mTOR Inhibition Enhances the Procoagulant State of Renal Transplant Recipients

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Submitted
ABSTRACT

**Background** Renal transplant recipients are at increased risk of venous thromboembolic events, which is in part caused by their treatment with maintenance immunosuppressive drugs. Because we observed an increased incidence of venous thrombo-embolic events in renal transplant recipients treated with a mTOR inhibitor (mTORi), we aimed to identify possible prothrombotic mechanisms of this immunosuppressive drug.

**Methods** In a single center study, nested in a multi-center randomized controlled trial, we measured parameters of endothelial activation, coagulation and fibrinolysis in renal transplant recipients who received the mTORi everolimus (n=16, mTOR group) and compared them to a similar patient group, treated with a calcineurin inhibitor and/or mycophenolate sodium (n=20, non-mTOR group). All patients were at least 6 months following transplantation with a stable transplant function.

**Results** The use of a mTORi was associated with significantly higher levels of von Willebrand factor, prothrombin fragment 1+2, thrombin-activatable fibrinolysis inhibitor and plasminogen activator inhibitor-1 as compared to a non-mTOR based immunosuppressive regimen.

**Conclusion** Treatment with an mTORi leads to increased endothelial activation, thrombin formation and impaired fibrinolysis. Larger studies with clinically relevant end-points are required to establish the thrombosis risk.
INTRODUCTION

Chronic kidney disease (CKD) is associated with an increased risk of venous thrombo-embolism (VTE), especially in the presence of a nephrotic syndrome. The rate of VTE in the first years after kidney transplantation is also increased, mounting up to about 5-9%. This increase can be explained by several factors. Many renal allograft recipients still suffer from CKD showing a mean estimated creatinine clearance (eGFR) of about 55 ml/minute at 2 years following renal transplantation. Furthermore, renal transplant patients are at risk of developing proteinuria, caused in most cases by either recurrence of the original kidney disease or chronic allograft nephropathy.

Another risk factor for thromboembolic disease after renal transplantation is the maintenance immunosuppressive medication. Both steroids and calcineurin inhibitors have prothrombotic properties. Little is known about the possible prothrombotic side effects of the mTOR-inhibitors (mTORi) sirolimus and everolimus in allograft recipients. Like calcineurin-inhibitors (CNI), they have been associated with thrombotic micro-angiopathy, but the underlying mechanism is uncertain. Reduction in (local) production of vascular endothelial growth factor (VEGF) may play a pathogenetic role. Also a direct effect on prothrombotic genes encoding for PAI-1 and tissue factor (TF), has been observed. Recently we performed a multicenter randomized controlled trial (MECANO) studying early cyclosporin withdrawal after six months and comparing maintenance therapy with prednisolone/cyclosporine A (P/CsA) to prednisolone/mycophenolate sodium (P/MPS) or prednisolone/everolimus (P/EVL). Preliminary results have been published. Early in this study, we noticed cases of VTE in the everolimus treated patients. This observation, and the known effects on endothelial cells, raised our suspicion of a relationship between mTORi and the occurrence of thrombotic complications.

To study whether indeed treatment with mTOR inhibition leads to procoagulant alterations, we measured parameters of coagulation, fibrinolysis and endothelial activation in renal transplant recipients, participating in the afore mentioned trial, who received the mTOR inhibitor everolimus (EVL) and compared them to patients who were treated with a CNI and/or mycophenolate sodium (MPS).

PATIENTS AND METHODS

Patients

This study was conducted as part of a larger prospective, multicenter randomized trial (MECANO) 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 have been published previously (trial registration number: NTR567 (Dutch trial registry), ISRCTN69188731, www.trialregister.nl)\(^{15}\). 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 were (in the absence of rejection, proven by kidney biopsy) randomized to one of three immunosuppressive regimens: P/CsA, P/MPS and p/everolimus(EVL). Drug exposure of CsA and EVL was monitored by calculating the Area Under the Curve (AUC) at fixed moments. The target values of the AUC for CsA was 5400 \(\mu\text{g}\cdot\text{h}/\text{l}\) in the first 6 weeks and 3250 \(\mu\text{g}\cdot\text{h}/\text{l}\) thereafter. Target AUC for EVL was 150 \(\mu\text{g}\cdot\text{h}/\text{l}\). The primary outcome was interstitial graft fibrosis and 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 this form of double therapy resulted in an increase in severe acute rejection episodes.

