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Factors associated with proteinuria in renal transplant recipients treated with sirolimus

Adrian Liew,[1] Gilbert S. C. Chiang[2] and Anantharaman Vathsala[1]

1 Department of Renal Medicine, Singapore General Hospital, Singapore, Singapore
2 Department of Pathology, Singapore General Hospital, Singapore, Singapore

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Correspondence
A. Vathsala, Division of Nephrology, Department of Medicine, Yong Loo Lin School of Medicine, National University of Singapore, 5 Lower Kent Ridge Road, Singapore 119074, Singapore. Tel.: +65 67722544; fax: +65 67794112; e-mail: vathsala@nus.edu.sg

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Summary

Although sirolimus (SRL) use in renal allograft recipients (RTX) is associated with improved renal function, proteinuria develops in a significant proportion. 48 SRL-treated RTX were evaluated for development of proteinuria and stratified by level of proteinuria after SRL therapy. The Proteinuria Group ($n = 25$, 52.1%) had new-onset proteinuria or >25% increase in proteinuria following SRL conversion; the Nonproteinuria Group had stable proteinuria <0.5 g/day throughout. There was a higher proportion of male RTX and female donors to male recipients in the Proteinuria Group, (24% vs. 10%, $P = 0.008$). Calcineurin inhibitor- and statin usage were significantly higher in the Nonproteinuria Group (8% vs. 17%, $P = 0.046$; 28% vs. 83%, $P < 0.001$ respectively) whereas biopsy-proven acute rejection was higher in the Proteinuria Group (68% vs. 33%, $P = 0.037$). SDS-PAGE analysis of urine from 23 RTX in the Proteinuria Group demonstrated glomerular proteinuria in 100% and tubular proteinuria in 87%. While male gender and gender mismatch may impact on glomerular proteinuria through inadequate nephron dose and subsequent hyperfiltration, concurrent cyclosporine use may mitigate the development of proteinuria in SRL-treated patients, through afferent arteriolar vasoconstriction. Glomerular injury occurring following acute rejection may further contribute to glomerular proteinuria. Statins, through their anti-inflammatory and anti-fibrotic effects, may protect against development of proteinuria.

Introduction

Sirolimus (SRL) is a potent immunosuppressive agent that has been used successfully in renal transplant recipients (RTX). It modulates the activity of an intracellular kinase, the mammalian target of rapamycin, inhibiting interleukin-2 mediated signal transduction and arresting cell cycle in the G1-S phase. This results in blocking the response of T- and B-cell activation by cytokines, preventing cell-cycle progression and proliferation of lymphocytes.

There have been many studies demonstrating the efficacy of SRL in the prophylaxis of acute rejection (AREJ) in RTX [1–3]. In RTX receiving concomitant full dose cyclosporine (CsA) and prednisone (Pred), SRL has been demonstrated to reduce AREJ episodes by 10–15% when compared with combinations with azathioprine [1]. Likewise, SRL has been shown to be efficacious as a primary immunosuppressant when used in conjunction with mycophenolate (MPA) analogues and corticosteroids [2,3]. Clinical studies have also demonstrated the efficacy of SRL in ameliorating allograft dysfunction in RTX with underlying calcineurin inhibitor (CNI)-nephrotoxicity or chronic allograft nephropathy [4]. Thus *de novo* SRL-based immunosuppression, with or without concomitant use of CNI and conversion from CNI-based to SRL-based immunosuppression have been used increasingly in clinical renal transplantation over the last decade.

Nonetheless, despite its efficacy as an immunosuppressant, the development of significant proteinuria with SRL treatment is becoming a deterrent to its widespread use in renal transplantation. In one retrospective study of 68

RTX in whom SRL was substituted for a CNI, proteinuria was markedly increased at 3, 6, 12 and 24 months. Proteinuria was subsequently noted to be reversible in 19 patients from whom SRL was withdrawn [5]. Significant proteinuria has also been described in paediatric RTX and other organ transplant recipients after conversion to SRL [6–8].

