Sirolimus and Proteinuria in Renal Transplant Patients: Evidence for a Dose-Dependent Effect on Slit Diaphragm-Associated Proteins

Giovanni Stallone,[1] Barbara Infante,[1] Paola Pontrelli,[1] Maddalena Gigante,[1] Eustacchio Montemurno,[1] Antonia Loverre,[2] Michele Rossini,[2] Francesco Paolo Schena,[2] Giuseppe Grandaliano,[2] and Loreto Gesualdo[1]

Background. The mechanisms underlying the development of proteinuria in renal-transplant recipients converted from calcineurin inhibitors to sirolimus are still unknown.
Methods. This is a single-center cohort study. One hundred ten kidney transplant recipients converted from calcineurin inhibitors to sirolimus in the period from September 2000 to December 2005 were included in the study. All patients underwent a graft biopsy before conversion (T0) and a second protocol biopsy 2 years thereafter (T2), according to our standard clinical protocol. On the basis of the changes observed in proteinuria between T0 and T2 (median 70%), the patients were divided into two groups: group I (<70%) and group II (>70%). The authors blinded the sirolimus blood trough levels. We investigated in vivo the effects of sirolimus on nephrin, podocin, CD2ap, and actin protein expression. Slit diaphragm (SD)-associated protein expressions were evaluated in T0 and T2 biopsies. The same analysis was performed in cultured human podocytes treated with different doses of sirolimus (5, 10, 20, and 50 ng/mL).
Results. The SD protein expression in group II T2 biopsies was significantly reduced compared with the T0 biopsies and with T2 group I biopsies. In addition, sirolimus blood trough levels directly and significantly correlated with the SD protein expression at T2 graft biopsies. Group II patients presented significantly higher sirolimus blood levels than group I. In vitro study confirmed that sirolimus effect on podocytes was dose dependent.
Conclusions. Our data suggest that sirolimus-induced proteinuria may be a dose-dependent effect of the drug on key podocyte structures.

Keywords: Sirolimus, Slit diaphragm proteins, Renal allograft.

(*Transplantation* 2011;91: 997–1004)

Nephrotic range proteinuria was reported in 64% of renal transplant recipients converted from a calcineurin inhibitor (CNI)-based to a sirolimus-based immunosuppressive regimen (*1*). The mechanism of this event is still unknown. Several studies emphasized the role of podocytes in the pathogenesis of proteinuria and glomerular damage (*2*, *3*).

Podocytes are differentiated cells with a complex cytoarchitecture. They have a voluminous cell body and interdigitated foot processes, covering the glomerular basement membrane, connected by an electron-dense structure, the slit diaphragm (SD) (*3*). Proteinuria, the most common clinical manifestation of glomerular diseases, is invariably associated with podocyte foot-process effacement and flattening (*3*). The SD is a specialized cell-adhesion structure essential for glomerular ultrafiltration that differentiates from typical junctional complexes during development and share similar-

This work was supported by Ministero dell'Università e della Ricerca Scientifica (PRIN 2005 granted to L.G.), the University of Foggia (ex 60%, 2005, granted to L.G.), and by Ministero della Salute (Ricerca Finalizzata 2006, granted to G.G.).
G.S. and B.I. equally contributed to this study.
G.S., G.G., F.P.S., and L.G. received honorary fees for lectures and advisory boards from Wyeth, Novartis, Astellas, and Roche; and L.G., G.G., and F.P.S. have received an unrestricted research grant from Wyeth, Astellas, Roche, and Novartis.
[1] Section of Nephrology, Department of Biomedical Sciences, University of Foggia, Italy.
[2] Department of Emergency and Organ Transplantation, Nephrology, Dialysis and Transplantation Unit, University of Bari, Italy.
Address correspondence to: Giovanni Stallone, M.D., Division of Nephrology, Department of Biomedical Sciences, University of Foggia, Viale Luigi Pinto, 1, 71100 Foggia, Italy.
E-mail: g.stallone@unifg.it or stallonegio63@gmail.com

G.S. and B.I. designed research and wrote the article; G.G. analyzed data and revised the article; M.R. and A.L. performed histological analysis; E.M. and M.G. performed in vitro study; P.P. analyzed data and supervised results; and F.P. S. and L.G. critically revised the article.
Supplemental digital content is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).
Received November 23, 2010. Revision requested December 12, 2010.
Accepted January 21, 2011.

ISSN 0041-1337/11/9109-997
DOI: 10.1097/TP.0b013e318211d342

ities with tight junctions (*4*). SD, like other adhering junctions, includes immunoglobulin (Ig) and cadherin superfamily cell-adhesion receptors. Nephrin and its homologue Neph1 are members of the Ig superfamily and have been suggested to form the framework of the SD through homophilic interactions between podocytes (*5–8*). Nephrin is crucial for podocyte maturation and glomerular function, because its mutation is associated with congenital Finnish type nephrotic (*5*, *9*). Mutations in several other SD-associated proteins lead to the loss of podocyte foot-process architecture and the development of nephrotic syndrome (*10–14*).

Recent studies suggest that nephrin, podocin, and CD2-associated protein (CD2ap) might participate in a common cell-signaling pathway. Huber et al. (*15*) demonstrated that both nephrin and CD2ap interact in vivo with the p85 regulatory subunit of phosphoinositide-3-OH-kinase (PI3K). PI3K is the first signaling protein demonstrated to interact with the cytoplasmic surface of SD–protein complex in vivo. Nephrin and CD2ap recruit PI3K to the plasma membrane and, together with podocin, stimulate PI3K-dependent activation of the serine-threonine kinase, AKT. These findings reveal a novel role for the SD proteins and demonstrate that nephrin, CD2ap, and podocin, in addition to their structural functions, initiate signal transduction in podocytes (*15*, *16*).

Sirolimus is an immunosuppressive drug currently used in the antirejection regimen in kidney transplantation (*17*). Sirolimus inhibits mammalian target of rapamycin (mTOR), a serine/threonine-kinase, lying immediately downstream of AKT, which modulates proliferation and clonal expansion of activated T cells (*18*). In addition to its immunosuppressive actions, sirolimus inhibits growth factor-mediated proliferation of several nonimmune cells (*19*).

To date, there are no reports in vivo on the effect of sirolimus on SD-associated protein function and structure. Moreover, little is known about the molecular mechanisms leading to proteinuria in renal transplant patients receiving sirolimus. Thus, our work focused on the effects of sirolimus on nephrin, podocin, CD2ap expression, and their interaction with the podocyte cytoskeleton, in the attempt to define the influence of this drug on SD functions and whether these effects might be dose dependent.