The present study is a sub study nested in the multicenter trial: renal transplant recipients recruited at one side only (AMC) participated. From February 2008 until December 2009, blood samples were collected from all consecutive patients who were admitted for either a protocol renal biopsy or for protocol drug level monitoring. To study whether treatment with mTOR inhibitors leads to procoagulant alterations, we compared the patients treated with everolimus (mTOR-group) to those who were treated either with CsA or MPS or a combination of the latter two (non-mTOR group). We measured parameters of coagulation, fibrinolysis and endothelial activation. Patients treated with vitamin K antagonists were excluded (n = 1).

**Measurements**

Blood was drawn between 8 and 10 am and anticoagulated with sodium citrate (final concentration, 0.32%). Plasma samples were centrifuged twice at 4°C, 2000 rpm for 20 minutes and frozen at −80°C until assays were performed. To assess the various contributors to haemostasis: endothelial activation, thrombin/ fibrin formation, anticoagulation and fibrinolysis; the following parameters were measured: von Willebrand factor, vascular endothelial growth factor (VEGF), activated partial thromboplastin time (APTT), prothrombin time (PT), prothrombin fragment F1+2 (F1+2), endogenous thrombin potential (ETP), activated protein C (APC) resistance, protein C, protein S, thrombin-activatable fibrinolysis inhibitor (TAFI), plasminogen activator inhibitor-1 (PAI-1), plasmin-antiplasmin complexes (PAP) and D-dimer.

Von Willebrand factor antigen (vWF-Ag) was determined with a home-made ELISA using antibodies from DAKO (Glostrup, Denmark), reference values (rv)
50-150%. VEGF was measured by ELISA (R&D systems), rv: <115 pg/ml. PT and APTT were performed on an automated coagulation analyzer (Behring Coagulation System) with reagents and protocols from the manufacturer (Siemens Healthcare Diagnostics, Marburg, Germany), rv 25.0 – 38.0 and 10.7-12.9 sec, respectively. The plasma concentrations of F1+2 were measured by ELISA (Siemens), rv 53 – 271 pmol/l.

The Calibrated Automated Thrombogram® assays the generation of thrombin in clotting plasma using a micro titer plate reading fluorometer (Fluoroskan Ascent, ThermoLab systems, Helsinki, Finland) and Thrombinscope® software (Thrombinscope BV, Maastricht, The Netherlands). The assay was carried out as described by Hemker et al.16 and the Thrombinscope® manual.

Coagulation was triggered by recalcification in the presence of 5 pM recombinant human tissue factor (Innovin®, Siemens), 4 μM phospholipids, and 417 μM fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland). Fluorescence was monitored using the Fluoroscan Ascent fluorometer (ThermoLabsystems, Helsinki, Finland), and the ETP was calculated using the Thrombinscope® software (Thrombinscope BV). Reference values for ETP were 1155-2606 nM.min.

Resistance to activated protein C was determined by testing the effect of activated protein C on the endogenous thrombin potential (ETP) with the CAT assay. The sensitivity to APC (Enzym Research Laboratories) of each plasma sample was determined in both the presence and absence of ~4 nM APC (final concentration). The APC concentrations used were adjusted to maintain a residual thrombin generation activity of approximately 10% in normal pooled plasma. Normal pooled plasma was run in parallel on each plate. The normalized ratio (APCsr) was determined by dividing the APCsr of an individual by the APCsr of the pooled plasma. A normalized APC sensitivity ratio >1.0 reflects an APC resistant phenotype. Reference value was <1.6.

Protein C was determined using the Coamatic protein C activity kit from Chromogenix (Mölndal, Sweden), rv 70 -120%. Total protein S antigen was assayed by ELISA using antibodies from DAKO (Glostrup, Denmark). Free protein S was measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. Reference values were 63-137% and 25-130%, respectively. TAFI activity levels were determined with a chromogenic assay (Pefakit TAFI, Pentapharm LTD, Basel, Switzerland) by converting TAFI into its active form and subsequently measuring the carboxypeptidase activity. Measurements were run on a BCS coagulation analyzer (Siemens), rv 64 – 125%. PAI-1 antigen was assayed by ELISA (Innotest PAI-1, Hyphen BioMed, Andrésy, France), rv: < 100 ng/ml. PAP complexes were determined by ELISA (DRG, Marburg, Germany), rv 47 – 563. D-dimer levels were determined with a particle-enhanced immunoturbidimetric assay (Innovance D-Dimer, Siemens), rv: < 1.00 mg/l fibrogen activity units (FEU).

