Sirolimus and proteinuria in kidney transplantation

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Introduction and outline of the thesis

INTRODUCTION

Kidney transplantation is the preferred treatment option for most patients suffering from end stage renal disease (ESRD), since transplantation is associated with improved quality of life and reduced mortality compared to either haemodialysis or peritoneal dialysis.[1] Since the first successful kidney transplantation in the Netherlands in 1966, results have improved substantially with current one-year graft survival rates around 86% for recipients of a deceased donor kidney and 95% for recipients of a living-donor kidney (NOTR, Dutch Organ Transplant Registry) (Figure 1). This improvement in first year graft survival is mainly attributed to development of more potent immunosuppressive drugs and the associated decrease in acute rejection episodes. At the beginning of the transplant era, the combination of prednisone and the anti-metabolite azathioprine was the standard immunosuppressive regimen. The calcineurin inhibitors (CNI) were introduced in the late seventies (cyclosporine) and early nineties (tacrolimus), and the anti-metabolite mycophenolate mofetil has been increasingly used in multidrug immunosuppression protocols since the mid-nineties. In parallel, the incidence of rejection episodes within the first three months after transplantation has decreased from 50-60% in the early eighties to 10-20% in most centres nowadays (NOTR). In the last decade even newer immunosuppressive agents have been introduced such as sirolimus or Belatacept. Although some may offer hope to further decrease the incidence of acute rejections, it is likely that the newer agents are best suited as alternatives for the current immunosuppressive drugs, and chosen based on the side effect profile.

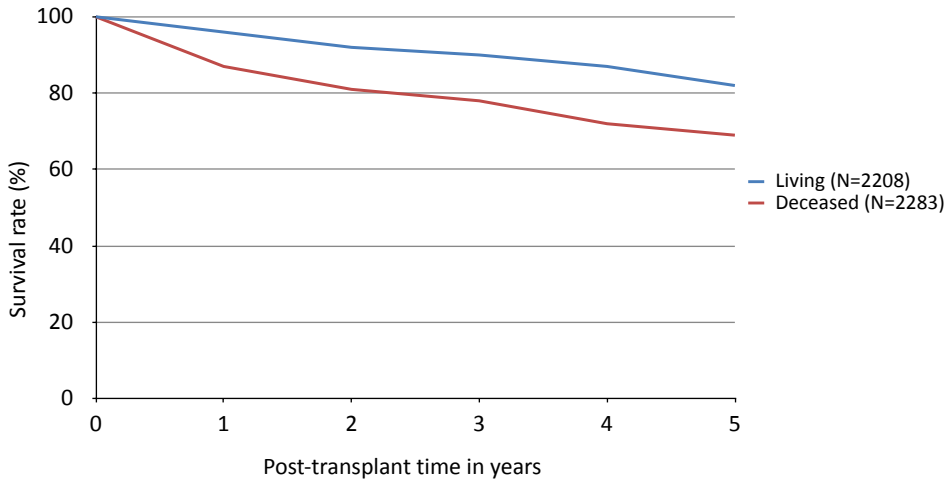

Figure 1. Graft survival rates of kidney transplantations performed between 1-1-2005 and 1-1-2011, stratified by donor type, in the Netherlands

Despite the excellent results of kidney transplantation, many unsolved problems remain.

1. A major drawback of kidney transplantation is the need for immunosuppressive therapy. Although immunosuppressive drugs are the cornerstone of therapy and pivotal to obtain good outcome of kidney transplantation, their use is accompanied with severe side effects such as:
   a. Nephrotoxicity is a major side effect of the widely used CNI, and contributes to graft function loss. The development of effective, non-nephrotoxic immunosuppressive drugs may help to avoid CNI as part of the immunosuppressive scheme and thus circumvent this problem. Sirolimus is suggested as a good candidate to replace the CNI.
   b. The use of immunosuppressive therapy after kidney transplantation is largely responsible for the increased incidence of cancer, particularly skin cancer. Newer drugs, with lower carcinogenic potential but equal immunosuppressive potency, may help to overcome this problem. Again, sirolimus may play a role in the newer immunosuppressive regimens.
2. The short-term results of kidney transplantation are excellent, but the long-term results have not been improved to the same extent. Thus, in many patients the clinical course after kidney transplantation is characterized by a slow, but progressive and unrelentless deterioration of graft function. One possibility to improve the long-term results of kidney transplantation is to develop biomarkers that would allow predicting late allograft loss, thus allowing to identify and treat patients at highest risk in an early stage.
3. Another problem with kidney transplantation is the shortage of organs and the long waiting time. Transplantation with a kidney of a living-donor may circumvent this problem, but the question is whether this procedure is safe for the donor, particularly in the light of the now broadly used laparoscopic nephrectomy.

BACKGROUND

Chronic allograft nephropathy and calcineurin inhibitor nephrotoxicity

The term "chronic allograft nephropathy (CAN)" is often used to define the process that is responsible for the relentless progressive kidney failure, often associated with proteinuria and hypertension that is observed in many patients after kidney transplantation and responsible for late graft failure, accounting for up to 40% of graft losses. A kidney biopsy shows a histological picture characterized by an obliterative vasculopathy, glomerulosclerosis and interstitial fibrosis with tubular atrophy. Histological changes are already present before clinically apparent renal dysfunction.[2] The pathogenesis of CAN is multifactorial, and includes (humoral) rejection, hypertension, hyperlipidaemia, aging, and smoking. Of special concern is the contribution of the CNI to the development of CAN. CNI have added greatly to the reduction in acute rejections after solid organ transplantation. The use of CNI is however hampered by several side effects, including acute and chronic nephrotoxicity, hypertension and hyperlipidaemia, thereby increasing the risk for the development of graft failure and for cardiovascular disease. The nephrotoxicity of CNI became apparent since its first use in transplantation in the early 1980s. In the Canadian multicentre study the serum creatinine at both 1 and 3 years was significantly higher in cyclosporine-treated than in the azathioprine-treated patients.[3] Several years later, Myers et al.[4] reported chronic renal insufficiency in cardiac transplant recipients that had received cyclosporine, some of whom subsequently required haemodialysis. The renal histological findings of arteriolar hyalinosis, glomerulosclerosis and interstitial fibrosis and tubular atrophy were attributed to CNI nephrotoxicity. Similar histologic findings were reported by Nankivell et al.[2] By 10 years virtually all patients were reported to have CNI nephrotoxicity and declining renal function. Both cyclosporine and tacrolimus result in increased synthesis of the fibrogenic cytokine transforming growth factor FGF-β1, which has been implicated as a key factor in the pathogenesis of chronic CNI nephropathy.[5,6]

With improvements in short-term outcomes, attention has increasingly turned to strategies that optimize long-term allograft survival by minimizing the side effects of immunosuppressants. Thus, while many transplant centres begin maintenance immunosuppression with a combination of three drugs, efforts are now focused on immunosuppressive strategies with CNI minimization or elimination that can optimize long-term graft function without increasing rejection rates.

Malignancy

The reported cancer risk in renal transplant recipients (RTR) is two- to six-fold greater than in the general population.[7-9] Cutaneous squamous cell carcinomas (SCCs) are the most common post-transplantation cancers, occurring 65 to 250 times more often than in the general population and are a serious complication of long-term organ transplantation.[10-12] Moreover, SCCs appear to be more aggressive in transplant recipients. They often grow rapidly, recur locally in 13% of patients,[13] and metastasize in 5 to 8 percent of patients.[14] The incidence of skin malignancies in RTR increases progressively with intensity and duration of immunosuppression and therefore the overall time elapsed after transplantation. Duration of immunosuppressive therapy influences SCC risk, with compelling evidence for the carcinogenic mechanisms associated with cyclosporine[15-17] and azathioprine.[18]

RTR should be educated in using sunscreen and should be strongly discouraged from smoking, as this is a risk factor for SCC and many other malignancies. Still, these preventive measures are insufficient. Recent guidelines from the American Academy of Dermatology have reviewed treatment options for SCC.[19] Topical therapies include 5-fluorouracil, imiquimod, cryosurgery or photodynamic therapy in areas of pre- or early-malignant change. Adjuvant radiotherapy may play a role in selected cases. This is usually undertaken in conjunction with a reduction in immunosuppression if SCC is recurrent or metastatic. Systemic retinoids have been demonstrated in small short-term prospective and in long-term retrospective studies to play a role in the secondary prevention of SCC, but are teratogenic and poorly tolerated at higher doses.

Sirolimus

Sirolimus is a macrocyclic compound with potent immunosuppressive properties. It binds to the same FK-binding protein as tacrolimus. The sirolimus-FK-binding protein complex has no effect on calcineurin, and instead inhibits the mammalian target of rapamycin (mTOR), a cytosolic enzyme that regulates growth and proliferation of lymphocytes during the G1 phase of the cell cycle. When used in combination with cyclosporine and steroids, sirolimus has been proven to be safe and efficacious in reducing acute rejection rates in clinical trials,[20,21] which served as the basis for the initial approval of the product in the United States and elsewhere. Continuation of dual therapy with sirolimus and steroids was shown to be effective in preventing acute rejection episodes and allowed successful withdrawal of cyclosporine 3 months after renal transplantation, a regimen that formed the basis for regulatory approval in the European Union.[22] Moreover, sirolimus has been reported to be less nephrotoxic than CNI.[23,24] Thus the introduction of sirolimus could facilitate withdrawal or avoidance of CNI, thus preventing CNI induced graft function deterioration. Moreover, it has been

hypothesized that sirolimus may prevent CAN by inhibiting the expression of growth factor mRNAs, or by inhibiting the proliferation of smooth muscle cells involved in the characteristic vasculopathy.[25] Sirolimus has also important anti-neoplastic properties, and may be a drug that can be safely continued to prevent allograft rejection in transplant recipients who have developed a malignancy.[26]

Common dose-dependent side effects of sirolimus include thrombocytopenia and hyperlipidaemia. Idiosyncratic reactions include aphtous ulcer formation, interstitial pneumonitis, and rash. The anti-proliferative effects of the drug may be responsible for impaired wound healing, including a relatively high incidence of lymfoceles.

Some reports showed that (CNI) treated kidney transplant recipients developed a significant increase of proteinuria when switched to sirolimus. The pathogenesis of this proteinuria is unknown, but has (partly) been attributed to the hemodynamic renal effects of CNI withdrawal.[27-29]

Biomarkers of renal function deterioration

The short-term results of kidney transplantation are excellent, but the long-term results have not improved to the same extent. Thus, in many patients the clinical course after kidney transplantation is characterized by a slow, but progressive and unrelentless deterioration of graft function. In the first ten years after transplantation, CAN is the most prevalent cause of allograft dysfunction, though its pathogenesis remains elusive.[30] Already before clinically apparent kidney dysfunction, histological changes are present.[3] Although any anatomical compartment can be involved in CAN, interstitial accumulation of extracellular matrix in association with progressive tubular atrophy is mostly observed.[31] One possibility to improve the long-term results of kidney transplantation is to develop biomarkers that would allow predicting late allograft loss, thus allowing to identify and treat patients at highest risk in an early stage. It is known that deterioration of kidney function is best correlated with tubulointerstitial injury. Tubular injury is characterized by increased urinary losses of low-molecular weight proteins (LMWPs). Of the LMWPs α_1-microglobulin and β_2-microglobulin, it is known that they are readily filtered by the glomerulus and reabsorbed and catabolized by proximal tubular cells. When the proximal tubular handling of these LMWPs is disturbed, they appear in the urine.[32] LMWPs and IgG are valuable in predicting the progression of renal function decline in patients with proteinuric glomerular diseases such as focal segmental glomerulosclerosis or membranous nephropathy.[33,34] However, little is known about the possible value of measuring these proteins in the transplantation setting.