RESULTS

In Vivo Study

Of 110 patients, 58 presented an increase in proteinuria less than 70% during the 2 years of observation (group I), whereas 52 patients developed an increase in urine protein excretion above 70% (group II). The main features of the patients' population are summarized in Table 1. During the whole period of observation, mean sirolimus blood trough levels (TL) was 6.6±1.1 ng/mL in group I (range, 5–9 ng/mL) and 11.7±1.2 ng/mL in group II (range, 10–14 ng/mL) (P=0.01). Chronic allograft nephropathy grading, according to Banff 1997 criteria (*20*), was similar in the two groups at conversion and at the end of follow-up. Also, the incidence and extent of specific glomerular lesions were similar in the two groups (69% of group I patients showed 25% of sclerotic glomeruli with presence of double contours in 20% of them, whereas 31% showed between 30 and 50% of glomerular sclerosis with the presence of double contours in 61% of them; 79% group II patients showed 25% of sclerotic glomeruli with presence of double contours in 17% of them; and 21% with glomerular sclerosis between 30% and 50% and presence of double contours in 71% of them). No statistically significant differences were observed in acute rejection rate, graft function, and survival between the two groups. The incidence of adverse events was similar in the two groups except for hyperlipidemia, which was significantly higher in group II.

TABLE 1. Clinical features of the two groups of patients

	Group I (N=58)	Group II (N=52)
Donor age (yr)	49±6.2	50±7.6
Recipient age (yr)	51±8.1	49±10.6
HLA MM	3±1.2	3±1.0
CIT (hr)	14±5.6	16±7.9
Transplant time (mo)	21±6.1	19±4.1
sCr (mg/dL) at conversion	1.9±0.4	2.1±0.7
sCr (mg/dL) at 2 yr	1.8±0.4	2.0±0.9
Sirolimus TL (ng/mL)	6.6±1.1	11.7±1.2[a]
CAN grading (Banff '97) at conversion		
Grade I	38	34
Grade II	12	13
Grade III	8	5
CAN grading (Banff '97) at 2 yr		
Grade I	39	34
Grade II	13	12
Grade III	6	6
Foot processes effacement at conversion (%)	23±13	21±15
Foot processes effacement at 2 yr (%)	27±11	73±18[b]
Proteinuria g/24 h at conversion	0.6±0.3	0.7±0.3
Proteinuria g/24 h at 2 yr	1.0±0.4	3.2±0.8[c]
Acute rejection		
Preconversion (%)	6.9	7.7
Postconversion (%)	0	0
Adverse events		
Hyperlipidemia (%)	53.4	73.0[d]
Anemia (%)	15.5	19.2
Infection (%)	0	0
Oedema (%)	0	0
Oral aphta (%)	3.4	3.8

[a] P=0.01 vs. group I.
[b] P=0.001 vs. group I.
[c] P=0.01 vs. group I and P=0.001 versus group II patients at conversion.
[d] P=0.02 vs. group I.
Values are given as mean±SD.
HLA MM, HLA mismatches; CIT, cold ischemia time; sCr, serum creatinine; CAN, chronic allograft nephropathy.

Sirolimus Modulates SD-Associated Proteins Expression and Distribution in Graft Tissues

We analyzed the effect of sirolimus on the main SD-associated proteins and on podocyte cytoskeleton in graft biopsies at baseline (Fig. 1A, E, I, M) and 2 years after conversion (Fig. 1). In group I patients, we observed a slight reduction in synaptopodin, nephrin, podocin, and CD2ap

