GFR meets mTOR: value of different methods to measure and estimate GFR & (side) effects of mTOR inhibition in renal transplantation
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Prolonged Treatment with Everolimus Does Not Induce Podocyte Damage and Leaves the Glomerular Basement Membrane Intact

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Submitted
ABSTRACT

Background Inhibitors of the mammalian target of rapamycin (mTOR) were introduced as non-nephrotoxic immunosuppressive drugs; but have been associated with variable degrees of proteinuria. The aim of the present study was to compare proteinuria in renal transplant recipients (RTR) on a maintenance regimen with a CNI to those with the mTOR-inhibitor everolimus, and relate the presence of proteinuria to the histopathological findings of the glomerulus in 2-year protocol biopsies.

Methods In a single center study, nested in a multi-center randomized controlled trial, we determined eGFR, proteinuria and renal biopsy data (light –and electron microscopy) of RTR who received prednisolone/everolimus (P/EVL) (n=16) and compared them to a similar patient group treated with prednisolone/cyclosporine A (P/CsA) (n=7). All patients were two years after renal transplantation and had been on the above described maintenance immunosuppression for 18 months.

Results Renal function at two years after transplantation did not differ between patients who received P/EVL or P/CsA (eGFR 45.5 vs 45.7 ml/min/1.73m²); proteinuria was borderline increased in the P/EVL vs the P/CsA group (0.29 vs 0.14 g/24h, p = 0.06). There were no differences in light microscopy changes according to Banff 2005 classification, nor in electron microscopic findings. We could not demonstrate increased podocyte effacement or changes in the glomerular basement membrane thickness in the P/EVL treated patients.

Conclusion Although there was a marginal increase of proteinuria in patients treated with P/EVL, long-term treatment with everolimus left the glomerular basement membrane and the podocytes unaffected upon light and electron microscopy.
INTRODUCTION

With the introduction of calcineurin-inhibitors (CNI, cyclosporine and tacrolimus) one year renal transplant survival has improved significantly. This improvement has mainly been caused by a decrease in the incidence of early acute rejection episodes. Disappointingly, however, long-term renal graft survival has not improved to the same extent and chronic allograft dysfunction remains the dominant cause for late allograft failure. Chronic allograft nephropathy, defined by interstitial fibrosis and tubular atrophy, is, paradoxically, associated with calcineurin-inhibitors. The mammalian target of rapamycin (mTOR) inhibitors, sirolimus and everolimus, are potent immunosuppressant drugs, with the promise of no or only minor nephrotoxic effects, and other beneficial effects such as on the vessel wall and oncogenesis. Several studies, however, have reported proteinuria after the switch from a CNI-based immunosuppressive regimen to sirolimus. Controversy exists whether this increase in proteinuria results primarily from withdrawing the CNI or from the consequence of direct toxicity of sirolimus on podocytes and/or glomerular basal membrane. The latter is at least supported by new onset proteinuria in patients not on a CNI-based regimen and the development of focal glomerular sclerosis following de novo use of sirolimus. On the other hand, beneficial effects have been described in experimental animals as well as in humans. The mechanism of the sirolimus-induced proteinuria is not clearly defined. Sirolimus has been shown to reduce the in vitro expression of the slit-diaphragm proteins nephrin and transient receptor potential cation channel 6 as well as the cytoskeletal adaptor protein Nck. Moreover, sirolimus has been shown to induce microscopic morphological changes in the cytoskeleton of immortalized podocytes. Injury of the slit-diaphragm and the actin cytoskeleton of podocytes as well as interference with the interaction of podocytes with the glomerular basement membrane can result in proteinuria and morphological alterations such as foot process effacement. Remarkably, although multiple reports exist on the incidence of de novo or significantly increased proteinuria during treatment with sirolimus, only few reports have been published on everolimus. Therefore the effects of everolimus on proteinuria are not well clarified. Successful conversion from cyclosporine to everolimus has been described, without the occurrence of nephrotic range proteinuria, although it was accompanied by a slightly increased proteinuria.

A complicating factor in comparing the effects of sirolimus and everolimus may be the difference in drug exposure. Trough levels of sirolimus in the aforementioned studies were between 8–15 ng/ml, whereas the trough levels of everolimus varied between 3 – 10 ng/ml. Yet, one study in a liver transplant recipient with diabetic nephropathy reported a decrease in sirolimus-induced proteinuria after switch to everolimus, with comparable trough level of sirolimus and everolimus.