Although SRL-based immunosuppression can potentially ameliorate allograft dysfunction and thereby improve allograft survival, proteinuria *per se* has been demonstrated to have a negative impact on allograft survival as in native kidney disease. In chronic glomerular disease, other than those that manifest highly selective proteinuria such as minimal change disease, the greater the proteinuria, the greater the risk of irreversible and progressive decline in glomerular filtration rate (GFR) [9,10]. Proteinuria, particularly heavy and nonselective, has been suggested to be nephrotoxic through a variety of mechanisms [11–13]. Thus, there is strong evidence that proteinuria is both a marker for and a mechanism of kidney disease progression. Consistent with this hypothesis are clinical studies showing that proteinuria reduction is associated with slower decline of subsequent GFR [10]. Studies on proteinuria in RTX receiving CNI-based therapy have likewise been demonstrated to have a negative impact on allograft function and survival [14,15].

Extrapolating data from these studies, the development and persistence of proteinuria with SRL treatment may have a detrimental effect on renal allograft function. Boratynska *et al.* [16] reported in a small case series that the development of nephrotic range proteinuria after conversion to SRL from CsA was associated with progressive deterioration of graft function. Letavernier *et al.* [5] suggested in a retrospective study that proteinuria above 0.3 g/day before the switch from CNI to SRL, correlated significantly with a decrease in renal function thereafter.

The aims of this study were to determine the parameters that predisposed to the development of proteinuria in patients treated with SRL, as well as to identify the protective factors in patients who remained proteinuria-free during SRL treatment. In addition, utilizing SDS-PAGE analysis of urine specimens, we sought to characterize the pattern of proteinuria in proteinuric patients.

Materials and methods

Of the 937 adult RTX on follow-up between 2000 and 2006 at the Singapore General Hospital, 48 who had received SRL-based immunosuppression for at least 6 months were included in the study population. RTX had been administered SRL either as *de novo* therapy in combination with full dose CsA and Pred for prophylaxis of rejection or following conversion from CNI-based therapy together with Pred, with or without an anti-metabolite. SRL doses were adjusted to trough SRL levels, measured using Abbott's IMx® System (Abbott Park, Illinois, USA) that operates on Microparticle Enzyme Immunoassay Technology. For the *de novo* group, target trough levels were 8–20 ng/ml; for the converted group, target trough levels were 5–8 ng/ml.

Proteinuria was quantified with 24-h urine collections and/or urine protein–creatinine ratios from early morning urine specimens. As part of the routine evaluation of proteinuria, early morning urine samples were collected from patients with proteinuria >0.5 g/day for SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) analysis, which was performed on the PhastSystem (Pharmacia, Uppsala, Sweden) using PhastGel Gradient 8-25 and PhastGel SDS Buffer Strips in accordance with the manufacturer's instructions. The staining intensity of protein bands indicated the amount of protein loss in the urine; the presence of low-molecular-weight (LMW) protein bands indicated tubular proteinuria, a correlate of tubular dysfunction, while the presence of albumin and high-molecular-weight (HMW) protein bands was a marker for glomerular proteinuria and damage. Following separation of urinary protein by SDS-PAGE, protein bands were scored based on their intensity, where a higher score indicating a more intense band, denoted in turn a greater amount of proteinuria. LMW protein and HMW proteins were ascribed *T*-scores (tubular dysfunction) and *G*-scores (glomerular damage) respectively based on the intensity of staining. Allograft biopsies were performed when clinically indicated and prior to conversion; biopsies were graded based on the Banff 97 working classification of renal allograft pathology [17].

Clinical and laboratory information of donors and RTX, extracted from electronic databases and from the patients' medical records, were collected retrospectively and were analysed for the study population. Renal function was estimated using the equation derived by Nankivell *et al.* [18]. All patients were followed up until 1 June 2007. The study was approved by the Institutional Review Board of Singapore General Hospital.

The study population was stratified into two groups based on their level of proteinuria after the initiation of SRL. The Proteinuria Group ($n = 25$) included RTX with new-onset proteinuria or >25% increase in proteinuria, in comparison to baseline, following SRL therapy. The Non-proteinuria Group ($n = 23$) were recipients with proteinuria of <0.5 g/day before and after starting SRL. Clinical parameters including patient demographics, co-morbidities, immunosuppression, concomitant medications and laboratory parameters such as nature and severity of proteinuria and lipid levels, obtained from retrospective chart review, were compared between groups.