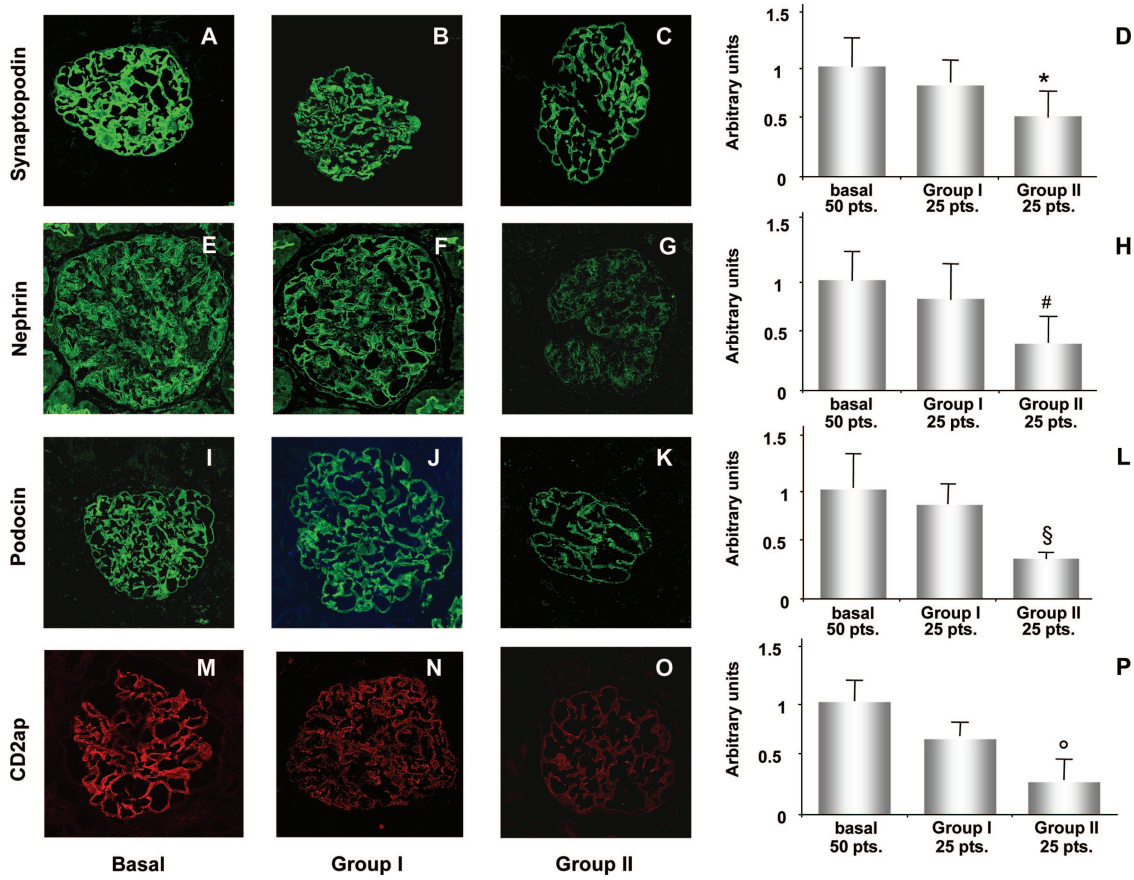


FIGURE 1. In vivo effect of sirolimus (SRL) on synaptopodin (A–D), nephrin (E–H), podocin (I–L), CD2ap (M–P) expressions and distribution on graft tissues at conversion (A, E, I, M) and 2 years thereafter (B, C, F, G, J, K, N, O) in patients from group I (B, F, J, N) and group II (C, G, K, O). Specific immunofluorescence signal was quantified as described in Materials and Methods section and expressed as arbitrary units/pixel (D, H, L, P). *P=0.004 group II vs. group I and P=0.0004 group II vs. basal; #P=0.003 group II vs. group I and P=0.0005 group II vs. basal; §P=0.001 group II vs. group I and P=0.0002 group II vs. basal; °P=0.005 group II vs. group I and P=0.0001 group II vs. basal. For this analysis, we evaluated 25 biopsies of group I patients and 25 for group II patients.

protein expressions when compared with baseline (Fig. 1B, F, J, N). Interestingly, in group II, synaptopodin, nephrin, podocin, and CD2ap protein expressions were reduced (Fig. 1C, G, K, O) compared with baseline. Specific signal quantification demonstrated that all the differences observed were statistically significant (Fig. 1D, H, L, P).

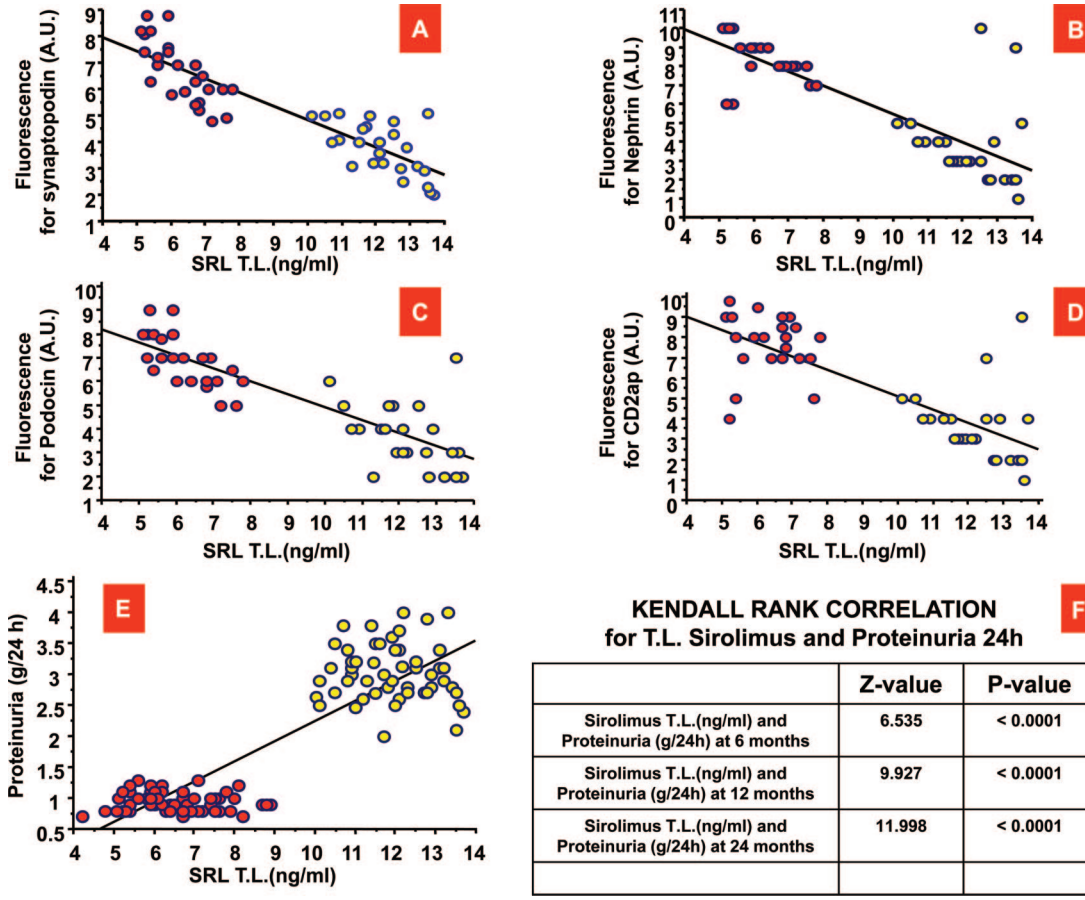
Interestingly, synaptopodin, nephrin, podocin, and CD2ap specific-immunofluorescence signals were inversely and significantly correlated with sirolimus TL at the time of second biopsy (R^2=0.625, P<0.0001; R^2=0.452, P<0.0001; R^2=0.526, P<0.0001; R^2=0.388, P<0.0001, respectively) (Fig. 2A–D). In addition, we observed a significant correlation between sirolimus TL and proteinuria (R^2=0.597, P<0.0001; Fig. 2E). Finally, we performed a Kendall rank correlation for sirolimus TL and proteinuria (Fig. 2F) at 6, 12, and 24 month after conversion. At all time points, sirolimus TL was significantly associated with urine protein excretion rate (Fig. 2F).

Electron microscopy study (Fig. 3) showed a different grade of podocyte damage in the two groups of patients. In group I, we observed a mild, segmental foot processes effacement with occasional early microvillous transformation and club-shaped foot processes (Fig. 3B–D). In group II, we noted an extensive foot processes effacement, resembling those observed in minimal change nephropathy and idiopathic focal segmental glomerulosclerosis (Fig. 3E–F). These podocyte alterations were absent at baseline (Fig. 3A, B).