Living-donor transplantation

The gap between the demand and supply of deceased donor kidneys continues to grow. Living-donor programs have gradually become an attractive strategy to expand the donor pool for kidney transplantation. Renine, a Dutch registry of data concerning dialysis and kidney transplantation patients, shows that nowadays over 50% of all renal transplantations are living-donor transplantations (Figure 2). Grafts from living-related donors display superior function and longer survival than those obtained from deceased donors.[35,36] As the beginning of living-donor kidney transplantation, physicians have expressed concern about the possibility that unilateral nephrectomy can be harmful to a healthy individual although survival and the risk of ESRD in carefully screened kidney donors appear to be similar to those in the general population.[37] Living-donor surgery has changed radically in the past decade as laparoscopic techniques have supplanted open nephrectomy. Laparoscopic donor nephrectomy was found to be associated with reduced analgetic consumption, shorter hospital stay, and faster return to normal physical functioning as compared to the open technique.[38] Whether the elevated intra-abdominal pressure during the laparoscopic procedure can be harmful to the donated or remaining kidney is not known.

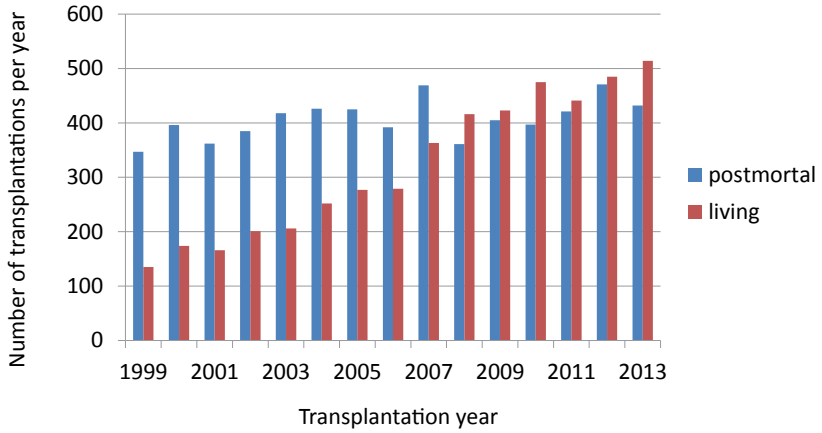

Figure 2. Number of transplantations per year, grouped per donor type, in the Netherlands

The studies described in this thesis address the abovementioned problems and were aimed at answering the following questions:

1. Can sirolimus improve outcome after kidney transplantation?
   a. With the use of sirolimus, is it possible to perform kidney transplantations using a CNI-free immunosuppressive regimen, thus avoiding CNI nephrotoxicity and CAN-associated graft failure? We specifically evaluated the effects of sirolimus on proteinuria.
   b. Does sirolimus reduce the rate of skin malignancies?
2. Can deterioration of kidney function after kidney transplantation be predicted?
3. Is laparoscopic donor kidney nephrectomy safe?

OUTLINE OF THE THESIS

We have studied whether the nephrotoxicity that occurs under the current standard immunosuppressive regimen with tacrolimus, low-dose steroids and MMF can be decreased by a regimen with sirolimus, daclizumab, low-dose steroids and MMF without an increased incidence of acute rejections (**Chapter 2**).

In **Chapter 3** we report the evolution of proteinuria in RTR in whom azathioprine was replaced by sirolimus. Based on the findings in this study, we performed experimental studies to examine the effect of sirolimus on proteinuria in a mouse model (**Chapter 4**).

In light of the significant morbidity and mortality of cutaneous invasive SSCs in RTR we performed a randomized, prospective, multicentre study to examine whether conversion to sirolimus-based immunosuppression from standard immunosuppression can reduce the recurrence rate of these skin cancers (**Chapter 5**).

We investigated whether graft function deterioration and graft loss can be predicted by measuring urinary protein markers (**Chapter 6**).

As the beginning of living-donor kidney transplantation, physicians have expressed concern about the possibility that unilateral nephrectomy can be harmful to a healthy individual. To investigate whether the elevated intra-abdominal pressure during laparoscopic donor nephrectomy causes early damage to the remaining kidney, we evaluated sensitive urine biomarkers after laparoscopic donor nephrectomy (**Chapter 7**).

In **Chapter 8** we summarize the studies and discuss the implications for future research and patient care.

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Chapter 2

Inferior results with basic immunosuppression with sirolimus in kidney transplantation

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Neth J Med 2007; 65:23-28.

ABSTRACT

Background

The introduction of sirolimus has provided the opportunity to develop an immunosuppressive regimen without the nephrotoxic calcineurin inhibitors.

Methods

We conducted a first trial in 30 renal allograft recipients. Ten patients were followed prospectively and received sirolimus, to achieve a target blood level of 10 to 15 ng/ml, induction therapy with one dose of daclizumab, low-dose steroids and mycophenolate mofetil. We compared this group with a historical control group of 20 patients who received our standard treatment consisting of tacrolimus, low-dose steroids, and mycophenolate mofetil.

Results

After a mean follow-up of 15 weeks, seven patients developed an acute rejection in the sirolimus group (70%) compared with three patients in the tacrolimus group (15%) ($p<0.01$).

Because of this unacceptable high rate of acute rejections we conducted a second prospective pilot study in nine patients. These patients received sirolimus in combination with two doses of daclizumab, high-dose steroids and mycophenolate mofetil.

No rejections occurred under this immunosuppressive regimen; however, many immunosuppression-related adverse events were seen.

Conclusion

The present study demonstrates an unacceptable high rate of acute rejections (70%) in patients treated with sirolimus, daclizumab, mycophenolate mofetil and low-dose prednisolone. No rejections but many adverse events were seen when sirolimus was given in combination with high-dose steroids.

INTRODUCTION

Immunosuppressive regimens including calcineurin inhibitors have greatly improved the results of kidney transplantations. Tacrolimus in combination with mycophenolate mofetil (MMF) and prednisolone decreased the number of acute rejection episodes within the first three months after transplantation to 15 to 20%. The incidence of graft failure from intractable acute rejections within one year after transplantation has dropped under the current regimen to below 5%. Therefore, tacrolimus combined with MMF and prednisolone is the standard regime in the first four months after transplantation in our centres. However, calcineurin inhibitors are nephrotoxic, which may eventually lead to loss of graft function. Long-term results are therefore disappointing. The introduction of sirolimus has provided the opportunity to develop an immunosuppressive regimen without nephrotoxic calcineurin inhibitors.[1]

Obviously, removing calcineurin inhibitors from the immunosuppressive regime should not lead to a higher percentage of rejections. On the other hand, the additional amount of immunosuppression needed beside sirolimus to prevent acute rejection should not lead to an unacceptable amount of immunosuppression-related adverse events. Recently, Flechner et al.[2] demonstrated in kidney transplant recipients that treatment with sirolimus, prednisolone, MMF, and additional IL-2 receptor blocker (basiliximab) was accompanied with an acute rejection percentage of 6.4%. However, the additional immunosuppression given, high-doses of steroids and two induction therapies, is much more than we are used to giving in combination with tacrolimus.

The main purpose of our study was to investigate whether the nephrotoxicity that occurs under the current standard immunosuppressive regimen with tacrolimus, low-dose steroids and MMF can be decreased by a regimen with sirolimus, daclizumab, low-dose steroids and MMF without an increased incidence of acute rejections.

MATERIALS AND METHODS

Patients

We included primary and secondary adult (age above 18 years) renal allograft recipients in Nijmegen and Utrecht. Exclusion criteria consisted of HLA-identical living-donor kidney; haemolytic uraemic syndrome as original renal disease; pregnancy or lactation; total white blood cell count $<3*10^9$/l or platelet count $<100*10^9$/l or haemoglobin level <5 mmol/l; current panel reactive antibodies (PRA) (last screening sample) >85%; the use of non-registered medication during the last four weeks preceding transplantation and during the study; a renal allograft transplant as part of a multiorgan transplantation; or treatment with CYP3A4 inhibitors or inductors. All recipients had a negative visual

complement dependent cytotoxicity crossmatch. Flow cytometry T-cell crossmatching did not take place.
The patients who gave their informed consent were prospectively followed and treated with a calcineurin inhibitor free immunosuppressive protocol including sirolimus, daclizumab, MMF, and low-dose steroids. This group was compared with a historical control group consisting of patients who met the same inclusion and exclusion criteria and had been treated directly before the start of the study with our standard immunosuppressive regimen including the calcineurin inhibitor tacrolimus, MMF and low-dose steroids.
The study was approved by both ethical committees of the participating centres and performed in accordance with the standards of the Declaration of Helsinki.

Immunosuppressive protocol and methods

First study

The patients in the calcineurin inhibitor free intervention group were treated with sirolimus at a loading dose of 15 mg prior to transplant surgery. As soon as a patient was capable of taking oral medication a second loading dose of 12 mg was given, followed by a daily dose of 6 mg, to achieve a target blood level of 10 to 15 ng/ml. The target trough level remained steady throughout the study.
The patients in the sirolimus treatment arm also received daclizumab during the transplant surgery intravenously at a dose of 1 mg/kg. At weekly intervals during the first ten weeks following transplantation, the coverage of IL-2 receptors was measured by flow cytometry.[3] If free IL-2 receptors were detected on the lymphocytes (reappearance of $CD3^{pos}CD25^{pos}$ lymphocytes) in the first four weeks, an extra dose of daclizumab at 1 mg/kg was given.
The steroid regimen in the sirolimus treatment arm consisted of 100 mg prednisolone intravenously on day 0 (day of transplantation); on day 1 to 5 prednisolone 4 times 25 mg orally/iv. From day 6 till week 17 the steroids were slowly reduced from the starting dose (determined by weight: > 70 kg: 25 mg; 50 to 70 kg: 20 mg; <50 kg: 15 mg) to zero.
Patients in the historical control group were treated with tacrolimus at a dose of 0.2 mg/kg/day orally, divided over the morning and evening doses, to be started on day 1 or 2 after transplantation. The target blood level in the first 14 days was between 15 and 20 ng/ml, from week 3 to 7 between 10 and 15 ng/ml and starting from week 7 the trough level should be 6 to 10 ng/ml.
The steroid regimen in the tacrolimus treatment group consisted of 100 mg prednisolone intravenously on day 0 (day of transplantation); on day 1 and 2 prednisolone 25 mg four times orally/iv. From day 3 till week 17 the steroids were slowly reduced from the starting dose (determined by weight: > 70 kg: 25 mg; 50 to 70 kg: 20 mg; <50 kg: 15 mg) to 0.1 mg/kg.

All patients were given MMF 750 mg twice daily from day 1 or 2 onwards. For patients with a body weight of ≥90, the dose was 1000 mg twice daily. In case of leucopenia or abdominal complaints, the dose was lowered (the minimal dose is 250 mg twice daily). All patients in whom a rejection was suspected underwent renal transplant biopsy, which were scored according the BANFF97 criteria.[4] The primary study endpoints were the difference in renal function and the number of acute rejections between both treatment groups.