Statistical analysis

All data are expressed as mean and standard deviation. Means of normally distributed data were compared by Student's *t*-test. Nonparametric tests were used when data were not normally distributed, such as the case with proteinuria. Proportions were compared by chi-squared test.

Results

Characteristics of the study population

The study population was predominantly Chinese who had undergone a deceased donor renal transplant and had glomerulonephritis as a cause of their end-stage renal failure (Table 1). Of note were the similar proportion of males and females and the 10.4% prevalence of post-transplant diabetes mellitus in the overall study population. While seven RTX (14.6%) received *de novo* SRL-based immunosuppression as part of a clinical trial, the remainder (41 of 48, 85.4%) received SRL following conversion from CNI-based therapies. No implant biopsies were available for the study population.

Among the 41 RTX undergoing conversion to SRL at a mean interval of 57.4 months post-transplant, 40 RTX had undergone an allograft biopsy within the 4 weeks prior to conversion; the majority underwent conversion for CNI nephrotoxicity (Table 2). Five of these RTX had interstitial nephritis secondary to BK virus infection while six had histological evidence of AREJ prior to conversion. Of the latter, four had been treated for AREJ prior to conversion, but had persistent allograft dysfunction following treatment of rejection while the remaining two had not been treated for AREJ in view of the presence of concomitant Epstein–Barr virus-related tumours. 83% of the converted patients had minimal proteinuria prior to conversion to SRL, while 56% had Nankivell clearance <40 ml/min. Thus, the 41 RTX converted to SRL had no significant proteinuria for 4–5 years following transplantation, prior to conversion to SRL.

In the Proteinuria Group, proteinuria rose from 0.35 g/day at baseline, to a peak of 2.02 g/day over an interval of 5 months from SRL initiation. Four patients (8.3%) developed nephrotic syndrome over the course of follow-up. Proteinuria in the Nonproteinuria Group was 0.31 g/day at baseline and remained stable at 0.21 g/day at a mean interval of 12.2 months post-SRL therapy. The characteristics of the Proteiniuria and Nonproteinuria Groups are shown in Table 3.

Gender and body mass index

As shown in Table 3, a greater proportion of RTX who developed proteinuria with SRL therapy were males (76% in Proteinuria Group compared with 26% in Nonproteinuria Group, $P < 0.001$). Further analysis of the gender mismatch data in the study population demonstrated that there was a significant proportion of female donor-to-male recipients in the Proteinuria Group (24% vs. 10%, $P = 0.008$). Conversely, significantly more male donor-to-female recipient were noted in the Nonproteinuria Group (60% vs. 16%, $P < 0.001$). In contrast, there were no significant differences in body mass index (BMI) between the groups (Table 3).

Table 1. Recipient-, donor- and transplant characteristics of study population.

Parameter	Value
Recipient characteristics	
Recipient age (years)	42.5 ± 9.0
Recipient gender (M:F)	25:23 (52%:48%)
Recipient race	
Chinese	40 (83.3%)
Indian	4 (8.3%)
Malay	4 (8.3%)
Aetiology of end-stage renal failure	
Glomerulonephritis	44 (91.7%)
Presumptive	33 (68.8%)
Biopsy-proven	11 (22.9%)
Reflux nephropathy	3 (6.3%)
Stone disease	1 (2.0%)
Proportion on haemodialysis prior to transplant	46 (95.8%)
Interval to transplant (years)	5.6 ± 3.4
Donor characteristics*	
Type of donor	
Deceased	38 (79.2%)
Living related	7 (14.6%)
Living unrelated	3 (6.2%)
Donor age (years)	45.8 ± 10.8
Donor gender (M:F)	32:13 (71%:29%)
Donor co-morbidities	
Hypertension	8 of 43 (18.6%)
Diabetes mellitus	2 of 43 (4.7%)
Transplant-related factors	
HLA mismatch*†	Median 2
Panel reactive antibodies (PRA), proportion ≥ 25%*†	8 of 42 (19.0%)
Incidence of rejection	
Prior to sirolimus therapy	20 of 37 (54.1%)
Following sirolimus therapy	12 of 48 (25.0%)
Proportion with post-transplant diabetes mellitus	5 (10.4%)

*Five recipients underwent deceased-donor transplantation at other centres. As clinical characteristics of the donors and some transplant-related factors were unknown, these patients have been excluded for these analyses.