Sirolimus Inhibits p70S6 Kinase-Phosphorylation and WT1 Expression But Does Not Modulate CD10 Expression in Graft Biopsies

We analyzed the effect of sirolimus on phospho-p70S6-kinase expression on graft biopsies at baseline (see **Figure 1A, Supplemental Digital Content 1**, http://links.lww.com/TP/A393) and 2 years after conversion (see **Figure 1B and C, Supplemental Digital Content 1**, http://links.lww.com/TP/A393). In both groups, we observed a reduction in p70S6 kinase phosphorylation. This reduction was significantly greater in group II patients (see **Figure 1D, Supplemental Digital Content 1**, http://links.lww.com/TP/A393).

We, then, analyzed a marker of podocyte differentiation on graft tissue. WT1 expression in group I biopsies was slightly reduced compared with baseline (see **Figure 1F and 1E, respectively, Supplemental Digital Content 1**, http://links.lww.com/TP/A393). On the contrary, in group II



KENDALL RANK CORRELATION for T.L. Sirolimus and Proteinuria 24h F

	Z-value	P-value
Sirolimus T.L.(ng/ml) and Proteinuria (g/24h) at 6 months	6.535	< 0.0001
Sirolimus T.L.(ng/ml) and Proteinuria (g/24h) at 12 months	9.927	< 0.0001
Sirolimus T.L.(ng/ml) and Proteinuria (g/24h) at 24 months	11.998	< 0.0001

FIGURE 2. Correlation between intensity of slit diaphragm (SD) proteins specific immunofluorescence in graft biopsies and sirolimus (SRL) blood trough levels (TL) (A–D). The specific immunofluorescence was quantified as described in the Materials and Methods section; synaptopodin, R^2=0.625, P<0.0001 (A); nephrin, R^2=0.452, P<0.0001 (B); podocin, R^2=0.526, P<0.0001 (C); CD2ap, R^2=0.388, P<0.0001 (D). Correlation between sirolimus TL and proteinuria (R^2=0.773, P<0.0001) (E). Kendall rank correlation for sirolimus TL and proteinuria (F) at 6, 12, and 24 months after conversion. All time points considered were statisticaly significant (F).

biopsies, we observed a greater reduction in WT1 expression compared with baseline (see **Figure 1G, Supplemental Digital Content 1**, http://links.lww.com/TP/A393), suggesting a dose-dependent effect of sirolimus on podocyte differentiation. Specific signal quantification demonstrated that all the differences observed were statistically significant (see **Figure 1H, Supplemental Digital Content 1**, http://links.lww.com/TP/A393).

In the attempt to demonstrate that the effect of sirolimus was specific for SD proteins, we examined the expression of another membrane-associated protein, CD10. Interestingly, the expression of this aminopeptidase was not influenced by sirolimus. Indeed, we did not observe any difference between T0 and T2, in group I or II (see **Figure 1I–L, Supplemental Digital Content 1**, http://links.lww.com/TP/A393).

Sirolimus Causes a Dose-Dependent Reduction in CD2ap, Nephrin, and Podocin Protein Expression in Cultured Human Podocytes

The in vivo observation of CD2ap, nephrin, and podocin protein expression modulation by sirolimus was confirmed in vitro in a podocyte cell line. We investigated the protein expression of the main components of podocyte SD by western blotting. This approach confirmed that sirolimus induces a significant and dose-dependent reduction in nephrin, podocin, and CD2ap protein levels (Fig. 4).

DISCUSSION

In this study, we demonstrated that sirolimus inhibits in vivo and in vitro, in a dose-dependent manner, the expression of the main components of podocyte cytoskeleton and SD. In addition, we demonstrated that, in vivo, this effect is specific for podocyte SD proteins, because the expression of CD10 on graft biopsies was not influenced by sirolimus treatment. Moreover, our results confirm that sirolimus induces, in a dose-dependent manner, a reduction in WT1, a transcription factor essential for maintaining podocyte integrity. Finally, we demonstrated a direct correlation between SD proteins expression in graft biopsies and sirolimus blood TLs. Our in vivo data suggest an association between these molecular effects of sirolimus and the development of clinically significant proteinuria. Interestingly, in vitro the reduced expression of SD proteins induced by sirolimus also seems to be dose dependent. Moreover, our in vitro data confirm other studies in primary cultures of human podocytes that explored the effects of sirolimus on several podocyte markers (*21*).

