CsA + SRL association presented a clear increase
of ED1+ cells in the interstitium, mainly located in areas of tubular damage. In contrast, the Tac + SRL association did not induce inflammatory cell recruitment, with similar ED1+ cells to Tac monotherapy (Figure 4). When CsA exposure was prolonged to 70 days, a numerical increase in ED1+ cells was induced. Neither Tac nor the association with SRL modified the number of ED1+ cells (data not shown).

To evaluate tissue inflammatory mediators, TNF-α and IFN-γ mRNA expression were measured. At 30 days, all monotherapy groups presented similar values to non-treated animals. Interestingly, the CsA + SRL association produced an overexpression of both cytokines, while the Tac + SRL association did not change the TNF-α or IFN-γ expression (Figure 5). The study at 70 days, showed an apparent increase in TNF-α and a less evident increase in IFN-γ in the Tac + SRL association. Interestingly, the TNF-α values of the Tac + SRL association at 70 days was half the CsA + SRL values at 30 days (data not shown).

**Evaluation of pro-fibrotic mechanisms in the different associations**

The CsA + SRL association produced an important increase in α-SMA+ cells, which were diffusely distributed in the interstitium. The myofibroblasts were mainly found in the areas with tubular atrophy. This suggests that there is a relation between structural tubular damage and the appearance of α-SMA+ cells. There were few α-SMA+ cells in non-treated, monotherapy or Tac + SRL groups (Figure 6). Prolongation to 70 days did not modify the α-SMA expression in neither of the groups.

**Fig. 4.** Evaluation of infiltrating renal ED1+ cells. Macrophages infiltrated the interstitium in CsA + SRL groups \((P < 0.05; \text{CsA} \pm \text{SRL versus Tac} \pm \text{SRL and monotherapy groups})\). A representative image showed renal ED1+ cells in both associations (magnification ×400).

**Fig. 5.** Cytokine mRNA quantification at 30 days. (A) TNF-α and (B) IFN-γ. (a) \(P < 0.01\) CsA + SRL versus Tac + SRL and monotherapy groups in all the cytokines studied. (b) \(P < 0.05\) CsA + SRL and Tac + SRL versus monotherapy groups.

Monotherapy or Tac + SRL groups at 30 days showed no changes in renal TGF-β1 values, like non-treated animals. However, when SRL was associated with CsA, this growth factor was greatly increased (\(P < 0.05\)). At 70 days
α-SMA ± cells, evaluation of interstitial α-SMA staining. CsA ± SRL kidneys had increased α-SMA staining in the interstitium whilst Tac ± SRL showed lower interstitial α-SMA in kidney (P < 0.05; CsA ± SRL versus Tac ± SRL). Immunostaining photomicrography shows α-SMA expression in a representative sample from each association of the experimental groups (magnification ×400).

TGF-β1 quantification at 30 and 70 days. (a) P < 0.01 CsA ± SRL versus Tac ± SRL and monotherapy groups at 30 days. (b) P < 0.05 Tac ± SRL versus monotherapy groups at 70 days.

VEGF and Pgp in the interaction of sirolimus and anticalcineurinics

Monotherapy with CsA or Tac did not modify VEGF mRNA expression. In contrast, and as expected, all animals receiving SRL presented a significant decrease in renal VEGF expression. Surprisingly, the association of SRL with CsA or Tac did not alter the tissue expression of this growth factor. (Figure 8).

As expected, kidneys exposed to CsA presented high Pgp protein overexpression. When SRL was added, similar band intensity was seen in the western blot (Figure 9A). Tac monotherapy and TAC + SRL showed middle increase Pgp

Fig. 6. α-SMA ± cells, evaluation of interstitial α-SMA staining. CsA ± SRL kidneys had increased α-SMA staining in the interstitium whilst Tac ± SRL showed lower interstitial α-SMA in kidney (P < 0.05; CsA ± SRL versus Tac ± SRL). Immunostaining photomicrography shows α-SMA expression in a representative sample from each association of the experimental groups (magnification ×400).

Fig. 7. TGF-β1 quantification at 30 and 70 days. (a) P < 0.01 CsA ± SRL versus Tac ± SRL and monotherapy groups at 30 days. (b) P < 0.05 Tac ± SRL versus monotherapy groups at 70 days.

Fig. 8. VEGF mRNA quantification at 30 days. (a) P < 0.05 CsA + SRL and Tac + SRL versus control and monotherapy groups; (b) P < 0.05 SRL versus control and monotherapy groups.

of treatment TGF-β1 values in monotherapy groups were not modified, but Tac + SRL significantly increased these values at 30 days. However, this increase was not as strong as in the CsA + SRL group at 30 days (Figure 7).
Pgp intensity of the western blot analysis of the Pgp, (± monotherapy groups.

The usual approach to minimizing CNI nephrotoxicity consists of reducing doses and simultaneously introducing the mTOR inhibitors have potent immunosuppressive effects, with a different mechanism of action from CNIs [27–29].

mTOR inhibitors have potent immunosuppressive effects, with a different mechanism of action from CNIs [27–29]. The usual approach to minimizing CNI nephrotoxicity consists of reducing doses and simultaneously introducing the synergistic immunosuppressant, SRL. At the present time, there is reliable clinical [30] and experimental [31] evidence on the renal toxicity of the CsA and SRL association. Tac

Far from the functional deleterious effect, which is potentially reversible, the crucial clinical implication of CNIs with the mTORi association is the development of structural damage. In the present study, animals under CsA + SRL displayed severe histological damage as expected in this model. Thus, kidneys exposed to CsA + SRL showed intense diffuse tubular atrophy, tubular cytoplasm shrinkage and vacuolization following a diffuse striped-like distribution. As seen in the results, these histological features agree with functional data. They appeared soon after toxic exposure and had deleterious effects. Interestingly, the addition of SRL to Tac produced a different pattern. There was noticeably less widespread damage. Damage was mainly located around the juxtaglomerular apparatus, involving the distal convoluted tubule. When the follow-up was extended, both of the CNIs in monotherapy produced an increase in diffuse tubular atrophy. This agrees with other studies in the literature using this model [2,35]. When exposure to Tac + SRL was prolonged, the tubular damage did not achieve the severity of CsA + SRL in the short term, again indicating that this association has less toxic impact.