Recently, we performed a prospective multicenter randomized trial in renal transplant recipients withdrawing ciclosporin and/or mycophenolate mofetil from a triple immunosuppressive regimen at 6 months after transplantation.
Patients continued on double therapy consisting of prednisolone and everolimus or prednisolone and cyclosporine. In this study protocol biopsies were performed just prior to randomization and at 2 years after transplantation.22

Here, we describe a subset of these patients in whom we studied the amount of proteinuria in relation to the changes in the glomerulus upon light and electron microscopy comparing patients treated with either a CNI (cyclosporine A) or the mTOR-inhibitor everolimus.

METHODS

Patients

This study was a sub-study nested in the larger prospective, multi-center randomized MECANO trial studying the effects of withdrawal of cyclosporine A (CsA) from an immunosuppressive regimen containing an IL-2 antagonist (basiliximab), CsA, prednisolone (P) and mycophenolate sodium (MPS) early after transplantation. Three university hospitals in the Netherlands participated in this trial from January 2005 until September 2009: the Academic Medical Center in Amsterdam (AMC), the Leiden University Medical Center and the University Medical Center in Groningen. Institutional review board approval has been obtained. The study was conducted in accordance with the declaration of Helsinki. Informed consent was obtained from every patient. The details and results of an interim analysis of this trial were previously published (trial registration number: NTR567 (Dutch trial registry), ISRCTN69188731, www.trialregister.nl)22. In short, renal allograft recipients, receiving their first or second kidney transplant, were treated with quadruple immune suppression consisting of prednisolone, CsA, MPS and basiliximab. After 6 months, patients (in the absence of rejection upon protocol transplant biopsy) were randomized to one of three immunosuppressive regimens: P/CsA, P/MPS and P/everolimus(EVL). Drug exposure of CsA, MPS and EVL was monitored by calculating the Area Under the Curve (AUC) at pre-fixed time-points. The target values of the AUC12 for CsA was 5400 μg*h/l in the first 6 weeks and 3250 μg*h/l thereafter. The AUC12 target for MPS was > 40 μg*h/l during time of triple therapy and 70-85 μg*h/l from month 7 on. Target AUC12 for EVL was 150 μg*h/l. The primary outcome was interstitial graft fibrosis and arteriolar hyalinosis. Secondary outcome was, among others, graft rejection. Patients who received a third or fourth transplant were excluded, as were patients with > 50% panel reactive antibodies. The P/MPS arm was prematurely halted because of an increase in severe acute rejection episodes.

For this sub-study, all patients of one center (AMC), who underwent a renal biopsy at 2 years after transplantation, who received either P/CsA or P/EVL during the complete study period and of whom material for light and electron microscopy was available were included. We compared renal function, proteinuria, urine sediment abnormalities and renal biopsy data of patients treated with P/EVL to those treated with P/CsA.
Measurements

Plasma creatinine was measured with an enzymatic PAP+ (phenol/4-aminoantipyrine) assay on a Roche Modular analyser (Roche, Almere, the Netherlands).

Estimated GFR was calculated using the abbreviated MDRD formula:
\[
\text{GFR} = 175 \times \left(\frac{\text{Pcr}}{88.4}\right)^{1.154} \times \text{age}^{0.203}, \text{ (female: multiply result by 0.742, black: multiply result by 1.210)}.
\]

Total urine protein was measured using a turbidimetric (Roche Diagnostics) assay. Proteinuria is reported as total urine protein/24 hours.

AUC\textsubscript{12} for CsA and EVL were calculated from blood samples drawn at C0, 1, 3, 4, 5 and 6 hr after administration. Everolimus levels were determined by immunoassay (Innofluor® Certican® Assay System) according to manufacturers’ instructions (Seradyn Inc, IN, USA).

Light microscopy (LM)

Biopsies were formalin fixed and paraffin embedded. Haematoxylin and Eosin, Periodic Acid Schiff and Jones’ Silver stainings were performed on all biopsies. Six and 24 months post-transplant protocol biopsies were scored according to the Banff 2005 working classification for allograft pathology by two observers in a simultaneous manner (SF and JK). Only biopsies with the minimal biopsy requirements of at least 7 glomeruli and 1 artery were included in this study.