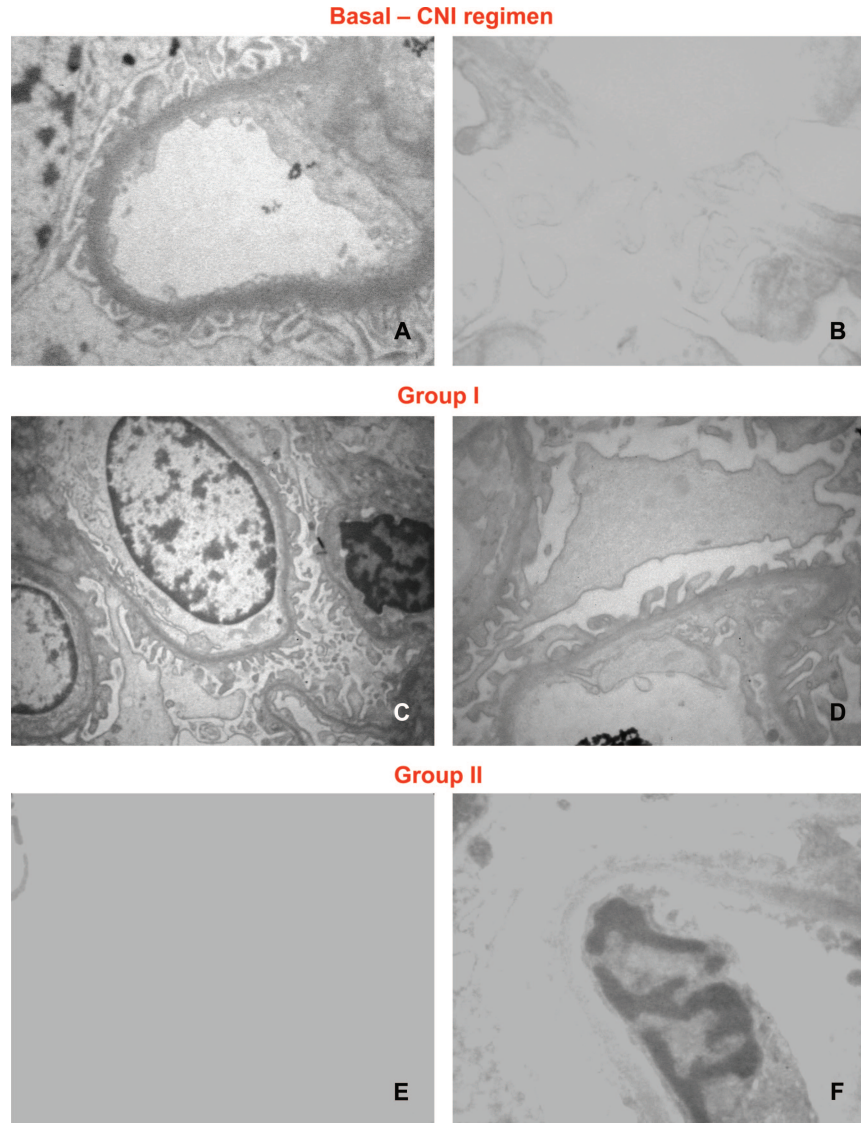


FIGURE 3. Microscopy electron images of podocytes at baseline (A, B) and 2 years after conversion to sirolimus treatment (C–F) in group I (C, D) and II (E, F). (A) Glomerular filtration barrier in a patient on CNI regimen. Minimal effacement is seen with minimal microvillous transformation (MVT). Transmission electron microscopy (TEM) ×5000. (B) Individual slit diaphragms are well oriented with minimal distortion. TEM ×9000. (C) Mild, segmental foot processes effacement in podocyte from a patient in low-dose group, with early MVT. TEM ×5000. (D) Mild foot processes effacement with occasional club-shaped foot processes. TEM ×6000. (E) Extensive foot processes effacement in podocyte from a patient in high-dose group. Frequent blebbing is also seen on podocyte surface. TEM ×5000. (F) TEM ×8000.

It is well known that the podocyte SD has a crucial role in glomerular filtration. Alteration of its protein components are involved in the pathogenesis of proteinuria. Mutations or inactivation of SD proteins cause proteinuria. In particular, podocin, a hairpin-shaped integral membrane protein with both ends directed into the intercellular space, interacts with the intracellular domains of nephrin and CD2ap (*22*, *23*). Severe proteinuria develops in podocin-knockout mice (*24*). CD2ap interacts with the intracellular domains of nephrin and podocin and it is also involved in SD signaling (*25*). Our data demonstrated that high doses of sirolimus induced a permanent alteration of SD and cytoskeleton causing a derangement of the proteins that form the glomerular filtration barrier. In addition, our results showed that the reduction in nephrin, podocin, and CD2ap is associated with the derangement of the stress fibers forming the podocytes cytoskeleton. Our data would support the observation of Letavernier et al. (*26*) that sirolimus at high dose may induce de novo focal segmental glomerulosclerosis, a glomerular disease characterized by significant podocyte alterations. Moreover, our observation also supports a recent in vitro study, suggesting the involvement of mTOR in SD proteins expression (*27*).

Recently, a case report (*28*) suggested a tubular mechanism for increased proteinuria in kidney transplanted patient who received sirolimus as a standard therapy. This observation suggests that sirolimus may induce severe proteinuria through a reduction of proximal tubular protein reabsorption. Moreover, it has been suggested that one of the possible pathogenic mechanisms of mTOR inhibitors-associated proteinuria might involve the vascular endothelial growth factor. An unbalance of the vascular endothelial growth factor system has been shown to play a role in the induction of proteinuria (*29*). Recently, Faul et al. (*30*) demonstrated that cyclosporine A (CsA) presents an antiproteinuric effect independent of its immunosuppressive function in T cells, resulting directly from the stabilization of the podocyte actin cytoskeleton. We cannot exclude the coexistence of all these mechanisms.

Proteinuria contributes to progression of renal disease by causing/exacerbating tubulointerstitial lesions (*31*). Re-