†As one recipient underwent transplantation in 1983, prior to routine testing for HLA-DR and PRA, this patient was excluded from these analyses.

Table 2. Baseline parameters prior to conversion to sirolimus.

Parameter	Value
Indication for sirolimus	
De novo	7 (15%)
Conversion	41 (85%)
Interval between baseline allograft biopsy and conversion to sirolimus (days)	31 ± 25
Histological features on preconversion allograft biopsy (n = 40)*	
Calcineurin inhibitor nephrotoxicity†	18 (45%)
Chronic allograft nephropathy†	8 (20%)
BKV nephropathy	7 (18%)
Acute rejection‡	6 (15%)
Glomerular disease	2 (5%)
Others	5 (13%)
Interstitial fibrosis scores (ci)	Median 2
Interval from transplant to sirolimus conversion (months)	57.4 ± 61.4
Nankivell clearance prior to conversion to sirolimus (ml/min)	33.3 ± 12.0
Proteinuria prior to conversion to sirolimus (g/day)	0.28 ± 0.32
Reasons for conversion to sirolimus	
Calcineurin inhibitor nephrotoxicity‡	20 of 41 (49%)
Prolonged delayed graft function‡§	8 of 41 (19%)
BKV infection¶	9 of 41 (22%)
Epstein–Barr virus related tumours**	4 of 41 (10%)

*As multiple pathologies were reported on allograft biopsies, the percentages do not add up to 100%.

†Three out of 26 patients with calcineurin inhibitor (CNI) nephrotoxicity or chronic allograft nephropathy on allograft biopsy were converted to SRL because of concomitant Epstein–Barr virus (EBV) related-tumours, while one had concomitant BK viraemia.

‡Four out of six patients with AREJ on allograft biopsy were treated for rejection and were converted to SRL because of persistent delayed graft function following treatment of AREJ. They had no repeat biopsy following treatment of rejection. Two had concomitant EBV related tumours and were thus not treated for the AREJ.

§Four out of eight patients with prolonged delayed graft function had acute tubular necrosis (n = 2), focal areas of infarction (n = 1) or non-diagnostic biopsies (n = 1).

¶Two patients had BK viraemia in the absence of BKV nephropathy and were converted to SRL. CNI toxicity and acute tubular necrosis were noted in each of these patients respectively.

**One patient had no allograft biopsy prior to conversion. Two had CNI toxicity and AREJ on biopsy while one had CNI toxicity only.

Impact of baseline renal parameters on proteinuria following sirolimus therapy

The proportion of patients with glomerulonephritis as underlying disease was not significantly different between the groups (Table 3). Likewise, among converted patients, the median creatinine clearance before starting SRL was not significantly different between the groups (Table 3). After initiation of SRL, the median creatinine clearance expectedly increased in both groups of patients, but remained statistically not significant (Proteinuria Group: 35 ml/min, Nonproteinuria Group: 40 ml/min; $P = 0.614$). There was also no correlation between Nankivell clearance and level of proteinuria in either group (Proteinuria Group: $r = -0.387$, $P = 0.06$; Nonproteinuria Group: $r = 0.273$, $P = 0.18$; Spearman), suggesting that the degree of proteinuria was not related to the preconversion, postconversion renal function nor the change in creatinine clearance.

CNI toxicity was the predominant histological finding on the biopsies, accounting for 52% and 41% of the patients in the Proteinuria and Nonproteinuria Groups respectively. The single patient who declined a preconversion biopsy had features of CNI toxicity on a subsequent postconversion biopsy. Notably, none of the patients in the Nonproteinuria Group had evidence of *de novo* or recurrent glomerular disease in the baseline biopsy; two patients (9%) in the Proteinuria Group had glomerular disease on the baseline biopsy (diabetic glomerulosclerosis of donor origin and early membranous nephropathy, with no proteinuria at time of SRL conversion, respectively).