Second study

Because of the unacceptably high rate of acute rejections in the above-described patients treated with sirolimus (see results) we conducted a second prospective pilot study in nine patients. They received sirolimus and MMF following the same protocol as described above. Besides the daclizumab given during the transplant surgery, they received an additional dose daclizumab of 1 mg/kg ten days after transplantation. The steroid regimen consisted of 500 mg methylprednisolone intravenously on day 0 (day of transplantation) to 2, and then oral prednisolone from 120 mg to 30 mg by day 8, 27.5 mg by day 21, 25 mg by day 30, tapered by 2.5 mg each month to a maintenance of 7.5 mg daily.

RESULTS

First study

Ten patients included in the sirolimus group were compared with 20 patients who were treated with tacrolimus. Patient characteristics are summarised in table 1. Apart from more older donors and an unfavourable donor type profile in the sirolimus group, no significant differences were found. After a mean follow-up of 15 weeks, seven patients in the sirolimus group had developed an acute rejection (70%; 95% confidence interval 42 to 98%). This was significantly more than the 15% rejection rate in the control group ($p<0.01$; Fisher's exact test). Characteristics of the rejection episodes that occurred in the sirolimus group are mentioned in table 2. In four patients the renal allograft function recovered after three pulses of solumedrol alone. Two patients required a second course of solumedrol and one patient required antithymocyte globulin (ATG) after the solumedrol treatment before renal function improved. All patients were converted to tacrolimus and returned to a stable allograft function, with a mean serum creatinine of 159 µmol/l at one year after transplantation.

Four rejection episodes occurred within two weeks after transplantation. One of them was not biopsy proven because of the absence of renal tissue in the biopsy. In one of these patients there appeared to be no IL-2 receptor blockade because the patient did

not receive any daclizumab by mistake. In all the patients who received daclizumab, the IL-2 receptor was fully blocked at two and three months after transplantation after one dose of daclizumab.

Three rejections occurred between 8 and 15 weeks after transplantation. In all these cases the trough sirolimus level appeared to be below the target range at the time of rejection. The mean sirolimus trough levels were within the target range in the different time periods (table 3), but 21% of the measurements were below target. This was comparable with 19% of the measurements below target in the tacrolimus treatment group.

One sirolimus-treated patient had a serious wound-healing problem. Two of the three rejections in the tacrolimus group occurred within one week after transplantation. The third rejection occurred after 11 weeks. All of the patients required ATG after the course of solumedrol. One of them died as a consequence of this therapy.

Table 1. Demographics of the first study

	Sirolimus (n=10)	Tacrolimus (n=20)	P
Recipients			
Gender (M:F)	7:3	10:10	NS
Age (years)(mean ± sd)	54 ± 14	46 ± 13	NS
Age >65 years	3	1	NS
Donors			
Gender (M:F)	2:8	8:12	NS
Age (years)(mean ± sd)	52 ± 15	47 ± 12	NS
Age >65 years	3	0	0.03
Secondary transplant	1	1	NS
PRA			NS
0%	10	18	
>0 and <50%	0	2	
HLA mismatches (mean ± sd)	2.8 ± 0.9	2.5 ± 1.5	NS
Donor type			<0.05
Low-risk (HB+LR)	3	14	
High-risk (NHB+LUR)	7	6	

M=male; F=female; PRA=panel reactive antibodies; HLA=human leucocyte antigen; NHB=non-heart beating; HB=heart beating; LR=living related; LUR=living unrelated; NS=not significant.

Table 2. Sirolimus-treated patients with acute rejections in the first study (n=7)

Rejections	1	2	3	4	5	6	7
Week after KTx	2	1	14	15	8	2	1
Donor type	NHB	HB	LUR	LUR	LUR	LR	LR
HLA mismatches (A-B-Dr)	1-0-0	0-2-1	1-1-1	1-1-2	0-1-2	1-1-1	1-1-1
Sirolimus level at rejection (ng/ml)	12	11	7.3	6.8	7.3	23	12
IL-2R blockade at 3 months	No	Yes	Yes	Yes	Yes	Yes	Yes
Doses MMF at rejection mg/day	1500	1500	1500	1500	1500	1500	2000
Steroid dose at rejection mg/day	20	25	2.5	2.5	7.5	22.5	25
Banff score:							
First biopsy	IIa+ ATN	Ib	Ib	IIa	Ib	No renal tissue	IIa
Second biopsy	IIa		Ia				
Therapy	3g Sol (twice)	3g Sol	3g Sol (twice)	3g Sol	3g Sol	3g Sol ATG	3g Sol
Creatinine one year after transplantation (µmol/L)	230	168	130	160	147	126	150

KTx=kidney transplantation; NHB=non-heart beating; HB=heart beating; LUR=living unrelated; LR=living related; HLA=human leucocyte antigen; IL-2R blockade=interleukin 2 receptor blockade; MMF=mycophenolate mofetil; ATN=acute tubular necrosis; Sol=solumedrol; ATG=antithymocyte globulin.

Table 3. Sirolimus and tacrolimus trough levels in the first and second study

	0-14 days	2-7 weeks	7 weeks-3 months
Tacrolimus trough level (ng/ml):			
- Target	15-20	10-15	5-10
- Actually reached (mean ± SEM)	16.4 ± 0.9	12.3 ± 0.4	9.3 ± 0.3
Sirolimus trough level (ng/ml):			
- Target	10-15	10-15	10-15
- Actually reached (mean ± SEM):			
- First study	13.4 ± 1.1	14.9 ± 1.0	11.8 ± 1.0
- Second study	10.8 ± 0.8	15.6 ± 0.9	13.1 ± 0.9

SEM=standard error of the mean.

Second study

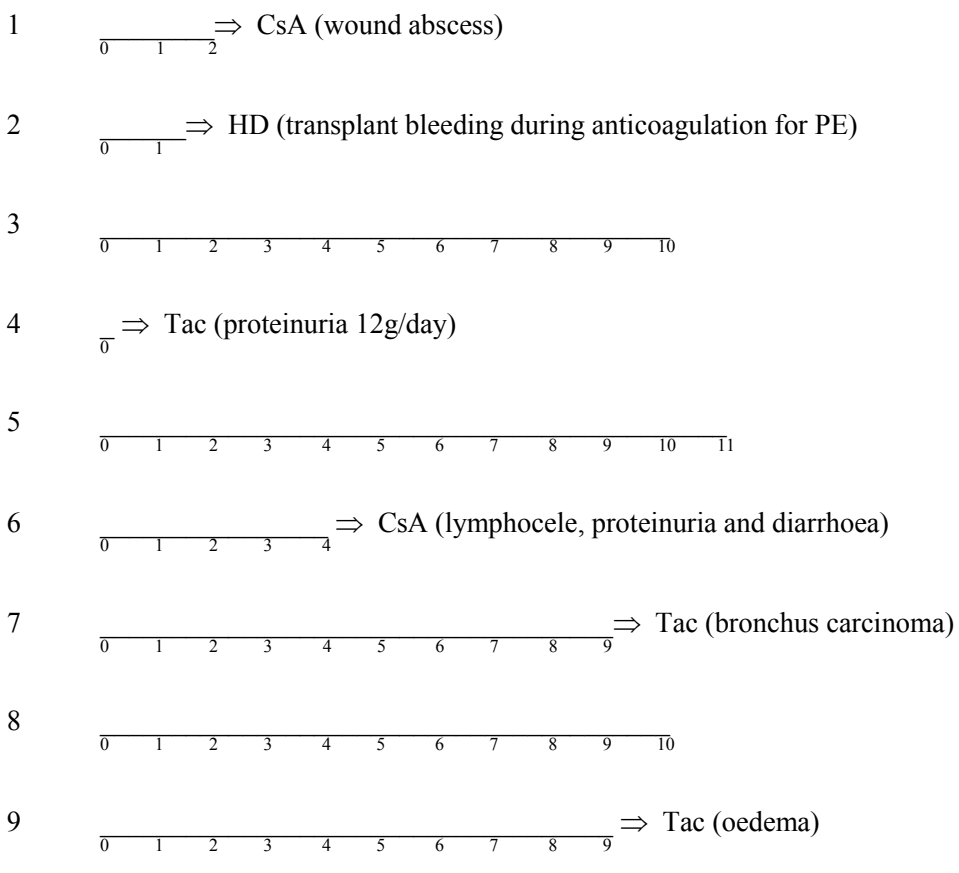
No acute rejections occurred in the second sirolimus treatment group (n=9) with high-dose additional immunosuppression after a mean follow-up of ten months. On the contrary, many serious adverse events were seen in this group, as summarised in table 4. Six patients (67%) suffered delayed wound healing, with a secondary wound infection in three of them. Operative abscess drainage was necessary in one of them. Four patients (44%) developed a lymphocele requiring drainage. In one patient a secondary infection developed in the lymphocele. One patient developed a pulmonary embolus and thereafter, during anticoagulation therapy, a bleeding in the transplant. After insertion of a vena cava filter, a vena cava inferior syndrome occurred and because of continuous bleeding in the kidney transplant a transplantectomy was performed and haemodialysis was restarted. Three patients (33%) developed proteinuria after transplantation. One of them is the above-described patient with pulmonary embolus. Another patient developed proteinuria of 12 g/day one week after transplantation. A kidney biopsy showed tubulointerstitial damage without glomerular damage. The proteinuria disappeared within one month after switching to tacrolimus. The third patient with proteinuria developed proteinuria till 1.5 g/day, which also disappeared after switching to cyclosporine. Three patients developed diarrhoea (33%), two of them requiring hospitalisation.

Table 4. Adverse events in sirolimus-treated patients (second study)

Patient no.	1	2	3	4	5	6	7	8	9
Acute rejection	-	-	-	-	-	-	-	-	-
Graft loss	-	+	-	-	-	-	-	-	-
Surgical complications									
- Delayed wound healing	+	+	+	-	+	+	+	-	-
- Haematoma	+	-	-	-	-	-	-	-	-
- Wound abscess/infection	+	+	-	-	+	-	-	-	-
- Lymphocele	-	-	+	-	+	+	+	-	-
Hypercholesterolaemia (>6 mmol/l)	-	-	-	-	+	+	+	+	-
Hyperglycaemia (fasting glucose>7 mmol/l)	+	+	-	-	-	+	-	-	-
Pulmonary embolus	-	+	-	-	-	-	-	-	-
Proteinuria (>1g/day)	-	+	-	+	-	+	-	-	-
Candidiasis (oral)	+	-	-	-	-	-	-	-	-
Diarrhoea	-	-	+	-	+	+	-	-	-

Three patients could be maintained on the sirolimus regimen during the mean follow-up period of ten months. The other six patients were switched to another immunosuppressive regimen because of severe complications. The time till the switch of immunosuppression and the main reason for switching is shown in figure 1. Two patients were switched to cyclosporine (after two and four months), three patients were switched to tacrolimus (one after one week and two after nine months), and one patient restarted haemodialysis after nephrectomy (seven weeks after transplantation).

Figure 1. Time frame (in months) for sirolimus-treated patients: reason for switch of immunosuppression (second study)



CsA=cyclosporine; HD=haemodialysis; PE=pulmonary embolus; Tac=tacrolimus.