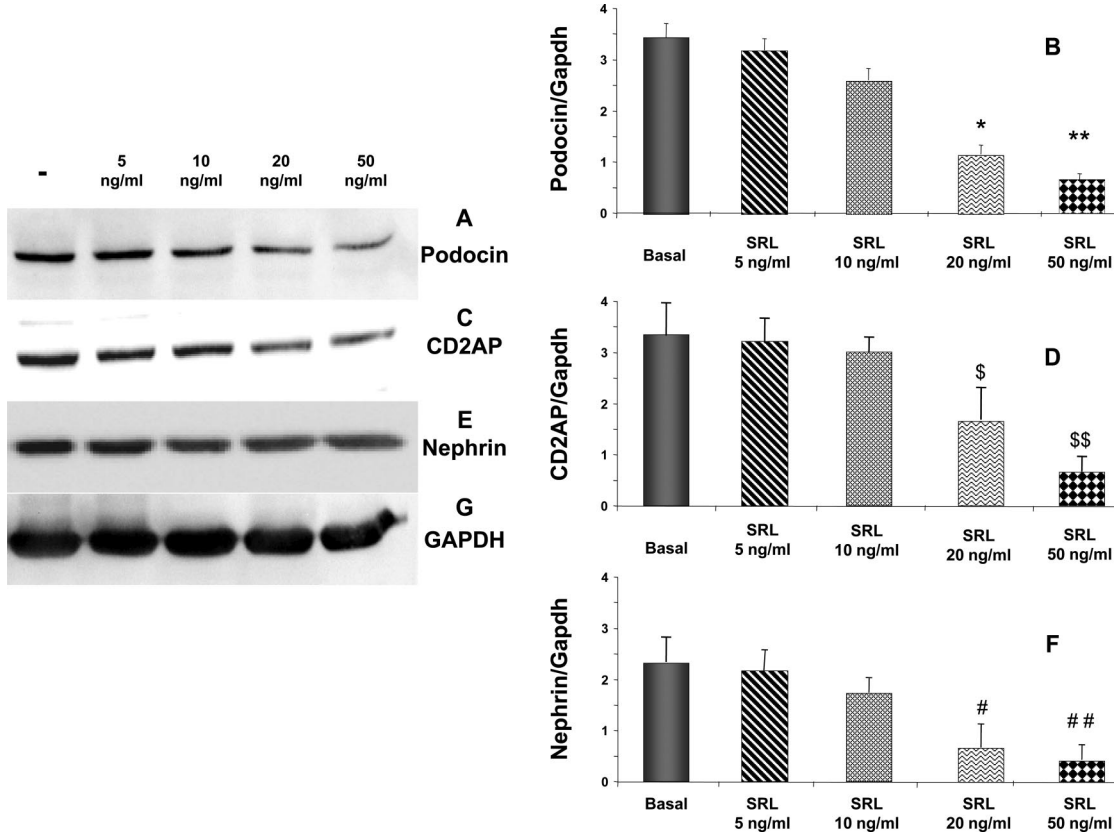


FIGURE 4. Effect of sirolimus (SRL) on podocin (A, B), CD2ap (C, D), and nephrin (E, F) protein expression in cultured human podocytes analyzed by western blotting. Cultured human podocytes were incubated in the absence or in the presence of sirolimus at the final concentration of 5,10, 20, and 50 ng/mL. Specific signal was quantified as described in the Materials and Methods section and normalized to GADPH expression (G). *P=0.003 vs. sirolimus 5 and 10 ng/mL and P=0.001 vs. basal; **P<0.0001 vs. 5 and 10 ng/mL and vs. basal; $$P$=0.002 vs. sirolimus 5 and 10 ng/mL and vs. basal; $$$P$<0.0001 vs. sirolimus 5 and 10 ng/mL and vs. basal; #P=0.03 vs. sirolimus 10 ng/mL; P=0.002 vs. sirolimus 5 ng/mL and vs. basal; ##P<0.0001 vs. sirolimus 5 and 10 ng/mL and vs. basal.

cently, sirolimus was shown to reduce the extent of interstitial fibrosis and the expression of profibrotic genes in an experimental model of progressive renal damage induced by proteinuria and reduced renal mass (*32*, *33*). Hyperplasia of proximal tubular epithelial cells has been observed in both proteinuric animals and nephrotic patients (*34*), and it is proposed to represent a factor favoring progression of renal disease. Sirolimus also blocks proliferation of renal epithelial cells in response to proteinuria (*35*). Thus, sirolimus may also act beneficially at this level. The results of Bonegio et al. (*32*) indicate that sirolimus may exert its beneficial effects in proteinuric nephropathy at doses considerably lower than those required for the prevention of transplant rejection. This is the crucial point of our data. Indeed, it is conceivable that all the potential side effects of this drug are dose dependent, although there are no evidence that indicate a correct dose of sirolimus in transplant recipients to avoid side effects and to assure a safe immunosuppression (*36*). We have previously demonstrated that sirolimus blood TLs lesser than 10 ng/mL ensure an excellent graft function without acute rejection episodes (*37*, *38*).

The main point of strength of our study also represents its main limit. Indeed, our observation is directly related to a clinical setting. However, working with human subjects, we cannot definitively prove our suggestion and neither affirm that it is the only mechanism inducing proteinuria in these patients. Then, we observe an association between the development of proteinuria and higher sirolimus blood TL, and we can only suggest that there is a cause–effect relationship. Although our data are supported by the in vitro observation, we must take into consideration that in vitro data do not always accurately reflect in vivo conditions.

In conclusion, we demonstrated that sirolimus alters the structure and function of SD proteins and causes proteinuria in a dose-dependent manner. Our data would suggest that sirolimus-induced proteinuria, observed during conversion from CNIs- to sirolimus-based immunosuppression, might be avoided aiming at lower blood TL of the drug.

MATERIALS AND METHODS

Patients

This is a single-center cohort study on all patients converted from CNIs to sirolimus in the outpatients clinic of the University of Bari and Foggia Transplant Centres. In the period from September 2000 to December 2005, 110 kidney transplant recipients were converted from CNIs to sirolimus. All patients underwent a graft biopsy before and 2 years after conversion, according to our standard clinical protocol. All patients gave their informed consent. The patients included in the study received a kidney transplant between 12

and 36 months before conversion, presented stable renal function, a PRA less than 20%, histologic diagnosis of chronic allograft nephropathy, according to the Banff 1997 criteria (*20*), a preconversion proteinuria less than 1 g/24 hr and received our standard immunosuppressive regimen, including corticosteroids (prednisone), CsA (Neoral, Novartis), or tacrolimus (Prograf, Astellas), and mycophenolate mofetil (MMF; Cell-Cept, Roche). All patients at the time of conversion received a standard dose of angiotensin-converting enzyme inhibithors (ramipril 5 mg/day). The patients were then divided according to the increase of proteinuria throughout the period of observation. Specifically, the patients who showed an increase in proteinuria less than 70% (the median of the changes in proteinuria between pre- and posttreatment period) were included in group I. Group II included all patients who presented an increase in proteinuria higher than 70%. Two authors blinded to sirolimus TL performed the group assignment. To this purpose, proteinuria levels were measured three times over 1 month immediately before and 2 years after conversion and the changes were calculated on the mean of the three pre- and posttreatment determinations. The study was approved by our internal institutional review board.

Immunosuppressive Regimens

Before Conversion

Seventy-four patients received 2.9 to 4.1 mg/kg per day of CsA in two equally divided doses; 36 patients received 0.1 to 0.2 mg/kg per day of tacrolimus in two equally divided doses; all patients received 500 mg of MMF two times per day; 5 mg/day of prednisone was administered to all patients.

After Conversion

CNIs were abruptly withdrawn and sirolimus was introduced with an initial loading dose of 0.1 mg/kg for the first day and a maintaining dose of 0.02 to 0.04 mg/kg per day thereafter, aiming at blood TL of 6 to 14 ng/mL. In all patients, the dose of MMF and prednisone was maintained.

Renal Allograft Biopsy

Renal specimens were obtained by needle-core biopsies performed under ultrasonographic guidance, fixed in 4% formaldehyde, and then processed for routine histologic staining. Portions of cortex were fixed in 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), processed, and embedded in Epon resin. Thin sections were examined using a ZEISS910 transmission electron microscope (Zeiss, Arese, Italy). Semiquantitative scoring for acute and chronic tubular, interstitial, vascular, and glomerular alterations was performed according to Banff 1997 criteria (*20*). To limit imprecision in score estimation, only biopsy specimens with at least 8 to 10 glomeruli and at least one artery per section were considered as representative. All biopsies satisfied listed criteria. Renal tissue for immunofluorescence, confocal laser scanning microscopy, immunohistochemistry, and electron microscopy studies was available for 25 patients/group. Extension of foot processes effacement was semiquantitatively assessed on at least 2 glomeruli/biopsy (avoiding partially/completely scarred glomeruli) and the results were given averaging at least 5 capillary loops/glomerulus.