Electron microscopy (EM)

After fixation in Karnovsky fixative, the material was post fixed in 1% osmiumtetroxide and block-stained with 1% uranyl acetate. After one-step dehydration in dimethoxypropane the tissue was embedded in epoxyresin LX-112. LM sections were stained with toluidine blue. EM sections were stained with tannic acid, uranyl acetate and lead citrate and examined in a Philips CM10 transmission electron microscope (FEI, Europe BV, Eindhoven, the Netherlands).

For this study, 10 capillary loops of one to two glomeruli in each patient were photographed in a random and unbiased fashion with a final magnification of 10500. The thickness of the glomerular basement membrane (GBM) of 10 different capillary loops was measured at their thinnest points (using Universal TEM Imaging Platfom Software (Soft Imaging System)). Median and range were calculated. GBM length was traced and measured in an image processing and analysis program (ImageJ, http://rsb.info.nih.gov/ij/). The number of podocyte foot processes along the GBM was counted by hand. A foot process was defined as any connected epithelial segment butting on the basement membrane, between two neighbouring filtration pores or slits.

Podocyte foot process effacement of each biopsy was expressed as foot processes width, which was calculated as follows: FPW = \(\pi/4 \times \left(\Sigma \text{GBM length}/\Sigma \text{foot process}\right)\).

Where ‘\(\Sigma \text{foot process}\)’ is the total number of foot processes counted along the GBM in all the available pictures from each biopsy. ‘\(\Sigma \text{GBM length}\)’ is the total GBM length measured in all the pictures available of each biopsy, and the correction factor...
of \( \pi/4 \) serves to correct for presumed random variation in the angle of section relative to the long axis of the podocyte\(^23\).

**Statistical analysis**
All statistical analyses were performed under non-Gaussian assumption. Differences in patient characteristics, median Banff scores and glomerular basement membrane thickness between P/CsA and P/EVL were calculated with the use of Mann-Whitney-U signed rank tests. A \( p < 0.05 \) was considered statistically significant. Analyses were performed with the use of SPSS 16.0 (SPSS Inc. Chicago, IL, United States of America).

**RESULTS**

**Clinical parameters**
In the total multicenter trial 361 patients were enrolled\(^22\), 224 underwent randomization. After two years, 74 patients had been treated with P/CsA during the complete study period, 25 with P/MPS and 58 with P/EVL. At that time-point, mean proteinuria did not differ between both groups (P/CsA 0.30 ± 0.32 g/24h, P/MPS 0.38 ± 0.47 g/24h and P/EVL 0.43 ± 0.48 g/24h). However, proteinuria had significantly increased in the P/EVL group since randomization at 6 months (proteinuria at 6 months 0.24 ± 0.17 g/24h, \( p < 0.001 \)), whereas it had remained stable in the P/MPS and P/CsA group. Estimated GFR was significantly better in the P/MPS and P/EVL versus the P/CsA treated patients (64.3 ± 33.2 and 58.7 ± 21.1 vs 49.2 ± ml/min/1.73m\(^2\), \( p =0.01 \) and 0.03, respectively) (van der Heide, manuscript in preparation).

Table 1 shows the characteristics of the patients included in this substudy, treated with either P/EVL or P/CsA, of whom material for light and electron microscopy was available at 2 years after transplantation. Twenty-three patients were included, all Caucasian. Sixteen received P/EVL and 7 P/CsA. Three biopsy-proven rejection episodes had occurred, all within 6 months after transplantation (i.e. before randomization to either P/EVL (n=1) or P/CsA (n=2). At two years after transplantation, proteinuria was borderline increased in the P/EVL group (\( p = 0.06 \)), as compared to the P/CsA group.

The incidence of diabetes mellitus, either de novo after transplantation or already pre-existent (defined as the need for either oral anti-diabetics or subcutaneous insulin) was comparable between both groups (P/EVL 4/16 (25%), P/CsA 2/7 (29%)). Time of exposition of the renal transplant to increased glucose levels was also similar in both groups (P/EVL 11.8 (9.0 – 25.4) months vs P/CsA 12.6 (9.1 - 16.0 months).

The median (range) AUC (at the day of kidney biopsy) of CsA (n = 6) was 2882 (2404 – 5852) µg*h/l and 164 (84 – 247) µg*h/l of EVL (n = 16). Median (range) trough levels of EVL were 10.4 (3.6-17.1) µg/l.