Impact of immunosuppressive therapy on proteinuria

A greater percentage of patients who did not develop proteinuria were using CsA concurrently (17.4% in Nonproteinuria Group and 8% in Proteinuria Group; $P = 0.046$; Table 4, Fig. 1). On the contrary, as shown in Fig. 1, the concomitant use of MPA was not significantly different between the groups. Although mean blood SRL trough levels in the Proteinuria Group was significantly higher (11.9 ± 2.97 ng/ml) than those in the Nonproteinuria Group (10.3 ± 1.74 ng/ml; $P = 0.032$), there was no correlation between levels and the degree of proteinuria (Proteinuria Group: $r = -0.094$, $P = 0.64$; Nonproteinuria Group: $r = -0.057$, $P = 0.84$; Spearman).

Blood pressure, lipid profile and diabetes mellitus

As indicated in Tables 2 and 3, blood pressure levels and the prevalence of diabetes mellitus did not differ between the groups. The low density lipoprotein (LDL) levels before and after the initiation of SRL in both groups were not dissimilar; nevertheless, there was a small, nonsignificant increase in the mean LDL levels in Group 1 after the start of SRL (Tables 3 and 4).

ACE inhibitors and statins use

The prevalence of angiotensin converting enzyme (ACE) inhibitors or angiotensin II receptor blockers (ARB) usage

Table 3. Risk factors for proteinuria in study population prior to sirolimus therapy.

Parameter	Proteinuria Group	Nonproteinuria Group	*P*-value
Recipient gender (M:F)	19:6 (76%:24%)	6:17 (26%:74%)	<0.001
Gender mismatch			
Female donor-male recipient	6 of 25 (24%)	2 of 20 (10%)	0.008
Male donor-female recipient	4 of 25 (16%)	12 of 20 (60%)	<0.001
Body mass index			
Male recipients	23.1 ± 3.1	20.6 ± 2.8	
Female recipients	18.1 ± 0.5	22.2 ± 4.1	
Aetiology of end-stage renal failure			
Glomerulonephritis	21 (84%)	23 (100%)	NS
Presumptive	15 (60%)	18 (78%)	
Biopsy proven	6 (24%)	5 (22%)	
Others	4 (16%)	0 (0%)	
Baseline immunosuppression in patients converted to sirolimus			
Tac–MPA–Pred	4 (17%)	2 (11%)	
Tac–Aza–Pred	2 (9%)	2 (11%)	
CsA–MPA–Pred	10 (44%)	7 (39%)	
CsA–Aza–Pred	6 (26%)	5 (28%)	
Thymoglobulin–MPA–Pred	1 (4%)	2 (11%)	
Incidence of rejection prior to sirolimus therapy	15/22 (68%)	5/15 (33%)	0.037
Interval to sirolimus therapy in converted patients (months)	55.1 ± 66.8	61.0 ± 53.8	
Baseline renal function prior to conversion to sirolimus (ml/min)	32.7 ± 13.2	34.2 ± 10.2	0.725
Baseline proteinuria prior to conversion to sirolimus (g/day)	0.36 ± 0.37	0.17 ± 0.18	NS
LDL levels presirolimus (mmol/l)	3.12 ± 0.76	3.37 ± 1.33	0.470

Table 4. Risk factors for proteinuria in study population after sirolimus therapy.

Parameter	Proteinuria Group	Nonproteinuria Group	*P*-value
Indication for sirolimus			
De novo	2 (8%)	5 (22%)	
Conversion	23 (92%)	18 (78%)	
Concomitant immunosuppressive therapy			
Cyclosporine	2 (8%)	4 (17%)	0.046
MPA	13 (52%)	9 (39%)	0.08
Concomitant nonimmunosuppressive therapy			
ACEI/ARBs	6 (24%)	8 (35%)	0.12
Statins	7 (28%)	19 (83%)	<0.001
Incidence of rejection following sirolimus therapy	6 (24%)	26%	0.868
Prevalence of diabetes mellitus	3 (12%)	2 (9%)	0.242
Average systolic BP (mmHg)	139.0 ± 11.5	136.7 ± 13.4	0.533
Average diastolic BP (mmHg)	81.2 ± 7.9	78.3 ± 7.0	0.194
LDL levels post-sirolimus (mmol/l)	3.74 ± 1.41	3.31 ± 1.06	0.251

was not dissimilar between both groups (Fig. 1). Hyperkalemia and blood pressure levels precluded the use of ACE inhibitors and ARBs in the remainder. In contrast, a significant proportion of SRL-treated patients who did not develop proteinuria were on statins (82.6% in Nonproteinuria Group and 28% in Proteinuria Group; $P < 0.001$). Statin usage was irrespective of the serum LDL levels, which was not disparate between the two groups.