Tissue Immunofluorescence and Confocal Laser Scanning Microscopy

Synaptopodin, Nephrin, CD2AP, Podocin, phospho-P70S6-kinase and CD10 protein expression and distribution in graft biopsies at conversion and 2 years thereafter were evaluated by indirect immunofluorescence and confocal microscopy analysis using specific antibodies. The sections were incubated for 1 hr in blocking buffer (2% bovine serum albumin, 0.5% fetal bovine serum) and then with the primary antibodies.

Synaptopodin was detected using a monoclonal antibody from Progen Biotechnik (Heidelberg, Germany) (ready to use, 1 hr at room temperature[RT]). Nephrin expression and distribution were evaluated using a rabbit polyclonal antibody (*39*) (1:1500 dilution, overnight at 4°C). Podocin and CD2ap expressions were investigated using a respective monoclonal and goat polyclonal antibody (Santa Cruz Biotechnology; 1:100 and 1:200 dilution, respectively, overnight at 4°C). Phospho-P70S6 kinase protein expression was investigated on paraffin-embedded renal biopsies using a goat polyclonal antibody (Santa Cruz Biotechnology; 1:100 dilution, overnight at 4°C). CD10 was detected on paraffin-embedded renal sections using a monoclonal antibody from Abcam (Cambridge, MA; 1:50 dilution, 1 hr at RT).

The immune complexes were then identified incubating the slides with the secondary antibodies Alexa-Fluor555 goat anti-mouse IgG-TRITC conjugate (1:400 dilution), Alexa-Fluor488 rabbit anti-goat IgG-FITC conjugate (1:400 dilution), Alexa-Fluor488 goat anti-mouse IgG-FITC conjugate (1:200 dilution), and Alexa-Fluor goat anti-rabbit IgG-FITC conjugate (1:300 dilution; Molecular Probes) for 1 hr at RT. Nuclei were stained with TO-PRO. The slides were then mounted in Gel/Mount (Biomeda, Milan, Italy) and sealed. Negative control was obtained incubating tissue sections with the blocking solution and then substituting the primary antibody with a nonimmune poly-IgG serum. The specific fluorescence was analyzed by confocal laser scanning microscopy using the Leica TCS SP2 (Leica, Wetzlar, Germany) equipped with argon–krypton (488 nm) and green–neon (543 nm) lasers. Mean fluorescence was measured by confocal laser scanning microscopy software and expressed as unit of fluorescence intensity/pixel.

Immunohistochemistry

WT1 protein expression was evaluated on paraffin-embedded kidney sections, using a specific mouse monoclonal anti-human WT1 antibody at 1:40 dilution. Immobilized mouse antibodies were detected by the Avidin Biotin Complex method with the Vectastain Avidin Biotin Complex system, according to the manufacturer's instructions (Vector, Burlingame, CA). Negative controls were obtained by omitting the primary or secondary antibodies and using nonimmune antiserum as first layer. The protein expression levels were assessed semiquantitatively by our pathologist blinded to the origin of the slides.

Cell Culture

An immortalized human podocyte cell line, obtained by infection of primary glomerular epithelial cells with a hybrid Adeno5/SV40 virus (*40*), was used for the experiments. The cell line was cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C with 5% CO_2.

Western Blot

Podocytes were plated in six-well dishes and grown to confluence. The cells were starved for 24 hr and, then, exposed to sirolimus (Alexis Biochemicals, San Diego, CA) for 48 hr at the final concentration of 5, 10, 20, or 50 ng/mL. At the end of the treatment, the cell monolayer was rapidly rinsed twice with ice-cold PBS and lysed in 100 μL of RIPA buffer (1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 1 mM sodium orthovanadate, 150 mM NaCl, 8 μg/mL leupeptin, 1.5% Nonidet P-40, 20 mM Tris-HCl, pH7.4). The lysates were centrifuged at 10,000*g* at 4°C for 5 min. The supernatants were collected and aliquots containing 40 μg of proteins from each lysate were subjected to SDS/PAGE and then electrotransferred onto nitrocellulose membrane (Hybond C, Amersham, UK). The filter was blocked overnight at RT with 2% bovine serum albumin in PBS containing 0.1% Tween-20 (TBS) and then incubated with the goat polyclonal antinephrin antibody (Santa Cruz, 1:200 dilution, overnight at 4°C) or the monoclonal anti-CD2ap antibody (Santa Cruz, 1:50 dilution, overnight at 4°C) or the goat polyclonal antipodocin antibody (Santa Cruz, 1:100 dilution, at RT for 2 hr). The membranes were washed twice in TBS and incubated for 1 hr at RT with HRP-conjugated goat anti-mouse-IgG or the rabbit anti-goat-IgG at 1:1500 dilution in TBS, respectively. The membranes were washed three times at RT in TBS and then once with 0.1%SDS in PBS. The enhanced chemiluminescence system (ECL, Amersham) was used for detection. The same membranes were then stripped and immunoblotted again with rabbit polyclonal anti-GAPDH-antibody (Santa Cruz, 1:1000 dilution, overnight at 4°C).

Statistical Analysis

Continuous variables were expressed as mean ± SD and compared by analysis of variance and paired Student's *t* test, as appropriate. Association between two continuous variables were investigated by Spearman and Kendal

rank correlations, as appropriate. A two-sided *P* less than 0.05 was considered statistically significant.

ACKNOWLEDGMENTS

The authors acknowledge Dr. Michelangelo Corcelli and Mr. Vincenzo Gesualdo (both from the Department of Emergency and Organ Transplantation, University of Bari) for their skilful technical help.