**Light microscopy**
Of the 23 patients, 16 of the 16 patients in the P/EVL group and 6 of the 7 patients in the P/CsA group had an adequate 6 months protocol biopsy. None of the Banff
Table 1. Characteristics of 23 renal transplant recipients 2 years after transplantation, treated with either prednisolone/everolimus (P/EVL) or prednisolone/cyclosporine (P/CsA). Data are expressed as median (range), unless otherwise reported. ns = not significant.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>P/EVL (n=16)</th>
<th>P/CsA (n=7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males/females</td>
<td>10/6</td>
<td>6/1</td>
<td>ns</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.2 (24.2 – 69.3)</td>
<td>44.0 (23.2 – 69.9)</td>
<td>ns</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>45.5 (20.4-102.8)</td>
<td>46.2 (38.7 – 57.7)</td>
<td>ns</td>
</tr>
<tr>
<td>Underlying renal disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Vascular</td>
<td>5</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>- Immunological</td>
<td>4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>- Urological</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>- Other</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>- Unknown</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Transplant type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Living/deceased</td>
<td>6/10</td>
<td>1/6</td>
<td>ns</td>
</tr>
<tr>
<td>Blood pressure</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systolic/diastolic (median/range)</td>
<td>132 (115-150)/75 (88)</td>
<td>140 (112-174)/80 (100)</td>
<td></td>
</tr>
<tr>
<td>Use of ACE/ARB</td>
<td>8/16 (50%)</td>
<td>3/7 (43%)</td>
<td>ns</td>
</tr>
<tr>
<td>Proteinuria (g/24h)</td>
<td>0.29 (0.12 – 0.84)</td>
<td>0.14 (0.08 – 1.04)</td>
<td></td>
</tr>
<tr>
<td>- 0 – 0.30 g (n)</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>- 0.30 – 1.0 g (n)</td>
<td>7</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>- &gt; 1.0 g (n)</td>
<td>0</td>
<td>1</td>
<td>0.06</td>
</tr>
<tr>
<td>- missing (n)</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Urine sediment abnormalities (n):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- None</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Erythrocytoria</td>
<td>10</td>
<td>7</td>
<td>ns</td>
</tr>
<tr>
<td>- Leukocyturia</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Erythrocyturia and leukocyturia</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| parameters were significantly different between P/EV: and P/CsA group at the time of randomization. Table 2 shows the median (range) Banff scores of the 16 patients with an adequate protocol biopsy at 24 months after transplantation (11/16 in the P/EVL group and 5/7 in the P/CsA group). None of the Banff parameters were significantly different between the P/EVL and P/CsA group.

Electron microscopy
Electron microscopy was performed in all patients. In 3/23 patients, no glomerulus was detected in the enclosed material. We measured basement membrane thickness and foot process width in 14/16 P/EVL patients and in 6/7 P/CsA patients (table 3 and figure 1). Basement membrane thickness did not differ between both groups. Podocyte effacement, expressed as foot process width, was similar as well.
Table 2. Banff scores at the 2-year post-transplant protocol biopsy, treated with either rednisolone/everolimus (P/EVL) or prednisolone/cyclosporine A (P/CsA). All scores are listed as median (range). ns = not significant

<table>
<thead>
<tr>
<th>Banff parameter</th>
<th>P/EVL (n = 11/16)</th>
<th>P/CsA (n = 5/7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage sclerosed glomeruli</td>
<td>0% (0-18%)</td>
<td>0% (0-14%)</td>
<td>ns</td>
</tr>
<tr>
<td>Tubulitis</td>
<td>0 (0-1)</td>
<td>0 (0-0)</td>
<td>ns</td>
</tr>
<tr>
<td>Interstitial inflammation</td>
<td>0 (0-2)</td>
<td>0 (0-1)</td>
<td>ns</td>
</tr>
<tr>
<td>Glomerulitis</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>ns</td>
</tr>
<tr>
<td>Arteritis</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>ns</td>
</tr>
<tr>
<td>Arteriolar hyalinosis</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>ns</td>
</tr>
<tr>
<td>Mesangial matrix increase</td>
<td>0 (0-1)</td>
<td>0 (0-1)</td>
<td>ns</td>
</tr>
<tr>
<td>Allograft glomerulopathy</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>ns</td>
</tr>
<tr>
<td>Vascular intima thickening</td>
<td>0 (0-3)</td>
<td>0 (0-1)</td>
<td>ns</td>
</tr>
<tr>
<td>Interstitial fibrosis/tubular atrophy</td>
<td>1 (0-3)</td>
<td>0 (0-3)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Figure 1. Representative electron microscopy images from patients treated with everolimus. A: Normal GBM (260nm) and normal podocytes. B: Thin GBM: (110nm) and podocyte effacement. C: Thick GBM (480nm). L: capillary lumen, US: urinary space. Bar: 1 μm.