SDS-PAGE

All the patients in the Proteinuria Group had demonstrable glomerular pattern of proteinuria on SDS-PAGE, with

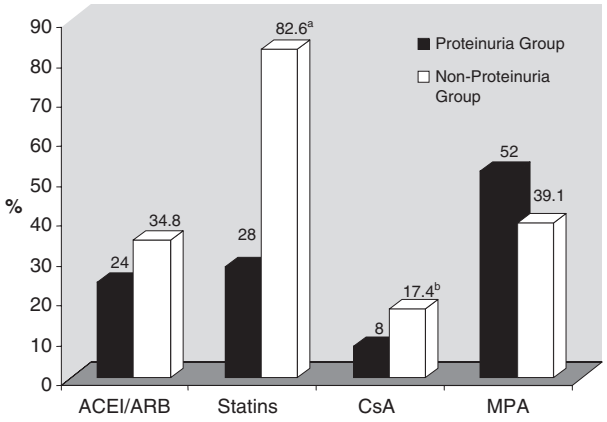

Figure 1 Concomitant medication usage in sirolimus-treated patients. [a]Statin use was significantly higher in the Nonproteinuria Group ($P < 0.001$). [b]Cyclosporine use was significantly higher in the Nonproteinuria Group ($P = 0.046$).

a mean and median *G*-score of 1.50 ± 1.64 and 1 respectively. Furthermore, 87% of the patients in Proteinuria Group had a tubular pattern of proteinuria by SDS-PAGE, with a mean and median *T*-score of 1.39 ± 1.22 and 1 respectively.

Discussion

The study presented herein demonstrated occurrence of proteinuria in 52% of the patients receiving SRL either as *de novo* therapy or following conversion, consistent with reported incidences in other studies (48.4–64%) [19,20]. Whilst a retrospective analysis, there were several interesting and important differences between proteinuric and nonproteinuric patients receiving SRL. These include: a greater proportion of male recipients receiving an allograft from a female donor in the Proteinuria Group, the predominance of glomerular origin of SRL-associated proteinuria, and the concurrent use of CsA and statins in the Nonproteinuria Group.

Gender mismatch between donor and recipient

It was noted that male RTX treated with SRL had a higher risk for the development of proteinuria. Indeed, gender mismatch between donor and recipient was significantly different between the two study groups, as male recipients receiving an allograft from a female donor had an increased risk for proteinuria with SRL therapy in contradistinction to the reduced risk of proteinuria for female recipients of an allograft from a male donor. These suggest that gender-related inadequacy between donor nephron supply and recipient functional demand is an important risk factor for proteinuria post-SRL.

These findings are in corroboration with clinical studies demonstrating that kidney transplants fare better in female- than in male recipients [21]. Other studies have likewise documented inferior short-term and long-term graft survival when kidneys from female donors were transplanted into male patients [22,23]. It has been postulated that worse outcome of kidney grafts coming from female donors was a result of nephron underdosing [22,23] due to the fewer number of nephrons in kidneys from female donors [24]. Nephron underdosing would be expected to increase the workload of remnant nephrons, with resultant glomerular hyperfiltration [24,25]. Indeed, the impaired baseline renal function (median GFR of 33 ml/min) in the study population, coupled with the significant glomerulosclerosis evident on baseline renal allograft biopsy, clearly indicate them to be at significant risk of hyperfiltration injury even prior to SRL therapy.

We postulate that the glomerular hyperfiltration that results from donor and recipient gender mismatch increases the risk for the development of proteinuria in SRL-treated RTX. Although obesity has been recognized to be an independent risk factor for albuminuria [26], and weight loss in obese subjects has been demonstrated to reduce urinary albumin excretion, the BMI in our study population was 21.8 ± 3.6, not considered as being in the obese category. Moreover, BMI was not significantly different between the two groups in our study population, indicating that glomerular hyperfiltration from gender mismatch, rather than from obesity, contributed to the evolution of proteinuria in patients on SRL therapy.