Plasma creatinine levels were measured with an enzymatic PAP+ (phenol/4-aminoantipyrine) assay on a Roche Modular analyzer (Roche, Almere,
The Netherlands). Estimated GFR (eGFR) was calculated using the abbreviated MDRD formula:

\[
GFR = 175 \times (\text{Pcr} \div 88.4)^{-1.154} \times \text{age}^{-0.203}, \text{ (female: multiply result by 0.742, black: multiply result by 1.210)}.
\]

**Statistical analysis**

Statistical analysis was performed using SPSS 16. Data are expressed as median (interquartile range) since not all parameters were normally distributed and numbers were relatively small. For comparison between groups in the substudy, we used Mann–Whitney U test, Chi-square test or Fisher-Freeman-Halton (using StatXact-3), when appropriate. We used the Spearman correlation coefficient to correlate parameters of coagulation with the AUC of CsA, EVL and MPS. Multivariable linear regression analysis was performed to assess the independent influence of various parameters (age, gender, days after transplantation and type of immunosuppression) on the contributors to coagulation. The normality assumption for the linear model was assessed by inspection of the residuals. Since for F1+2 and PAI-1 violations were observed, these variables were analyzed on a transformed scale using $1/F1+2$ AND $10\log(\text{PAI}-1)$. A p-value $\leq 0.05$ was considered statistically significant.

**RESULTS**

We studied 2 differently treated patient groups after renal transplantation (non-mTOR versus mTOR). Patient characteristics are shown in table 1. Forty-two blood samples were collected. After exclusion of 6 blood samples (3 due to clotting, 2 because of the use of oral anticoagulants and 1 because of a too small plasma volume), 36 samples (from 36 patients) were available for analysis. There were twenty patients in the non-mTOR group and 16 in the mTOR group. Of the 20 patients in the non-mTOR group, 11 were treated with triple therapy i.e. prednisolone/cyclosporine/mycophenolate sodium (P/CsA/MPS), 4 were treated with P/CsA, 4 with P/MPS and one was treated with P/tacrolimus. All patients in the mTOR group were on P/everolimus (EVL).

In 29/36 patients a protocol kidney biopsy was performed just following blood withdrawal (20 in the non-mTOR group and 9 in the mTOR group). In 2 cases (both in the non-mTOR group), in the protocol biopsy, a borderline acute cellular rejection was diagnosed, for which no additional treatment was given. They were not randomized to double therapy but continued triple therapy.

The demographic characteristics were similar between both groups, except for the time interval from transplantation to blood withdrawal: this was increased in the patients in the mTOR group as compared to patients in the non-mTOR group (median 754 days versus 570 days). None of the patients developed VTE during the study.
Table 1. Patient characteristics of 36 renal transplant recipients treated with either prednisolone/cyclosporine/mycophenolate sodium (non-mTOR group) or prednisolone/everolimus (mTOR group). Data are expressed as median (interquartile range). M/F = male/female. eGFR = estimated GFR.

<table>
<thead>
<tr>
<th></th>
<th>non-mTOR (n= 20)</th>
<th>mTOR (n = 16)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/F</td>
<td>17/3</td>
<td>11/5</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>58.2 (38.3 – 64.4)</td>
<td>51.3 (40.6 – 64.3)</td>
<td>NS</td>
</tr>
<tr>
<td>Underlying renal disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vascular</td>
<td>4</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>Immunological</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Urological</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>6</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transplant type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Living/deceased</td>
<td>6/14</td>
<td>7/9</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.6 (25.2 – 29.3)</td>
<td>24.8 (22.9 – 28.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Days after transplantation</td>
<td>570 (181 - 724)</td>
<td>754 (621 - 782)</td>
<td>P = 0.03</td>
</tr>
<tr>
<td>eGFR (ml/min)</td>
<td>42.9 (35.3 – 61.2)</td>
<td>50.3 (38.6 – 65.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Proteinuria (g/24h)</td>
<td>0.21 (0.13 – 0.45)</td>
<td>0.27 (0.19 – 0.43)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 2 shows an overview of the parameters of coagulation. When certain parameters could not be determined due to lack of material, the number of analyzed patients is reported. Figure 1 demonstrates the most important findings. vWF concentrations were elevated in both patient groups and the majority of patients had an abnormal D-dimer result: 24/35 (69%) > 0.5 mg/l and 9/35 (26%) > 1.0 mg/l. CRP did not differ between both groups: median (interquartile range) non mTOR group 1.2 (0.55 – 3.8) mg/l versus 2.2 (0.55 – 3.0) mg/l in the mTOR group (p = 0.67).

vWF, VEGF, F1+2, protein C, TAFI and PAI-1 were significantly higher in the mTOR treated patients as compared to the non-mTOR group. Multivariable linear regression analysis, including age, gender, days after transplantation and type of immunosuppression, showed that vWF and F1+2 were significantly associated with age and type of immunosuppression, VEGF with gender and immunosuppression. Protein C, TAFI and PAI-1 were affected by type of immunosuppression only.