Table 3. Glomerular basal membrane thickness and foot process width measured by electron-microscopy in 21/24 renal transplant recipients 2 years after transplantation, treated with either prednisolone/everolimus (P/EVL) or prednisolone/cyclosporine (P/CsA). Data are expressed as median (range). ns = not significant

<table>
<thead>
<tr>
<th></th>
<th>P/EVL (n=14/16)</th>
<th>P/CsA (n=6/7)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glomerular basement membrane thickness (nm)</td>
<td>244 (104 – 300)</td>
<td>255 (170 - 276)</td>
<td>ns</td>
</tr>
<tr>
<td>Foot process width (nm/foot process)</td>
<td>546 (421 – 753)</td>
<td>518 (424 - 580)</td>
<td>ns</td>
</tr>
</tbody>
</table>
DISCUSSION

In the total multi-center MECANO trial and in this single center substudy, we found no significant difference in proteinuria 2 years after transplantation in patients treated with prednisolone/everolimus as compared to patients treated with prednisolone/cyclosporine, although there was an increase in proteinuria in patients treated with everolimus 2 years versus 6 months after renal transplantation. However, here we show that this is not accompanied by histopathologic changes as detectable by detailed light and electron microscopic analysis.

From studies performed in animal models, the effect of mTOR inhibitors on glomerular cells appear to be heterogenous and context dependent. For example, in early diabetic nephropathy, characterized by hypertrophy of the glomerulus including the podocytes, mTOR inhibition has been shown to attenuate the progression of diabetic kidney disease by reducing glomerular hypertrophy, mesangial expansion and glomerular basement membrane thickening. On the other hand, mTOR inhibitors have been shown to induce changes in the cytoskeleton and slit-diaphragm. For instance, the expression of nephrin, one of the critical components of the slit-diaphragm was down-regulated. Moreover, mTOR inhibitors reduce VEGF expression, which is relevant for podocyte survival and differentiation. Based on these observations, we hypothesized that mTOR inhibitors could lead to proteinuria by interfering not only with the integrity of the slit-diaphragm but also with the synthesis of the GBM resulting in a thinner membrane. To our knowledge, no previous electron microscopy studies of GBM in protocol biopsies from renal transplant recipients treated with mTOR inhibitors have been reported. Yet, with detailed electron microscopy, we could not demonstrate any difference in the glomeruli between patients treated with EVL or CsA.

No nephrotic range proteinuria occurred in our substudy and since we found no histological differences between the P/CsA and P/EVL group, the observed difference in proteinuria might also be explained by the anti-proteinuric effect of calcineurin inhibitors, caused by a decrease in renal blood flow and stabilization of the actin cytoskeleton through inhibition of synaptopodin degradation. Moreover, we cannot exclude a tubular origin of the proteinuria as previously suggested by Straathof et al. Nevertheless, light microscopy did not reveal any tubular damage. Lastly, mTOR inhibitors could interfere with the expression of cubilin and megalin in the proximal tubular epithelial cells, resulting in decreased tubular receptor-mediated albumin endocytosis.

Multiple reports exist on the association of treatment with sirolimus and an increased incidence of proteinuria. However, only few data are available on possible proteinuric effects of everolimus. This might be explained by the longer use of sirolimus in renal transplantation, differences in actual exposure and/or physico-chemical properties. As already mentioned, the difference in trough levels that were achieved in the several studies may explain a discrepancy in side effects. In our study, we measured a median AUC of 164 μg*h/l, corresponding with trough levels...
of 10.4 (3.6-17.1) μg/l, comparable with most of the sirolimus studies. Thus, it has to be elucidated whether everolimus and sirolimus in equimolar concentrations yield similar effects regarding proteinuria. In an in vitro model of cultured rat brain cells, everolimus as compared to sirolimus exerted fundamentally different effects on cell metabolism.

In conclusion, prolonged treatment with everolimus did not induce any change in the glomerular basement membrane, in the podocytes or on overall histological examination, neither by light microscopic, nor by electron microscopic analysis. The observed small difference in proteinuria between everolimus and cyclosporine treated patients is most probably explained by the antiproteinuric properties of calcineurin-inhibitors.

REFERENCES