Glomerular proteinuria

A second finding of importance in this study was the identification of glomerular proteinuria in all patients in the Proteinuria Group. The mechanisms by which SRL induces proteinuria have remained elusive thus far; indeed, there is conflicting evidence on the origin of proteinuria, whether glomerular or tubular, in SRL-treated proteinuric patients. Franco *et al.* [27] demonstrated no specific glomerular changes on light microscopy in biopsies performed after the onset of proteinuria. Straathof-Galema *et al.* [28] used FITC-labelled anti-albumin antibodies and found complete absence of albumin in the proximal tubules in a patient who developed proteinuria with SRL use, thus postulating that reduced tubular reabsorption of proteinuria contributes to protein loss. An effect of SRL on tubular epithelial cell protein endocytosis and proliferation has been suggested from a rat study, corroborating these findings [29].

However, other studies have refuted a tubular mechanism, suggesting instead a glomerular origin for proteinuria in SRL-treated patients [30–32]. Glomerular

proteinuria has been postulated to occur through several mechanisms, including its association with post-transplantation glomerulonephritis, either *de novo* or as recurrence [30,31], and SRL-induced *de novo* focal segmental glomerulosclerosis [32]. In the latter study, Letavernier *et al.* showed evidence for podocyte dysregulation based on immunohistochemical examination of biopsies of patients with SRL-induced proteinuria; they showed absent or diminished expression of the podocyte-specific epitopes synaptopodin and p57 (reflecting de-differentiation) and neo-expression of cytokeratin and PAX2 (reflecting an immature fetal phenotype).

The results of the SDS-PAGE analysis of the urinary samples in our study patients demonstrated that SRL-induced proteinuria is universally glomerular. The higher incidence of AREJ prior to the use of SRL in the Proteinuria Group is similar to that reported in CsA-treated RTX [33]. Although the histopathological changes of chronic rejection, transplant glomerulopathy and endovasculitis were absent in patients with prior AREJ in the Proteinuria Group, glomerulosclerosis was invariably present in all patients with AREJ, suggesting the latter to be the primary reason for glomerular proteinuria in those with this finding in our study population. Glomerulonephritis, either recurrent or *de novo* was likewise an uncommon histopathological finding in the Proteinuria Group. The glomerular proteinuria as demonstrated herein may further result from nephron underdosing and gender mismatch and the ensuing glomerular hyperfiltration alluded to earlier.

Concurrent use of CsA

The finding of significantly higher concurrent CsA usage in the Nonproteinuria Group could further substantiate the hyperfiltration theory and glomerular origin of SRL-associated proteinuria suggested above. Indeed, many other investigators have concurred with the protective effect of CNI against proteinuria associated with SRL use, or the reversal of proteinuria with the switch from SRL back to a CNI [5,6,16,30]. The afferent arteriolar vasoconstrictive effect of CNI, and in turn the reduction of hyperfiltration, likely accounts for this protective effect against proteinuria. Indeed, Saurina *et al.* [34] demonstrated a tendency for intraglomerular pressure to increase and renal functional reserve to decrease, with an increase in proteinuria, in patients converted from CNI to SRL. Although the use of *de novo* SRL appeared to be protective against the development of proteinuria in our study population, this is likely related, in part, to the concomitant use of CsA in these patients.

Tubular proteinuria was also found in a large proportion of patients with proteinuria in our study population and may well be explained by evidence of tubular atrophy and interstitial fibrosis in all the allograft biopsies in the study population.

Concurrent use of statins

Finally, our present study suggests that statins had a protective effect on the development of proteinuria, one that was irrespective of their LDL-lowering action. Over the last two decades, numerous studies have demonstrated that statins diminish cardiovascular morbidity and mortality, largely through their ability to lower circulating LDL levels. However, more recent research has focussed on the substantial pleiotropic effects of HMG-CoA reductase inhibitors, not directly related to lipid lowering. It has been shown that statins exert anti-proliferative, anti-inflammatory and anti-fibrotic effects on a wide variety of tissues. Indeed, Katznelson *et al.* [35] suggested an approximate twofold reduction in the incidence of biopsy-proven AREJ episodes in RTX treated with pravastatin, while Tuncer *et al.* [36] reported similar decreased rates of biopsy-proven acute allograft rejection in both simvastatin- and pravastatin-treated groups.