REFERENCES

1. Izzedine H, Brocheriou I, Frances C. Post-transplantation proteinuria and sirolimus. *N Engl J Med* 2005; 353: 2088.
2. Somlo S, Mundel P. Getting a foothold in nephrotic syndrome. *Nat Genet* 2000; 24: 333.
3. Tryggvason K, Patrakka J, Wartiovaara J. Hereditary proteinuria syndromes and mechanisms of proteinuria. *N Engl J Med* 2006; 354: 1387.
4. Reiser J, Kriz W, Kretzler M, et al. The glomerular slit diaphragm is a modified adherens junction. *J Am Soc Nephrol* 2000; 11: 1.
5. Kestilä M, Lenkkeri U, Männikkö M, et al. Positionally cloned gene for a novel glomerular protein nephrin is mutated in congenital nephrotic syndrome. *Mol Cell* 1998; 1: 575.
6. Donoviel DB, Freed DD, Vogel H, et al. Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol Cell Biol* 2001; 21: 4829.
7. Khoshnoodi J, Sigmundsson K, Ofverstedt LG, et al. Nephrin promotes cell-cell adhesion through homophilic interactions. *Am J Pathol* 2003; 163: 2337.
8. Wartiovaara J, Ofverstedt LG, Khoshnoodi J, et al. Nephrin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography. *J Clin Invest* 2004; 114: 1475.
9. Doné SC, Takemoto M, He L, et al. Nephrin is involved in podocyte maturation but not survival during glomerular development. *Kidney Int* 2008; 73: 697.
10. Boute N, Gribouval O, Roselli S, et al. NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* 2000; 24: 349.
11. Shih NY, Li J, Karpitskii V, et al. Congenital nephrotic syndrome in mice lacking CD2-associated protein. *Science* 1999; 286: 312.
12. Ciani L, Patel A, Allen ND, et al. Mice lacking the giant protocadherin mFAT1 exhibit renal slit junction abnormalities and a partially penetrant cyclopia and anophthalmia phenotype. *Mol Cell Biol* 2003; 23: 3575.
13. Winn MP, Conlon PJ, Lynn KL, et al. A mutation in the TRPC6 caution channel causes familial focal segmental glomerulosclerosis. *Science* 2005; 308: 1801.
14. Reiser J, Polu KR, Möller CC, et al. TRPC6 is a glomerular slit diaphragm associated channel required for normal renal function. *Nat Genet* 2005; 37: 739.
15. Huber TB, Kottgen M, Shilling B, et al. Interaction with podocin facilitates nephrin signaling. *J Biol Chem* 2001; 276: 41543.
16. Huber TB, Hartleben B, Kim J, et al. Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol Cell Biol* 2003; 23: 4917.
17. Vasquez EM. Sirolimus: A new agent for prevention of renal allograft rejection. *Am J Health Syst Pharm* 2000; 57: 437.
18. Bierer BE, Mattila PS, Standaert RF, et al. Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or sirolimus. *Proc Natl Acad Sci U S A* 1990; 87: 9231.
19. Kim G, Jun JB, Elkon KB. Necessary role of phosphatidylinositol 3-kinase in transforming growth factor beta-mediated activation of Akt in normal and rheumatoid arthritis synovial fibroblasts. *Arthritis Rheum* 2002; 46: 1504.
20. Racusen LC, Solez K, Colvin RB, et al. Banff 97 working classification of renal allograft pathology. *Kidney Int* 1999; 55: 713.
21. Letavernier E, Bruneval P, Vandermeersch S, et al. Sirolimus interacts with pathways essential for podocyte integrity. *Nephrol Dial Transplant* 2009; 24: 630.
22. Sellin L, Huber TB, Gerke P, et al. NEPH1 defines a novel family of podocin interacting proteins. *FASEB J* 2003; 17: 115.
23. Schwarz K, Simons M, Reiser J, et al. Podocin, a raft-associated component of the glomerular slit diaphragm, interacts with CD2AP and nephrin. *J Clin Invest* 2001; 108: 1621.
24. Roselli S, Heidet L, Sich M, et al. Early glomerular filtration defect and severe renal disease in podocin-deficient mice. *Mol Cell Biol* 2004; 24: 550.
25. Schiffer M, Mundel P, Shaw AS, et al. A novel role for the adaptor molecule CD2-associated protein in transforming growth factor-beta-induced apoptosis. *J Biol Chem* 2004; 279: 37004.
26. Letavernier E, Bruneval P, Mandet C, et al. High sirolimus levels may induce focal segmental glomerulosclerosis de novo. *Clin J Am Soc Nephrol* 2007; 2: 326.
27. Vollenbroeker B, George B, Wolfgart M, et al. mTOR regulates expression of slit diaphragm proteins and cytoskeleton structure in podocytes. *Am J Physiol Renal Physiol* 2009; 296: F418.
28. Straathof-Galema L, Wetzels JF, Dijkman HB, et al. Sirolimus-associated heavy proteinuria in a renal transplant recipient: Evidence for a tubular mechanism. *Am J Transplant* 2006; 6: 429.
29. Diekmann F, Rovira J, Carreras J, et al. Mammalian target of sirolimus inhibition halts the progression of proteinuria in a rat model of reduced renal mass. *J Am Soc Nephrol* 2007; 18: 2653.
30. Faul C, Donnelly M, Merscher-Gomez S, et al. The actin cytoscheleton of kidney podocytes is a direct target of the antiproteinuric effect of cyclosporine A. *Nat Med* 2008; 14: 931.
31. Eddy A. Role of cellular infiltrates in response to proteinuria. *Am J Kidney Dis* 2001; 37: 25.
32. Bonegio RGB, Fuhro R, Wang Z, et al. Sirolimus ameliorates proteinuria-associated tubulointerstitial inflammation and fibrosis in experimental membranous nephropathy. *J Am Soc Nephrol* 2005; 16: 2063.
33. Torras J, Herrero-Fresneda I, Gulias O, et al. Sirolimus has dual opposing effects on proteinuric experimental nephropathies: Is it a matter of podocyte damage? *Nephrol Dial Transplant* 2009; 24: 3632.
34. Hebert LA, Agarwal G, Sedmak D, et al. Proximal tubular epithelial hyperplasia in patients with chronic glomerular proteinuria. *Kidney Int* 2000; 57: 1962.
35. Pallet N, Thervet E, Le Corre D, et al. Sirolimus inhibits human renal epithelial cell proliferation: Effect on cyclin D3 mRNA expression and stability. *Kidney Int* 2005; 67: 2422.
36. Stallone G, Infante B, Grandaliano G, et al. Management of side effects of sirolimus therapy. *Transplantation* 2009; 87: S23.
37. Stallone G, Infante B, Schena A, et al. Sirolimus for treatment of chronic allograft nephropathy in renal transplant patients. *J Am Soc Nephrol* 2005; 16: 3755.
38. Stallone G, Schena A, Infante B, et al. Sirolimus for Kaposi's sarcoma in renal transplant patients. *N Engl J Med* 2005; 352: 1317.
39. Ruotsalainen V, Ljungberg P, Wartiovaara J, et al. Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci USA* 1999; 96: 7962.
40. Conaldi PG, Bottelli A, Baj A, et al. Human immunodeficiency virus-1 tat induces hyperproliferation and dysregulation of renal glomerular epithelial cells. *Am J Pathol* 2002; 161: 53.