The median (range) AUC of CsA (n=13) was 3302 (1693 – 5852) µg*h/l, the median (range) AUC of EVL (n=13) was 188 (123 – 229) µg*h/l. We could not demonstrate a significant correlation between the AUCs of EVL and CsA and the level of the assessed parameters of coagulation, fibrinolysis and endothelial activation.
Table 2. Parameters of coagulation in renal transplant recipients treated with either prednisolone/cyclosporine/mycophenolate sodium (non-mTOR group) or prednisolone/everolimus (mTOR group). Data are expressed as median (interquartile range). Ns = not significant. vWF = von Willebrand factor, VEGF = vascular endothelial growth factor, APTT = activated partial thromboplastin time, PT = prothrombin time, F1+2 = prothrombin fragment F1+2, ETP = endogenous thrombin potential, APC = activated protein C, TAFI = thrombin-activatable fibrinolysis inhibitor, PAI-1 = plasminogen activator inhibitor-1, PAP complexes = plasmin-antiplasmin complexes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>non-mTOR (n= 20)</th>
<th>mTOR (n = 16)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td>192 (175 – 252)</td>
<td>220 (192 – 266)</td>
<td>NS</td>
</tr>
<tr>
<td>vWF (%)</td>
<td>178 (129 – 243)</td>
<td>315 (252 – 361)</td>
<td>p &lt; 0.0001</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>32.4 (20.6 – 38.2)</td>
<td>39.6 (29.2 – 58.4)</td>
<td>p = 0.02</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>32.4 (30.9 – 38.0)</td>
<td>33.1 (30.6 – 36.6)</td>
<td>NS</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>11.9 (11.6 – 12.9)</td>
<td>12.0 (11.7 – 12.7)</td>
<td>NS</td>
</tr>
<tr>
<td>F1 + 2 (pmol/l)</td>
<td>356 (307 – 463)</td>
<td>556 (383 – 791)</td>
<td>p = 0.03</td>
</tr>
<tr>
<td>ETP (nM.min)</td>
<td>1399 (1271 – 1583)</td>
<td>1477 (1346 – 1632)</td>
<td>NS</td>
</tr>
<tr>
<td>APC</td>
<td>0.58 (0.00 – 1.07)</td>
<td>0.65 (0.00 – 1.90)</td>
<td>NS</td>
</tr>
<tr>
<td>Protein C (%)</td>
<td>128 (109 – 163)</td>
<td>173 (152 – 196)</td>
<td>p = 0.009</td>
</tr>
<tr>
<td>Protein S (total) (%)</td>
<td>120 (106 – 125)</td>
<td>131 (111 – 159)</td>
<td>NS</td>
</tr>
<tr>
<td>Protein S (free) (%)</td>
<td>109 (95 – 123)</td>
<td>109 (94 – 121)</td>
<td>NS</td>
</tr>
<tr>
<td>TAFI (%)</td>
<td>102 (90 – 120)</td>
<td>122 (102 – 134)</td>
<td>p = 0.01</td>
</tr>
<tr>
<td>PAI-1 (ng/ml)</td>
<td>61 (30 – 90)</td>
<td>100 (48 – 149)</td>
<td>p = 0.05</td>
</tr>
<tr>
<td>PAP complexes (µg/l)</td>
<td>488 (365 – 736)</td>
<td>657 (435 – 752)</td>
<td>NS</td>
</tr>
<tr>
<td>D-dimer (mg/l)</td>
<td>0.78 (0.32 – 1.19)</td>
<td>0.76 (0.48 – 0.94)</td>
<td>NS</td>
</tr>
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</table>