Similarly, there is growing evidence from *in vitro* and *in vivo* experiments suggesting beneficial effects of statins in progressive renal disease [37–49]. Animal studies have suggested that statins may have potential utility as a therapeutic option in renal diseases that are characterized by inflammation and fibrosis. The protective effect of statins against the development of glomerulosclerosis has been demonstrated in rat studies, through the inhibition of transforming growth factor-β1 expression in glomeruli, prevention of intra-glomerular proteolytic activity in experimental nephrotic syndrome and protective effect against oxidative stress to the glomeruli [37–40]. In rat studies, pravastatin was similarly found to be renoprotective by attenuating ischemia-reperfusion injury [41], while treatment with rosuvastatin and cerivastatin were shown to prevent progressive renal inflammation and fibrosis via effects on oxidative enzymes and pro-inflammatory cytokines [42,43]. The findings from these experimental studies have also been corroborated in clinical studies in patients with native glomerular disease [44–46]. These mechanisms of renal injury amelioration as suggested by the experimental studies could explain the protective effect of statins noted on the development of glomerular proteinuria in SRL-treated RTX as demonstrated in this study.

In addition, the development of proteinuria has been correlated with podocyte injury. Indeed, Nakamura *et al.* [47] clearly demonstrated that the use of statins appeared to restore injured podocytes in patients with chronic

glomerulonephritis with proteinuria. This podocyte-restorative effect of statins was again suggested by Blanco *et al.* [48] in patients with type 2 diabetes. Experimental data in rat studies suggest that the beneficial effect of statins on podocytes can be attributed to direct modulation of excessive RhoA activity [49].

Another mechanism of statin-mediated amelioration of SRL-associated proteinuria may be its ability to modulate vascular endothelial growth factor (VEGF) levels as has been suggested in various studies [50,51]. The importance of VEGF in the pathogenesis of proteinuric kidney diseases has been previously elucidated. In studies by Quaggin where glomerular-selective deletion or over-expression of VEGF-A could lead to glomerular diseases in mice, tight regulation of VEGF-A signalling was suggested as being critical for establishment and maintenance of the glomerular filtration barrier [52]. Indeed, SRL use has been associated with both reduction and elevated expression of VEGF in experimental models and RTX. While Hochegger *et al.* [53] showed that the reduction of renal VEGF-A expression with SRL use, likely because of its harmful effects on podocytes or endothelial cells, resulted in worsening of glomerulonephritis, plasma concentrations and renal expression of VEGF was found to be elevated in a patient with SRL-associated proteinuria post-transplantation [54]. Because VEGF is a potent enhancer of vascular-wall permeability, the authors speculated that it may allow the development of glomerular proteinuria by altering glomerular permeability. Over-expression of VEGF in visceral and parietal glomerular cells has also been documented in rats with protein-overload nephrosis and collapsing glomerulopathy as seen in HIV nephropathy [55]. Thus multiple pathways of glomerular injury may be ameliorated by the use of statins in SRL-treated RTX.

Conclusions

In conclusion, our data suggests that proteinuria in SRL-treated RTX is both glomerular and tubular in origin. While AREJ continues to be a risk factor for proteinuria in SRL-treated RTX, the effects of nephron underdosing with its associated hyperfiltration may contribute significantly to the development of proteinuria. Proteinuria appears to be enhanced in the absence of concurrent CsA usage likely because of CsA-induced absence of afferent arteriolar vasoconstriction. While prospective studies are needed to confirm the beneficial effects of statins in ameliorating proteinuria in RTX receiving SRL-based therapy, our study suggests that statins, through their anti-inflammatory and anti-fibrotic effects, appear to be protective against the development of proteinuria, independent of their lipid-lowering action.

Authorship

AL: performed study, collected data, analysed data, wrote the paper. GSCC: reported histology of renal allograft biopsies. AV: designed study, analysed data, wrote the paper.

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