DISCUSSION

The use of calcineurin inhibitors has resulted in improved graft survival following kidney transplantation. However, this is associated with acute and chronic nephrotoxicity and may be an important contributor to the development of chronic transplant nephropathy and chronic graft loss.[5] Calcineurin inhibitor nephrotoxicity is becoming increasingly prevalent, and is virtually universal by ten years after transplantation and progressive despite mild to moderate reductions in calcineurin doses.[6] The introduction of sirolimus has provided the opportunity to develop an immunosuppressive regimen without nephrotoxic calcineurin inhibitors. Recently, Flechner et al.[2] demonstrated in kidney transplant recipients that treatment with sirolimus, prednisolone, MMF and additional IL-2 receptor blocker (basiliximab) was accompanied with an acute rejection percentage of 6.4 vs 16.6% in the control arm (cyclosporine, prednisolone, MMF and IL-2 receptor blocker). At 12 months their sirolimus-treated patients enjoyed significantly better creatinine clearances than their cyclosporine-treated patients (81.1 and 61.1 ml/min, respectively). However, the additional amount of immunosuppression given beside sirolimus is very high.

In our first study we achieved a rejection percentage of 70% in the sirolimus group compared with a 15% rejection rate in the tacrolimus group ($p<0.01$) within a mean follow-up of 15 weeks. Because of this unacceptably high rejection rate we ended the study prematurely and switched the patients to the standard immunosuppressive regimen including tacrolimus. To date, none of the patients have lost their grafts in the mean follow-up of 18 months. This high percentage of rejections cannot be explained by the fact that only patients with a high rejection risk were included in the sirolimus group. All rejections occurred in patients who underwent a first kidney transplantation with a PRA of 0% and there were no significant differences in the number of HLA mismatches and number of non-heart beating donors between the groups. However, when we divided the donors into a low-risk group (heart beating and living related donors) and a high-risk group (non-heart beating and living unrelated donors) significantly more patients with an unfavourable donor type were found in the sirolimus-treated patients. Although this can be partly responsible for the bad outcome in the sirolimus group we do not think this can totally explain the very high rejection rate of 70%.

Four of the seven rejections in the sirolimus group occurred within two weeks after transplantation. One of these rejection episodes occurred in a patient who did not receive any daclizumab by mistake. In all other patients the IL-2 receptor was fully blocked at two and three months after transplantation by one infusion of daclizumab during transplant surgery. Three of the seven rejections occurred between 8 and 15 weeks after transplantation. These three rejections occurred when the prednisolone

was reduced to below 10 mg/day, in accordance with the protocol. All patients used at least 1500 mg MMF during the study period. At the time of rejection the sirolimus levels appeared to be lower than the target level in all three of them. The sirolimus levels were below target in 21% of all measured levels in the sirolimus group, but were never measured below 6.8 ng/ml. In the tacrolimus group 19% of all measured levels were below the target level. Some fluctuation in (sirolimus) trough levels is inevitable, but we must conclude that this seems immediately catastrophic in our low immunosuppressive regimen of the sirolimus group. There have been reports of calcineurin inhibitor free therapy, even without using antibody induction, that describe lower rates of acute rejection than we found. Kreis et al.[7] using sirolimus, MMF and steroids reported an acute rejection rate of 27.5% one year after transplantation and Groth et al.[8] using sirolimus, azathioprine and steroids reported an acute rejection rate of 41% at one year. In comparison with our protocol the target trough sirolimus level amounted to 30 ng/ml for the first two months in both studies and they started with 500 mg of methylprednisolone tapered to a maintenance dose of 10 mg daily. In the Symphony trial standard immunosuppression with normal dose cyclosporine (target trough level 150 to 300 ng/ml) was compared with three regimens with low doses of either cyclosporine, tacrolimus or sirolimus in combination with MMF, daclizumab and corticosteroids in 1645 de-novo renal transplant patients. The rate of biopsy-proven acute rejections with low-dose sirolimus (target trough level 4 to 8 ng/ml) at one year (35%) was higher than the other groups (15 to 25%). The conclusion of this study was that the room for increasing sirolimus immunosuppression should be evaluated against the specific sirolimus toxicity profile.[9,10] Contrary to our study, Flechner et al. started with 500 mg methylprednisolone intravenously on day 0 to 2, and then oral prednisolone from 120 mg to 30 mg by day 8, and thereafter slowly tapered to a maintenance dose of 7.5 mg daily at eight months. Their mean trough sirolimus levels appeared to be 13.2 ± 7.9 ng/ml at one month after transplantation and 11.2 ± 5.8 ng/ml at three months after transplantation. They also gave a higher dose of MMF of 1 g twice daily instead of the 750 mg twice daily in our study and they used two gifts of basiliximab. These differences might explain the high rejection rate we found.

To prove this supposition we conducted a second prospective trial in nine patients. This protocol differed from the first by an additional dosage of daclizumab 1 mg/kg at ten days after transplantation and higher doses of MMF and steroids according to the Flechner protocol. No acute rejections occurred under this treatment regimen. On the contrary, many serious adverse events were seen, likely to be related to the combination of sirolimus and high-dose steroids. These findings are in accordance with Dean et al.[9] using sirolimus, six gifts of antithymocyte globulin induction, MMF, and prednisone. They achieved an acute rejection rate of 9% at one year, but a wound complication

rate of 35% in comparison with 10% in the tacrolimus control group. These adverse events and the interventions needed to treat them might also lead to a decline in renal function. This takes away the advantage of sirolimus, no nephrotoxicity, in the first place. However, the number of treated patients in our study is too small to compare renal function under the different regimens. In the Symphony trial where renal function was determined at 12 months they showed that low-dose tacrolimus was significantly superior to low-dose sirolimus with respect to glomerular filtration rate.[11] The results from our study showed that in order to replace a calcineurin inhibitor by sirolimus aiming to avoid calcineurin nephrotoxicity, higher additional immunosuppression is needed to prevent an unacceptable rejection rate. Because of the immunosuppression-related adverse events we experienced under such a regimen we do not think there should be a place for a sirolimus-based regimen without calcineurin inhibitor in the direct post-transplant period.

CONCLUSION

The present study demonstrates an unacceptably high rate of acute rejections (70%) in patients treated with sirolimus, daclizumab, MMF and low-dose prednisolone in the first months after transplantation and no rejections but many adverse events when sirolimus was combined with two times induction therapy and high-dose prednisolone.

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Chapter 3

Proteinuria following conversion from azathioprine to sirolimus in renal transplant recipients

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ABSTRACT

Recent studies have reported a significant increase of proteinuria in kidney transplant recipients who were switched from a calcineurin inhibitor (CI) to sirolimus. This has (partly) been ascribed to the hemodynamic renal effects of CI withdrawal. We have evaluated the evolution of proteinuria in renal transplant recipients who underwent conversion from azathioprine to sirolimus.

In a randomized, prospective, multicenter study called RESCUE (Recurrent cutanEous Squamous cell Carcinoma Under RapamunE) the efficacy and safety is investigated of conversion to sirolimus in stable renal transplant recipients with a cutaneous squamous cell carcinoma (SCC). In our center, 25 patients were included in this study of which 13 patients were randomized to continue their current immunosuppressive treatment and 12 to conversion to sirolimus.

After a mean follow-up of 360 days, mean proteinuria increased from 0.37±0.34 to 1.81±1.73 g/24h after conversion to sirolimus ($P<0.005$). In the control group there was no change in proteinuria. A significant increase of proteinuria was observed in all seven patients with proteinuria before conversion, whereas proteinuria remained absent in all patients without previous proteinuria. Two of the patients with proteinuria were converted from cyclosporine and five were converted from azathioprine to sirolimus. Sirolimus was discontinued in five patients with proteinuria, and in all of them proteinuria declined to baseline values.

Our study demonstrates that conversion from azathioprine to sirolimus after kidney transplantation may cause a reversible increase of proteinuria. Sirolimus-induced proteinuria therefore cannot be ascribed to the hemodynamic renal effects of CI withdrawal.

INTRODUCTION

Squamous cell carcinoma (SCC) is the most common form of malignancy after organ transplantation. The incidence of SCC increases with the duration of immunosuppressive therapy, ultimately affecting more than 50% of white transplant recipients.[1] Evidence suggests that sirolimus, an effective immunosuppressive drug with antiproliferative properties, may confer a decreased risk of malignancy. In a randomized study the incidence of cancer, particularly skin cancer, was lower in patients receiving sirolimus in comparison with other immunosuppressive therapies.[2] We initiated a randomized controlled study called RESCUE (Recurrent cutanEous Squamous cell Carcinoma Under RapamunE) to evaluate the efficacy of sirolimus in preventing new skin carcinomas in patients with at least one SCC in an earlier phase after transplantation.

Sirolimus has also been reported to be less nephrotoxic than other immuno-suppressive agents used in transplantation. However, recent reports indicate that calcineurin inhibitor (CI)-treated kidney transplant recipients may develop a significant increase of proteinuria when switched to sirolimus.[3-10] The pathogenesis of this proteinuria is unknown. In these studies most patients switched from CI to sirolimus because of progressive renal injury (chronic allograft nephropathy with or without CI nephrotoxicity). Therefore, it was not possible to distinguish between the hemodynamic renal effects of withdrawal of CI or a direct toxic effect of sirolimus.

We have evaluated the evolution of proteinuria in patients who participated in the above-mentioned randomized controlled trial. Most of these patients were switched from azathioprine to sirolimus, thus excluding any effects of CI withdrawal.

RESULTS

In our center 25 patients have been included in the RESCUE study until now. Thirteen patients were randomized to continue their current immunosuppressive treatment and 12 were randomized to conversion to sirolimus.

One patients converted to sirolimus dropped out because of the development of a hemolytic uremic syndrome. After a mean follow-up of 360 days the mean proteinuria level in the remaining 11 patients increased, whereas in the control group there was no difference in proteinuria at baseline and after a mean follow-up of 517 days (Table 1).

An increase in proteinuria was observed in all patients (n=7) with proteinuria before conversion (from 0.57±0.26 g/24h to 2.84±1.36 g/24h; $P<0.005$)(Figure 1), whereas proteinuria remained absent in all patients without proteinuria (n=4). Two of the patients with proteinuria were converted from cyclosporine to sirolimus and five patients were converted from azathioprine to sirolimus. There was no difference in arterial blood pressure before and after the switch to sirolimus and during sirolimus therapy no

angiotensin-converting enzyme inhibitor or angiotensin receptor blocker was added to the medication except for one patient. For this patient 100 mg losartan was started after which proteinuria did not increase further. The increase in proteinuria started within 3 months after conversion in six of the seven patients. Sirolimus was discontinued in five patients with proteinuria, and in all of them proteinuria declined to baseline values within 6 months.