DISCUSSION

In this pilot study in 36 renal transplant recipients we aimed to find an underlying mechanism for the increased incidence in thrombotic events in patients using mTOR inhibitors. We here demonstrate that von Willebrand factor concentration is elevated in all RTRs as is the F1+2 level, pointing to increased coagulation potential and activation of coagulation following renal transplantation. Indeed, the use of
mTOR inhibitors even further increased vWF levels as compared to a non-mTOR based immunosuppressive regimen. Furthermore, we observed higher F1+2, TAFI and PAI-1 concentrations in the patients on a mTORi. This indicates that treatment with mTORi (i.e. everolimus) leads to increased endothelial activation, thrombin formation and impaired fibrinolysis compared to treatment with a non-mTORi (i.e. CNI and/or MPS).
In our study the high levels of vWF in both groups, and especially in the mTOR group, are striking. Previous studies in renal transplant recipients treated with prednisolone, cyclosporine and/or mycophenolate mofetil, reported vWF values of around 200%, corresponding with our values in the non-mTOR group. To the best of our knowledge no studies on vWF levels in renal transplant recipients treated with mTOR inhibitors have been reported so far. The higher levels of vWF in the EVL group are compatible with either increased endothelial activation or increased release from activated platelets. The latter also applies to the higher levels of VEGF in the EVL group. VEGF has been shown to be crucial to preserve the integrity of the endothelium. In contrast to our findings, previous studies demonstrated that mTOR inhibitors decrease VEGF levels locally and systemically, possibly mediated through hypoxia inducible factor 1α (HIF1α). HIF1α is regulated via the mTOR pathway: mTOR inhibitors decrease the production of HIF1α and thereby VEGF. However, the fact that we found elevated levels of VEGF might be explained by VEGF release from platelets, reflecting an ex vivo phenomenon caused by activation of platelets by blood collection using a tourniquet (Niers et al, unpublished data). The higher concentrations in the mTOR treated group found by us could therefore point to increased platelet activity in these patients. Increased levels of F1 + 2 in the mTOR group indicate enhanced in vivo thrombin formation leading to fibrin generation and platelet activation. In addition, increased thrombin generation leads to augmented activation of TAFI which impairs fibrinolysis. Both TAFI and PAI-1, another inhibitor of fibrinolysis were increased in the mTOR compared to the non-mTOR treated patients.

The only significant difference between the treatment group was the time after transplantation. Previous studies have demonstrated a decrease in levels of prothrombotic markers and an improvement of endothelial injury markers after transplantation as compared to before or early after transplantation. However, in our study, time after transplantation in patients in the mTOR group was even longer as compared to the non-mTOR treated group.

A possible explanation for the increased procoagulant state in the mTOR-group could be up-regulation of tissue factor (TF) due to the inhibition of the mTOR pathway by everolimus. TF production is known to be stimulated via the VEGF/MAP kinase pathway and inhibited by the PI3K/Akt/mTOR pathway. Inhibition of the mTOR pathway by everolimus thus may lead to up-regulation of TF expression, by removing this additional negative feedback loop, which in turn might lead to activation of coagulation.

The main limitation of this substudy of the MECANO trial is the relatively small number of patients. None of the patients in our substudy developed a VTE during the study period. However, our initial observation of thrombosis in everolimus treated patients was recently confirmed after completion of the final analysis of the MECANO trial van der Heide, manuscript in preparation). Seven out of 96 (7.3%) everolimus treated patients developed a VTE (5 deep venous thrombosis...
and 2 pulmonary embolism) versus 1/39 (2.6%) (DVT) and 0/89 (0%) in the P/MPS and P/CsA treated patients, respectively, \( p = 0.02 \) (Fisher-Freeman-Halman test). This increased incidence of VTE is in line with a recently published study\(^{27}\). In this study significant more VTEs occurred in lung transplant recipients treated with a combination of prednisolone, tacrolimus and sirolimus than in those treated with prednisolone, tacrolimus and azathioprine (15/87 (17.2%) versus 3/94 (3.2%), \( p < 0.01 \)). Moreover, several case reports have been published about striking arterial or venous thromboembolic events in solid organ transplant recipients treated with an mTORi \(^{28-30}\). Unexpectedly, VTE is not described as an adverse event in many randomized controlled trials studying mTORi in renal transplantation\(^{31, 32}\) or for the treatment of renal cell carcinoma\(^{33, 34}\). According to the published incidence of VTE in RTR or in patients with a renal cell carcinoma, one would expect, even without the use of mTORi, an incidence of at least 5% in those trials\(^{4, 5, 35, 36}\). However, venous thrombosis as an adverse event is often underreported in clinical studies.

In conclusion, we found an increased procoagulant state in renal transplant recipients treated with a mTORi (everolimus) compared to those treated with a non-mTOR (i.e. CNI and/or MPS) based immunosuppressive regimen. Increased endothelial activation, thrombin formation and impaired fibrinolysis seem to be responsible, although we could not exclude a possible effect of platelet activation. This suggests an increased risk of thrombotic events in renal transplant recipients treated with mTOR inhibitors. Although more studies with hard endpoints are necessary to establish the clinical risk, in the meantime one should be cautious in prescribing mTOR inhibitors to patients with a history of venous or arterial thrombosis.

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