An important inflammatory component has been reported with monocyte/macrophage infiltration and tubular apoptosis, preceding tubulointerstitial fibrosis [2,25,36]. Our results show that CsA + SRL produced a notable increase in apoptosis and infiltrating macrophages together with renal tubular toxic damage, thus substantiating inflammation as a key event in the pathophysiology of this association. Accordingly, the inflammatory mediators TNF-α and IFN-γ were increased, as described in the CsA model [37,38]. Thus, Thomas et al. [3] showed that CsA nephropathy is associated with a marked increase in tubular and interstitial apoptosis, partially mediated by angiotensin II and nitric oxide inhibition. In fact, macrophages are recruited to ingest necrotic and apoptotic cells [39,40]. In contrast, none of these inflammatory or apoptotic events were observed in Tac + SRL, which produced similar results to both drugs in monotherapy. It can be suggested that Tac + SRL produces less apoptosis and probably less tubular necrosis, and thus the inflammatory feedback is weak.
The transformation of tubular cells to α-SMA+ cells is believed to be the progression to chronic renal fibrosis [41]. Ling et al. [42] reported a strong pathologic link between apoptosis/inflammation and tubulointerstitial fibrosis in chronic CsA nephotoxicity. As our results show, monocyte/macrophage infiltration is coupled with a clear overexpression of tubular α-SMA in CsA + SRL kidneys. Interestingly, despite the excess of apoptosis and myofibroblast transdifferentiation in the CsA + SRL, we found few features of fibrosis in the short term. Continuous upregulation of TGF-β1 in the kidney is a warning sign, since an excess of this mediator is related to the development of fibrosis [43]. In our study, CsA monotherapy presented an excess of this mediator is related to the development of fibrosis markers in the short term. Continuous upregulation of TGF-β1 in the kidney is a warning sign, since an excess of this mediator is related to the development of fibrosis [43]. In our study, CsA monotherapy presented an excess of this mediator is related to the development of fibrosis markers in the short term. These data reveal that Tac + SRL has less effect on kidney fibrosis markers in the short term than CsA + SRL, although the potential pro-fibrotic effect increases in the long-term.

We aimed to understand the mechanisms that explain the differences between the two associations. VEGF is overexpressed in CsA nephotoxicity, which suggests that it contributes to the repair process [46]. In contrast, VEGF has been shown to decrease in SRL therapy, usually related to its antiangiogenic effect [46,47]. As expected, a decrease in VEGF in animals exposed to SRL monotherapy was observed. Surprisingly, renal VEGF further diminished when it was associated with both CNI. We speculate that in the kidney the effect of SRL therapy predominates over that of CNI, leading to VEGF reduction as it has been suggested by Diekman [48]. This decrease in renal VEGF when SRL is associated with CNI may neutralize the potential benefit of VEGF overexpression in CsA exposure [47], thus worsening pro-fibrotic inflammatory mechanisms.

In addition, we explored the role of the Pgp detoxification system in both associations. It is well documented that CsA induces the overexpression of Pgp in vitro and in vivo [18], in a reversible and dose-dependent manner [49]. However, there is scarce information on the kinetics of Pgp in experimental Tac toxicity [22]. In our study, animals exposed to CsA alone or in association increased renal Pgp mRNA expression and protein content. As CsA inhibits Pgp activity [52,53], we speculate that renal cells may respond to Pgp protein overexpression. In fact, tubular Pgp accumulation reflects a protection mechanism against CsA-nephrotoxicity with intent to increase the efflux of metabolites out of the cells. In the association of SRL with Tac, renal Pgp mRNA experienced an evident increase, more than protein. Thus, some post-translational modifications could be present in this association, hampering protein synthesis. It has been described that CsA and Tac act as Pgp substrates and inhibitors at similar concentrations, [17], but unlike Tac, only CsA reaches inhibitory tissue concentrations in vivo [50].

In summary, the association of calcineurin-inhibitors with sirolimus showed a different functional, inflammatory and pro-fibrogenic profile. While the addition of SRL to high doses of CsA leads to severe renal dysfunction, the combination with high doses of Tac is less deleterious in the short term. Although this apparent preservation in renal function and less severe structure damage was seen at 30 days of follow-up, an important focal tubular atrophy was distinguished and profibrotic inflammatory mediators were manifested when treatment was prolonged to 70 days. Thus, despite the less nephrotoxicity and inflammation with tacrolimus instead of cyclosporine, clinicians must be cautious about the long-term effects of this association. Further studies are needed to unveiled the mechanisms involved and identify possible approaches, in order to clarify these differences in toxicity.

Acknowledgements. This work was supported by grants from the Instituto de Salud Carlos III/FIS (PI07/078, PI05/1049, SAF2004-04705). Núria Lloberas, Immaculada Herrero-Fresned and Inés Rama are researchers from ISCIII/FIS (CP06/00067, PI01/3071 and CM0300045 respectively). Gabriela Alperovich and Marcel-la Franquesa are fellows of the Fundació Catalana de Transplant. The authors are particularly grateful to Núria Bolaños for her generous and excellent technical assistance.

Conflict of interest statement. None declared.

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