Table 1. Demographics

	Sirolimus (n=11)	Control (n=13)	P
Gender (male:female)	5:6	9:4	ns
Age (years)	55 ± 10	57 ± 8	ns
Previous immunosuppressive therapy			
Cyclosporine	3	1	ns
Azathioprine	8	11	
MMF	0	1	
Proteinuria (g/24h)			
At baseline	0.37 ± 0.34	0.38 ± 0.63	ns
At follow-up	1.81 ± 1.73**	0.29 ± 0.35	<0.05
Creatinine (μmol/l)			
At baseline	109 ± 31	102 ± 20	ns
At follow-up	118 ± 37*	103 ± 16	ns

** P<0.005 versus baseline, * P<0.05 versus baseline.
Values are given as mean ± s.d., ns=not significant

In four of seven patients who developed proteinuria the excretion of low molecular weight proteins, α_1-microglobulin and β_2-microglobulin, in urine was determined. The mean excretion of α_1-microglobulin was 65 mg/10 mmol creatinine (range 29-133) and of β_2-microglobulin 6.1 mg/10 mmol creatinine (range 0.2-20.4). These values are elevated compared with normal values (<10 mg/ 10 mmol creatinine and <0.3 mg/ 10 mmol creatinine, respectively). The protein selectivity index, calculated as the clearance of immunoglobulin G divided by the clearance of transferrin, ranged from 0.16 to 0.31.
In the control group (n=13), there was no difference in serum creatinine at baseline and after a mean follow-up of 517 days (102±20 and 103±16 μmol/l, respectively). The mean creatinine level in the sirolimus group (n=11) slightly increased from 109±31 before to 118±37 μmol/l after conversion (P=0.049) (Table 1). In the five patients who were switched back to their previous immunosuppressive regimen because of proteinuria serum creatinine increased after the introduction of sirolimus (from 118±33 to 135±37 μmol/l; P<0.01) and stabilized after reconversion (131±34 μmol/l; P=0.19 compared with baseline).

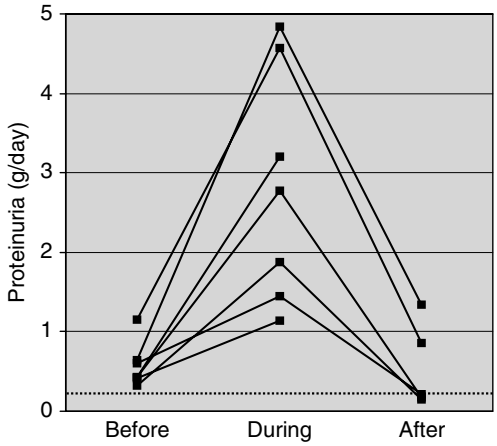

Figure 1. Evolution of proteinuria in the sirolimus-treated patients.
Individual data are given for seven patients with increased proteinuria after conversion to sirolimus. Sirolimus was subsequently withdrawn in five patients, accompanied by reduction of proteinuria in all. Upper reference value is indicated by the dotted line (0.2 g/24h=normal).

DISCUSSION

Our results demonstrate that conversion from azathioprine to sirolimus in stable renal transplant recipients with SCC may cause a reversible increase of proteinuria. This increase was seen only in patients with proteinuria at baseline.

Heavy glomerular proteinuria is an important and independent predictor of progressive renal damage. In the transplantation literature there is also ample evidence that persistent proteinuria is a strong risk factor for long-term allograft loss and lower patient survival from cardiovascular and all-cause mortality.[11]

Recent reports indicated that CI-treated kidney transplant recipients might develop a significant increase of proteinuria when switched to sirolimus.[3-10] Diekmann *et al*.[10] reported that a proteinuria below 800 mg/day at conversion from CI to sirolimus is the only independent predictor for positive outcome in chronic allograft dysfunction. In these studies most patients were switched from CI to sirolimus because of progressive renal damage (chronic allograft nephropathy with or without CI nephrotoxicity). The pathogenesis of this proteinuria is unknown, but has (partly) been ascribed to the hemodynamic renal effects of CI withdrawal. Morelon *et al*[3] suggested that CI withdrawal may lead to an increase in renal blood flow responsible for the development of previously masked proteinuria in patients with preexisting glomerular damage.

Our data clearly indicate that this is not a satisfactory explanation. Because in our study most of the patients who developed proteinuria were switched from azathioprine to sirolimus there must also be some direct nephrotoxic effect of sirolimus. Whether

sirolimus itself could affect glomerular permeability is not known. In vitro studies have demonstrated a tubulotoxic effect of sirolimus, especially in the setting of prior renal injury.[12] Straathof *et al.*[13] described a patient who developed heavy proteinuria during treatment with sirolimus. They showed that a decrease in tubular protein reabsorption contributed to the proteinuria. Experimental studies of Coombes *et al.*[14] have indicated that sirolimus causes a specific pattern of acute renal injury characterized by increased intratubular cast formation in protein overload nephropathy. The increased excretion of low molecular weight proteins α_1-microglobulin and β_2-microglobulin we found, might indicate that sirolimus promotes proteinuria by blocking the tubular protein reabsorption. Unfortunately, we have no information about the urinary excretion of small molecular weight proteins before conversion to sirolimus. Admittedly, we cannot exclude that tubular proteinuria is a consequence of tubular injury caused by glomerular protein leakage.

Sennesael *et al.*[15] showed in renal transplant patients, converted from CI to sirolimus, that proteinuria can be treated succesfully with angiotensin-converting enzyme inhibitors or angiotensin receptor blockers. In only one of our patients on sirolimus an angiotensin receptor blocker was started after which proteinuria did not increase further. Probably, we could have kept more patients on sirolimus if we had started renin-angiotensin blockade.

In conclusion, switching renal transplant recipients from azathioprine to sirolimus is associated with a reversible increase in proteinuria. The mechanism of this effect still remains unclear, but cannot be ascribed to the hemodynamic renal effects of CI withdrawal.

MATERIALS AND METHODS

In a randomized, prospective, multicenter study called RESCUE the efficacy and safety is investigated of conversion to sirolimus in stable renal transplant recipients with at least one cutaneous SCC. Patients are randomized to continue their current immunosuppressive regimen or conversion to sirolimus (trough levels of 5-10 ng/ml) and prednisolone. We evaluated the evolution of proteinuria in patients in our hospital who were included in the RESCUE study.

According to current guidelines we routinely used spot urine samples and calculated protein-creatinine ratios for the follow-up of our patients.[16] All patients collected one or more 24h urine samples during the study period. Since renal function was stable, proteinuria per 24h can be calculated from the formula: proteinuria (g/24h)=protein-creatinine ratio (g/10 mmol creatinine) * creatinine excretion (mmol/24h).

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Chapter 4

Sirolimus and proteinuria in Thy-1.1 transgenic mouse

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INTRODUCTION

Sirolimus, or rapamycine, is a macrolide produced by Streptomyces Hygroscopicus. It possesses immunosuppressive and antiproliferative properties by blocking the proliferative responses of different cell types to growth factors through inhibition of the mammalian target of rapamycin (mTOR) signalling cascade. Thus, sirolimus inhibits IL-2 induced lymphocyte proliferation, as well as the intima proliferation in the vasculature, which is a hallmark of chronic allograft nephropathy.[1] Since sirolimus is a strong immunosuppressive agent it may offer an opportunity to avoid exposure to calcineurin inhibitors (CNI) and thus prevent the associated nephrotoxicity.

In contrast, several studies showed that CNI-treated kidney transplant recipients developed significant proteinuria when switched to sirolimus.[2-6] It was suggested that this increased proteinuria is not caused by sirolimus per se, but rather the consequence of altered hemodynamics after CNI withdrawal. However, data from our centre also demonstrated an increase in proteinuria after conversion from azathioprine to sirolimus in stable renal allograft recipients.[7] This indicates that sirolimus itself may contribute to proteinuria.

Proteinuria can be a consequence of tubular and glomerular injury, and sirolimus thus could induce proteinuria by several mechanisms. In vitro studies using cultured mouse proximal tubular cells showed that sirolimus increases apoptosis and decreases the proliferative cell response.[8] It is possible that, by its inhibitory effect on cellular proliferation coupled with an increased rate of apoptosis, rapamycine might actually tip the delicate survival balance, worsening the tubulointerstitial damage after tubular cell injury. Indeed some studies have demonstrated prolonged periods of delayed graft function (acute tubular necrosis) after renal transplantation.[9]

Sirolimus induced proteinuria may thus be a consequence of tubular cell injury. Indeed, Straathof *et al*. described a transplant patient who developed heavy proteinuria during treatment with sirolimus. Histological analysis suggested that a decrease in tubular protein reabsorption contributed to the proteinuria.[10] However, another report provided no evidence for sirolimus-dependent reduction of protein reabsorption in proximal tubular cells.[11] Moreover, it is widely accepted that tubular diseases lead to a moderate proteinuria with a specific tubular profile. The described nephrotic range proteinuria in patients on sirolimus and the reduction observed after initiating angiotensin converting enzyme inhibitors rather suggest a glomerular origin of proteinuria.

Severe proteinuria is a hallmark of glomerular renal diseases. Proteinuria is generally attributed to a defect in the permselectivity of the glomerular filter. In recent years, many studies focused on the role of glomerular epithelial cells, the so-called podocytes, in the induction of proteinuria as well as in the development of focal glomerulosclerosis.[12-15] Several studies have shown that podocyte loss may be important in the initiation of

progressive glomerulosclerosis. Thus, also at the glomerular level sirolimus might cause injury by shifting the balance between apoptosis and repair. Letavernier *et al.* described focal segmental glomerulosclerosis (FSGS) lesions associated with nephrotic-range proteinuria within months after renal transplantation in patients who received sirolimus de novo after transplantation in the absence of CNI.[16] Thus sirolimus may induce podocyte injury and proteinuria in some patients due to unidentified mechanisms. Importantly, the clinical studies suggested that sirolimus induced proteinuria particularly in patients with pre-existing proteinuria.[17] The study of the role of sirolimus in inducing proteinuria and the possible pathogenic mechanisms would greatly benefit from experimental models. Therefore, we have initiated a set of pilot experiments with sirolimus in a mouse model of FSGS.

Kollias *et al.* generated transgenic mice that express the Thy-1.1 antigen on the podocytes.[18] These mice slowly and spontaneously develop albuminuria and focal glomerulosclerosis over a period of 26 weeks. Importantly, the process of FSGS development can be accelerated. Injection of anti-Thy-1.1 monoclonal antibodies in Thy-1.1 transgenic mice induces an acute albuminuria, which is followed by a rapid development of FSGS within 3 weeks after injection of the monoclonal antibody. This mouse model allowed us to investigate the effect of sirolimus in both non-proteinuric mice as well as in mice made proteinuric before start of sirolimus.

METHODS

Animals

Heterozygous Thy-1.1 transgenic mice were generated by injecting a hybrid human-mouse Thy-1.1 gene into pronuclei of zygotes of Thy-1.2 CBA x C57BL/10 mice.[18] These mice abnormally express the Thy-1.1 gene in podocytes, resulting in the presence of the Thy-1.1 antigen on podocytes. All mice were bred in our animal facility. Breading pairs always consist of a heterozygous (+/-) transgenic mouse and its non-transgenic (-/-) counterpart. To identify transgenic (+/-) and non-transgenic (-/-) mice, the presence of the transgene was examined by PCR on genomic DNA obtained from the tail, with a forward primer: 5'-CGCCTGAGTCCTGATCTCC-3' and a reverse primer: 5'-ACCTGCATCTTCACTGGGT-3'. The presence of the transgene resulted in a specific 834 bp band. As a positive control for the presence of amplifiable genomic DNA a primerset for aminopeptidase A (APA; EC 3.4.11.7), consisting of the forward primer: 5'-ACACAACCCCAGCTCCTTCC-3' and reverse primer 5'-TCTTCTGCAGCCTGGATCAC-3', was used. The amplification of the APA gene with these primers resulted in a 367-bp amplicon.

Sirolimus and the vehicle solution

The sirolimus suspension consisted of 1 mg/ml sirolimus in Phosal 50 PG. Phosal 50 PG is a standardized phosphatidylcholine concentrate with at least 50% phosphatidylcholine and propylene glycol and used as vehicle solution. Sirolimus suspension was further diluted with the vehicle solution till the daily dose to be given was dissolved in 0.1 or 0.2 ml solution. The suspension could be stored at 4 °C for two weeks.

Anti-Thy-1.1 mAb

For in vivo experiments a mouse anti-mouse Thy-1.1 mAb (19XE5: subclass IgG3) was used. 19XE5 was generated *in vitro*, by hollow fibre culture, purified by protein-A column affinity chromatography and concentrated (Nematology Department, Agriculture University Wageningen, the Netherlands). The mAb was decomplemented at 56°C for 45 min and sterilized by passage through a sterile 0.2 µm filter, and stored at –80°C.

Animal experiments

First experiment: Thy-1.1 male transgenic mice were studied from five weeks after birth (at this time point albuminuria was absent) until week 19. Thirty–five transgenic mice were randomised in five groups of 7 mice each. The first experimental group received 1 mg/kg/day of sirolimus orally by gavage (0.1 ml of a 4 times diluted suspension). The second experimental group received 4 mg/kg/day (0.1 ml) and the third group received 8 mg/kg/day of sirolimus (0.2 ml). The first control group daily received 0.1 ml of the vehicle solution alone and the second control group was untreated. Additionally, we studied a group of 7 non-transgenic mice treated with sirolimus (4 mg/kg/day). Albuminuria was measured at regular intervals. The 18-hour urine samples were collected by placing the animals individually in metabolic cages. The first measurement took place one week after the start of receiving sirolimus or vehicle solution. Thereafter albuminuria was measured every 2 weeks till the mice were 19 weeks old. At this time-point all mice were sacrificed and their kidneys were processed for microscopy. Before the mice were sacrificed blood pressure was measured and blood samples were drawn for measurement of serum creatinine levels.

Second experiment: In the second experiment thirty five-week old male Thy-1.1 transgenic mice received an intravenous injection with 2 mg anti-Thy-1.1 mAb (19XE5) in 0.1 ml 0.9% saline solution. In this experiment sirolimus 4 mg/kg/day (0.1 ml) or vehicle solution (0.1 ml) was started orally 11 days after injection of anti-Thy-1.1 mAb in mice who developed proteinuria (15 mice in each group). Albuminuria was measured in 18-hour urine samples collected at day 10, 17, 24 and 31 after anti-Thy-1.1 mAb injection. At day 31 all mice were sacrificed and kidneys were removed and processed

for histology. Before the mice were sacrificed blood pressure was measured and blood samples were drawn for measurement of serum creatinine levels.

Measurement of albuminuria

Albuminuria, as a sign of glomerular protein leakage was measured in urine samples obtained by placing the animals in individual cages during 18 hours. During their confinement in the cages, mice had only access to tap water. Urinary albumin excretion was measured by radial immunodiffusion, using goat antiserum against mouse albumin.[19]

Serum creatinine measurements

Serum creatinine levels were measured in serum sampled at the day the mice were sacrificed. Serum creatinine levels were measured using the Jaffe alkaline picrate method. Measurements were performed using the *AEROSET* creatinine assay and the *AEROSET* Clinical Chemistry System (Abbott Laboratories. Abbott Park, IL, USA).

Light microscopy, immunofluorescence, and immunohistochemistry

For light microscopy, kidney fragments were fixed in Bouin's solution, dehydrated, and embedded in paraplast (Amstelstad, Amsterdam, the Netherlands). 4 µm sections were stained with periodic acid-Schiff, and 2 µm sections with silver methenamine.[20] To obtain the FSGS-score, at least 60 glomeruli per mouse were evaluated for the presence of hypertrophy and hyperplasia of the glomerular epithelium, adhesions and/or sclerosis, and the percentage of abnormal glomeruli were calculated.

RESULTS

First experiment: Since some mice treated with the highest dose of sirolimus (8 mg/kg) died from aspiration, this experimental group was stopped. In addition, mice treated with sirolimus or vehicle solution gained less weight during this study, a reason to stop the study at 19 weeks.

Albuminuria was measured regularly in untreated transgenic control mice and in mice treated with various doses of sirolimus and vehicle solution only (Figure 1). As expected, the untreated transgenic control mice spontaneously developed proteinuria. Transgenic mice treated with sirolimus developed less proteinuria than untreated transgenic mice. This effect on proteinuria was dose-dependent and more obvious in mice treated with 4 mg/kg sirolimus than in mice treated with 1 mg/kg sirolimus. Also mice treated with vehicle solution only (Phosal 50) developed less proteinuria than untreated transgenic

mice. Non transgenic mice treated with 4 mg/kg sirolimus did not develop proteinuria. After 14 weeks of treatment all mice were sacrificed. Before the mice were sacrificed intra-arterial blood pressure was measured after cannulation of the femoral artery in anesthesized mice. We observed blood pressures in the normal range. In light microscopy very few focal sclerotic lesions were seen in all groups (0-5%). In the Thy-1.1 transgenic control group no more than 5% sclerotic lesions were seen.

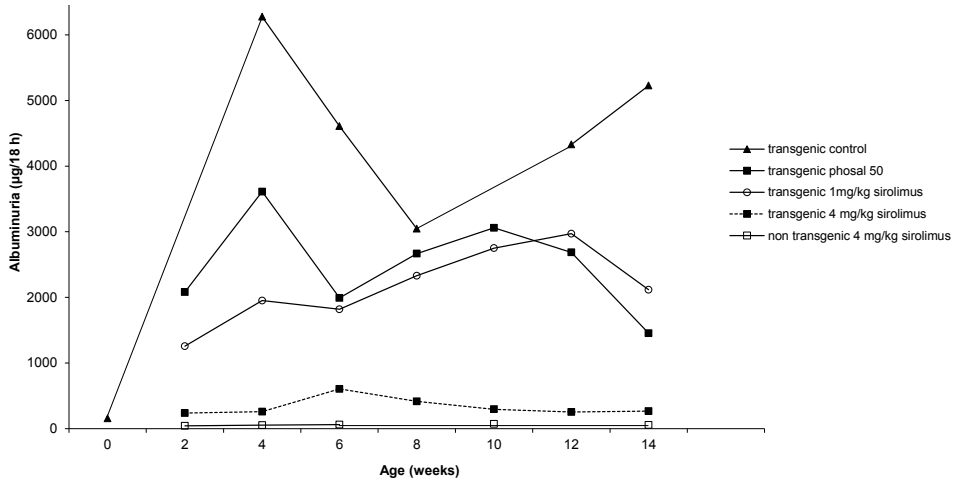

Figure 1. Albuminuria in sirolimus treated Thy-1.1 transgenic mice

Second experiment: In the second experiment thirty five-week old male Thy-1.1 transgenic mice received an intravenous injection with 2 mg anti-Thy-1.1 mAb (19XE5) in 0.1 ml 0.9% saline solution. In this experiment sirolimus 4 mg/kg/day (0.1 ml) or vehicle solution (0.1 ml) was started orally 11 days after injection of 2 mg anti-Thy-1.1 mAb in mice who developed proteinuria (15 mice in each group).
Albuminuria was measured in 18-hour urine samples collected at day 10, 17, 24 and 31 after anti-Thy-1.1 mAb injection. At all time points there was no difference in albuminuria between treatment groups (Table 1). At day 31 all mice were sacrificed and kidneys were removed and processed for histology. The percentage of normal glomeruli (without focal sclerotic or proliferative lesions) amounted 44 ± 23 % in the sirolimus treatment group and 46 ± 29 % in the vehicle solution treatment group (ns).

Table 1. Urinary albuminuria in male Thy-1.1 transgenic mice

Time	Albuminuria (µg/mmol creatinine)		
	Sirolimus 4 mg/kg/day	Vehicle solution	P value
Day 10	3929 ± 3377	4691 ± 2515	0.51
Day 17	4145 ± 3440	4023 ± 2392	0.34
Day 24	4213 ± 3221	4151 ± 2669	0.96
Day 31	2281 ± 1421	1752 ± 1012	0.32

Data are depicted as mean ± standard deviation.
The day of administration of the anti-Thy-1.1 antibodies is considered day 0. Sirolimus or vehicle solution was started on day 11 after injection of 2 mg anti-Thy-1.1 mAb in mice who developed proteinuria

DISCUSSION

We set up these experimental studies in an effort to develop a suitable model for investigation of sirolimus-induced proteinuria. We did not observe any increase in proteinuria after administration of sirolimus: neither in experiment 1 nor in experiment 2. We also did not detect differences in the occurrence of glomerulosclerosis. Thus, we were unable to replicate the finding of increased proteinuria observed in patients treated with sirolimus.

Of note, we even observed some reduction of proteinuria in transgenic mice treated with sirolimus, which could not be explained by differences in blood pressure. Thus, our data might suggest a dose dependent protective effect of sirolimus on proteinuria in male Thy-1.1 transgenic mice. The mechanisms responsible for this protective effect were not further evaluated.

In light microscopy very few focal sclerotic lesions were seen in all groups (0-5%). Since few control mice developed FSGS within the study period, no effect of sirolimus on development of FSGS could be demonstrated. Assmann *et al.* studied untreated transgenic mice and observed focal glomerulosclerosis in 17 +/- 6% of glomeruli at week 26.[21] Because in our study the body weight of the sirolimus treated mice decreased during the study period we sacrificed them at week 19. This might be the explanation for the very few glomeruli with focal sclerotic lesions that we observed. Although we expected that sirolimus might worsen damage if administered in mice with existing glomerular damage, our experiments could not confirm this hypothesis.

We thus were unable to create a mouse model that could replicate the finding of increased proteinuria that was observed in patients treated with sirolimus. Although the pathogenesis of proteinuria is likely multi-factorial and may involve tubular and glomerular damage, the latter seems much more likely. However, the pathways by which mTOR inhibition could induce podocyte injury remain speculative. The podocyte plays

a key role in both maintenance of the glomerular filtration barrier and in glomerular structural integrity. Various slit diaphragm and cytoskeletal proteins contribute to the maintenance of podocyte permeability functions, and podocyte secretion of growth factors such as vascular endothelial growth factor (VEGF) is necessary for endothelial cell survival. The podocyte itself also expresses numerous receptors and responds to many growth factors and metabolic products implicated in progressive kidney diseases. However, podocytes have limited proliferative capacity, and when glomerular growth and hemodynamic stresses exceed the ability of podocytes to undergo hypertrophy, they become irreversibly injured and disappear (apoptosis or detachment). Podocyte injury and loss contribute to proteinuria and progressive sclerosis. The studies by Gödel *et al.*[22] and Inoki *et al*.[23] demonstrated the importance of the mTOR pathway in podocytes. mTOR is a widely expressed protein kinase that mediates its functions in two complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)(Figure 2). The net consequences of mTOR activity for the podocyte and glomerular disease depend on the balance between these complexes. mTORC1 regulates proliferation and autophagy in response to various nutrients. mTORC1 is potently inhibited by the immunosuppressive drug sirolimus. mTORC2 controls cell survival, modulates the cytoskeleton and is largely (but not completely) sirolimus insensitive; its activation results in phosphorylation of AKT and protein kinase C (PKC).[24] Both mTORC1 and mTORC2 are needed for podocyte development and podocyte maintenance. The lack of mTORC1 in mouse podocytes induced proteinuria and progressive glomerulosclerosis, which was aggravated when mTORC2 was concomitantly inactivated.[22] Therefore, complete inhibition of mTORC1 activity with sirolimus treatment may worsen podocyte function and fail to yield a better clinical outcome. Intriguingly, podocytes seem to be particularly sensitive to mTORC1 deletion during glomerular development, indicating that mTORC1 is of particular importance during podocyte growth and adaptation. In podocyte specific mTORC2-deficient mice no obvious clinical, histological, or ultrastructural abnormalities were observed. However, podocyte specific mTORC2-deficient mice exposed to stress such as BSA overload developed significantly higher albuminuria than control littermates, suggesting that mTORC2 might play a role in podocyte adaptation and foot process reorganization in response to stress.

The activity of mTOR must be tightly regulated, since overactivation also causes damage. mTORC1 activity in mature podocytes is very low under basal conditions. It has been shown that podocyte mTOR activity is increased in glomerular diseases, likely in an attempt to maintain podocyte homeostasis. In response to mTOR activation, podocytes change in a fairly stereotypical manner with cell hypertrophy, foot process effacement, and eventually detachment from the glomerular basement membrane. This mTOR activation, which may provide some short-term benefits, ultimately causes proteinuria and glomerulosclerosis and facilitates disease progression. Curtailing

mTORC1 signalling in mice by genetically reducing mTORC1 in podocytes prevented glomerulosclerosis and significantly ameliorated the progression of glomerular disease in diabetic nephropathy.[22] The timing of mTOR inhibition in podocytes was also investigated. Adding rapamycin early prevented renal injury caused by mTORC1 activation. However, progression of sclerosis was not affected if rapamycin was given when injury induced by mTORC1 activation in podocytes was already moderate.[23] These results demonstrate the requirement for tightly balanced mTOR activity in podocyte homeostasis.

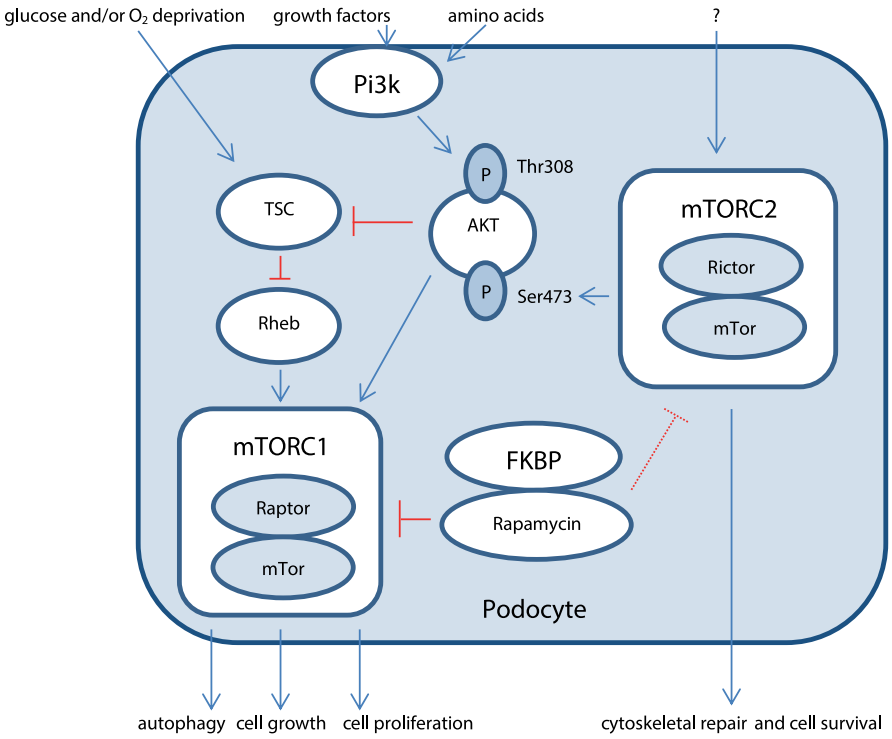

Figure 2. mTOR signaling in the podocyte

mTor is a component of two major intracellular signaling complexes: mTORC1 and mTORC2. These complexes contain two different scaffolding proteins (raptor and rictor) that "connect" them to different downstream targets. Podocyte maintenance is dependent on a fine-tuned balance of mTORC1 and mTORC2 activity. mTORC1 signaling is normally activated by growth factors and amino acids, which activate phosphatidylinositol-3-kinase (Pi3k), a lipid kinase. Pi3k activates AKT by phosphorylation it at Thr308. Rheb, a cytoplasmatic protein, activates mTORC1 and is regulated by the tuberous sclerosis complex (TSC). AKT activates mTORC1 by inhibition of TSC. AKT is also phosphorylated on Ser473 by mTORC2. The primary signals for mTORC2 activation are unknown. Rapamycin has an immediate inhibitory effect on mTORC1 and with chronic use may also inhibit mTORC2. Inhibition of mTORC1, especially during development or other physiologic or pathophysiologic growth, may cause podocyte injury. Added inhibition of mTORC2 activation causes more severe podocyte injury, sclerosis, and proteinuria, illustrating the dependence of normal podocyte function and structure on balance of the two complexes.

Canaud *et al.*[25] showed that upon nephron reduction podocytes are adapting to the stress of cell loss and undergo mTORC2-mediated phosphorylation of AKT at Ser473, delaying kidney disease progression by promoting podocyte survival and cytoskeleton integrity. Disruption of this adaptive mTORC2-AKT pathway via sirolimus resulted in podocyte apoptosis and foot process effacement after nephron reduction, leading to severe proteinuria and glomerulosclerosis in mice. Therefore, mTOR inhibitors should be used with caution in patients with nephron reduction to preserve the activity of the mTORC2-AKT axis and prevent podocyte apoptosis.

In experimental settings, the effects of sirolimus treatment on proteinuria are equivocal, with most studies reporting renoprotective effects[26-42] whereas some studies showed an increase in proteinuria[42-46] (Table 2).

In experimental models of reduced renal mass low-dose mTOR inhibition prevented progressive renal fibrosis when mTOR inhibition was started after the acute effects of renal ablation and reparation have taken place.[26-28] In contrast, when sirolimus was given in a high-dose early after surgery mTOR inhibition apparently inhibited the chronic glomerular repair reaction via inhibition of the proliferative but not apoptotic activity of the glomerular endothelial and mesangial cells. The inhibition of VEGF-mediated capillary and mesangial repair of ongoing complex glomerular injury and repair reaction seems to be critical for the adverse effects of mTOR inhibition.[43] While antiproliferative effects are beneficial for hyperproliferative lesions, during the acute phase of renal injury these antiproliferative effects seem to be problematic. The timely requirements of complex glomerular repair reaction, especially involving both endothelium and mesangium, being transiently inhibited by mTOR inhibition, seems to be critical for its adverse effects.

Beneficial effects of low-dose mTOR inhibition were also reported in diabetes induced by streptozotocin. Treatment reduced phosphorylation of AKT (pAKT) and normalized mTOR, suggesting that the mTOR pathway has an important pathogenic role in diabetic nephropathy.[29] Beneficial effects of mTOR inhibitors were also reported in other animal models such as anti-Thy 1 nephritis in rats[30,31], lupus nephritis[32-34] and membranous nephropathy.[35-37] Stratakis *et al.*[37] found that rapamycin monotherapy significantly improved proteinuria and histological lesions in experimental membranous nephropathy. This beneficial effect may be mediated by inhibition of the alloimmune response during the autologous phase of passive Heymann nephritis and by restoration of the normal expression of the podocyte proteins nephrin and podocin. In nephrotoxic serum nephritis, an anti-GBM nephritis model, the effect of rapamycin on proteinuria was influenced by timing of administration. When rapamycin was started before administration of the anti-GBM antiserum, mice were protected from glomerulonephritis, suggested by a dramatic decrease in albuminuria and reduced B and T cell responses.

Table 2. Characteristics of studies investigating the effect of mTOR inhibitor on proteinuria

Study	Animals	Model	Effect of mTOR inhibitor on proteinuria	Dose	Timing
Vogelbacher et al. 2007	Male Sprague-Dawley rats (uninephrectomy and infarction of 2/3 of the remaining kidney)	Renal mass reduction (chronic renal failure)	Increase	Everolimus 2.5 mg/kg/day orally (trough level 8.1 ± 1.9 ng/ml)	3 days after surgery (early)
Diekmann et al. 2007	Male Wilstar rats (right nephrectomy and 2/3 cryoablation of the left kidney)	Renal mass reduction (chronic renal failure)	Decrease	Sirolimus 3 mg/kg/week ip (high-dose) (trough level 33.7 ± 5.3 ng/ml)	6 weeks after surgery (late)
Rovira et al. 2009	Rats (right nephrectomy and 2/3 cryoablation of the left kidney)	Renal mass reduction (chronic renal failure)	Decrease	Sirolimus 1 mg/kg ip three times per week	2 weeks before and 12 weeks after surgery (early and late)
Kurdian et al. 2012	Male Wilstar rats 5/6 nephrectomy	Renal mass reduction (chronic renal failure)	Decrease	Everolimus 0.3 mg/kg/day orally	15 days after surgery (late)
Lloberas et al. 2006	Diabetic Sprague-Dawley rats (streptozotocin injection)	Diabetic nephropathy	Decrease	Sirolimus 1 mg/kg/day (low-dose)	16 weeks (late)
Wittmann et al. 2007	Chronic anti-Thy1 nephritis in Sprague-Dawley rats (anti-Thy1 after uninephrectomy)	MPGN	Decrease	Everolimus 2.5 mg/kg/day orally (trough level 7.6 ± 2.3 ng/ml)	2 weeks after disease induction (late)
Kramer et al. 2007	Anti-Thy1 induced chronic glomerulosclerosis in Male Wilstar rats (anti-Thy1 after uninephrectomy)	Mesangioproliferative nephropathy	Decrease	Sirolimus 2.5 mg/kg/day orally (low-dose) (trough level 1.4 ± 0.5 ng/ml)	10 days after antibody injection
Lui et al. 2008 (Lupes)	NZBW/F1 female mice	Lupus nephritis	Decrease	Sirolimus 3 mg/kg/day orally	12-weeks old mice
Lui et al. 2008 (NDT)	NZBW/F1 female mice	Lupus nephritis	Decrease	Sirolimus 3 mg/kg/day orally	6-months old mice

Table 2. Characteristics of studies investigating the effect of mTOR inhibitor on proteinuria *(Continued)*

Study	Animals	Model	Effect of mTOR inhibitor on proteinuria	Dose	Timing
Stylianou et al. 2011	NZBW/F1 female mice	Lupus nephritis	Decrease	Sirolimus 1 mg/kg/ every other day ip	Before proteinuria (4 months) and 1 week after severe proteinuria (5 months)
Kirsch et al. 2012	Nephrotoxic serum nephritis in male C57BL/6 mice	Anti-GBM nephritis	Increase	Sirolimus 0.5 mg/kg/day ip	14 days after disease induction
Hochegger et al. 2008	Nephrotoxic serum nephritis in male C57BL/6 mice (injection rabbit anti-mouse GBM antiserum)	Anti-GBM nephritis	Decrease	Sirolimus 1 mg/kg/day ip	Early (before immunization)
			Increase		Late (14 days after immunization)
Bonegio et al. 2005	Male Sprague-Dawley rats (anti-Fx1+ uninephrectomy)	Membranous nephropathy	Decrease	Sirolimus 0.5 mg/kg/day sc (low-dose)	1 week after nephrectomy
Naumovic et al. 2007	Heymann nephritis	Membranous nephropathy	Decrease	Sirolimus 1.5 mg/kg/day	Induction phase and evolving disease (early and late)
Strakatis et al. 2013	Heymann nephritis (anti-Fx1 in Sprague-Dawley rats)	Membranous nephropathy	Decrease	Sirolimus 0.5 mg/kg/day sc	1 week after anti-Fx1
Lui et al. 2009	Adriamycin nephropathy in male Balb/c mice	FSGS	Decrease	Sirolimus 3 mg/kg/day orally	At the time of adriamycin injection (early) or 1 week later.
Rangan et al 2007	Adriamycin nephropathy in male Wilstar rats	FSGS	No influence	Sirolimus 0.1 mg/kg sc	From day 14 (early)

Table 2. Characteristics of studies investigating the effect of mTOR inhibitor on proteinuria *(Continued)*

Study	Animals	Model	Effect of mTOR inhibitor on proteinuria	Dose	Timing
Ramadan et al. 2012	Adriamycin nephropathy in male Sprague-Dawley rats	FSGS	Decrease (early and low-dose)	Everolimus via drinking water: 20 mg/L (trough level 4.9 ± 0.6 ng/mL) or 100 mg/L (trough level 21.3 ± 4.4 ng/mL).	2 days before injection or 3 weeks after injection
Ito et al. 2011	PAN nephrosis in male Sprague-Dawley rats (puromycin aminonucleoside injection)	Minimal Change	Decrease	Everolimus 4 mg/kg/day orally	1 day before PAN injection
Torras et al. 2009	PAN nephrosis in male Sprague-Dawley rats (puromycin aminonucleoside injection)	Minimal change	Increase	Sirolimus 3 mg/kg/day orally (high-dose) (trough level 13 ± 1 ng/ml)	Directly after PAN injection
	Male Sprague-Dawley rats 5/6 nephrectomy	Renal mass reduction (chronic renal failure)	Decrease	Sirolimus 3 mg/kg/day orally (high-dose)	From the first day after surgery or from the eighth week after surgery
Ko et al. 2012	Male Fisher 344 and Lewis rats	Chronic allograft dysfunction	Increase	Sirolimus 1 mg/kg/day (versus Cyclosporine)	Directly after transplantation (early)
Stylianou et al 2012	Healthy Balb/c mice	Normal kidney	Increase in high-dose and 4-week groups	Sirolimus in different doses during 1 week: 1 mg/kg/day ip, 1.5 mg/kg/day ip, 3 mg/kg/day ip Sirolimus 1,5 mg/kg/day ip for different periods: 1 week, 4 weeks and 8 weeks	

In contrast, when rapamycin was started 14 days after infusion of the anti-GBM antiserum mice showed a significant increase in albuminuria and renal infiltration of inflammatory cells, and there were no differences in T and B cell responses. A significant decrease in vascular endothelial growth factor-A and an increase in IL-6 was detected indicating a disturbance of the endothelial cell/vascular endothelial growth factor system in the kidney.[47] In the same model Kirsch *et al.*[44] also found an increase in albuminuria when rapamycin administration was started 14 days after induction of nephrotoxic serum nephritis. This rapamycin-induced proteinuria seemed to be a result of the activation of the innate immune system rather than a direct toxicity to podocytes or glomerular endothelial cells.

In rats receiving a fixed high-dose of rapamycin, differences were found in the effects on renal function depending on the underlying injury. In minimal change disease, where podocyte structure has been damaged, rapamycin aggravates the disruption of the glomerular slit structure by lowering podocin expression, thus resulting in higher proteinuria. In contrast, in a chronic injury model rapamycin appears to have an important nephro-protective role associated with the maintenance of nephrin and podocin expression.[42]

In Adriamycin-induced nephrotic syndrome, early, and to a lesser extent late treatment, with a low but not a high-dose of everolimus was effective in reducing proteinuria in nephrotic rats. The mechanism may be via nephrin/podocin.[40]

In the glomerular compartment, others have demonstrated reduced VEGF, particularly in patients with significant proteinuria.[43] VEGF is synthesized by podocytes and has emerged during the past years as an essential autocrine/paracrine factor that promotes the survival of both endothelial cells and podocytes. Sirolimus has been demonstrated to inhibit production of VEGF through the inhibition of mTOR. A role for VEGF in the pathogenesis of chronic allograft dysfunction after kidney transplantation has been suggested, in particular, in promoting fibrosis.

Ko *et al.*[45] found that whilst both sirolimus and cyclosporine provided some protection against inflammation and fibrosis in a rat model of chronic kidney allograft dysfunction, sirolimus provided additional benefit in attenuating vasculopathy at the expense of proteinuria. Their observations of decreased expression of VEGF and VEGFR in glomeruli and vessels, plus inhibition of VEGF-stimulated proliferation of glomerular cells by sirolimus in vitro, suggest inhibition of VEGF signaling may be a key mechanism of both vascular protection and proteinuria in kidney transplant recipients.

Yan *et al.*[48] demonstrated the possible linkage of an energy-consuming process in glomerular podocytes to the mechanism of proteinuria. Puromycin aminonucleoside nephrosis, a rat model of minimal change disease, revealed the activation of the unfolded protein response (UPR) in glomerular podocytes to be a cause of proteinuria. Of note,

activation of mTORC1 was related to energy consumption and increased UPR response. Indeed, pre-treatment of puromycin aminonucleoside treated podocytes with mTOR inhibitor everolimus decreased UPR activation and energy consumption. This finding was further confirmed, also in a model of minimal change disease, by Ito *et al.*[41] They demonstrated that the mTORC1 inhibitor everolimus completely inhibited proteinuria through a reduction in both mTORC1 and UPR activity paralleled by preserved nephrin expression in glomerular podocytes. They concluded that mTORC1 activation may perturb the regulatory system of energy metabolism primarily by promoting energy consumption and inducing the UPR, which underlie proteinuria in minimal change disease.

It is apparent that our current understanding of the clinical significance and causative mechanisms of mTOR inhibitor-associated proteinuria is far from complete. The effect of mTOR inhibition on proteinuria in patients with kidney disease is probably influenced by multiple factors such as the nature and presence of pre-existing renal damage, timing of administration, dosage of rapamycin used and prior exposure to a calcineurin inhibitor. Additional basic and clinical studies are warranted to clarify the issue of rapamycin-associated proteinuria.

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Chapter 5

Two-year randomized controlled prospective trial converting treatment of stable renal transplant recipients with cutaneous invasive squamous cell carcinomas to sirolimus

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ABSTRACT

Purpose

In light of the significant morbidity and mortality of cutaneous invasive squamous cell carcinomas (SCCs) in renal transplant recipients, we investigated whether conversion to sirolimus-based immunosuppression from standard immunosuppression could diminish the recurrence rate of these skin cancers.

Patients and methods

In a 2-year randomized controlled trial, 155 renal transplant recipients with at least one biopsy-confirmed SCC were stratified according to age (< 55 v ≥ 55 years) and number of previous SCCs (one to nine v ≥ 10) and randomly assigned to conversion to sirolimus (n=74) or continuation of their original immunosuppression (n=81). Development of a new SCC within 2 years after random assignment was the primary end point.

Results

After 2 years of follow-up, the risk reduction of new SCCs in the multivariable analysis was not significant, with a hazard ratio (HR) of 0.76 (95% CI, 0.48-1.2; P=0.255), compared with a non-sirolimus-based regimen. After the first year, there was a significant 50% risk reduction, with an HR of 0.50 (95% CI, 0.28 to 0.90; P=0.021) for all patients together and an HR of 0.11 (95% CI, 0.01 to 0.94; P = 0.044) for patients with only one previous SCC. The tumor burden of SCC was reduced during the 2-year follow-up period in those receiving sirolimus (0.82 v 1.38 per year, relative risk 0.51; 95% CI, 0.32 to 0.82; P=0.006) if adjusted for the number of previous SCCs and age. Twenty-nine patients stopped taking sirolimus because of various adverse events.

Conclusions

Conversion to sirolimus-based immunosuppression failed to show a benefit in terms of SCC-free survival at two years.

INTRODUCTION

The reported cancer risk in renal transplant recipients (RTRs) is two- to six-fold greater than in the general population.[1-3] Cutaneous squamous cell carcinomas (SCCs) are the most common post-transplantation cancers, occurring 65 to 250 times more often than in the general population.[4-6] Invasive SCC may derive from SCC in situ (Bowen's disease) or other intra-epidermal precursor lesions (actinic keratoses) and may metastasize.[4] The cumulative incidence of cutaneous SCC ranges from 2 to 24% after 5 years post-transplantation.[5,7] Once an individual develops a first SCC, the risk of developing subsequent independent SCCs is high.[8,9] In addition to the clinical and economic burdens of multiple SCCs, transplantation skin cancers carry a worse prognosis than those from immunocompetent persons, with more aggressive behavior and increased mortality resulting from metastatic disease.[10]

Causes of cutaneous SCCs in RTRs include: exposure to ultraviolet radiation, reduced immunosurveillance, skin type, age at transplantation, and human papillomavirus infection.[4,11-13]The intensity and duration of immunosuppressive therapy influence SCC risk, resulting in increased incidence in cardiac compared with renal or liver transplant recipients.[14,15] There is also compelling evidence for carcinogenic mechanisms associated with cyclosporine [16-18] and azathioprine.[19]

The antiproliferative response of mammalian target of rapamycin (mTOR) inhibitors may confer a lower risk of the development of malignancy, as shown in small pilot studies and short-term registry analyses.[20-22] Sirolimus interferes with intracellular proteins, with influences on angiogenesis, cell growth, division, and survival.[23] In addition, sirolimus has been shown to inhibit the ultraviolet B activation of metalloproteinases that may promote cancer formation and premature skin aging.[24] Prospective randomized studies comparing efficacy of sirolimus with that of other immunosuppressive regimens have indicated a tendency for fewer skin tumors developed in the sirolimus group as a secondary outcome measure.[20,22] Until recently, there has been a lack of prospective randomized studies of mTOR inhibitors evaluating the recurrence rate of cutaneous SCC as the primary outcome measure. A study in Australia included 86 patients with either SCC or basal cell carcinoma (BCC).[25] At 1 year, there was a reduction in formation of new SCCs, of which most were SCC in situ. The incidence and pattern of skin tumors in Australia, however, is different from these in Western Europe because of differences in ultraviolet light exposure, which may affect the influence of sirolimus conversion.[12] In the RESCUE (Recurrent Cutaneous Squamous Cell Carcinoma Under Rapamune) study, a 2-year randomized, prospective, open-label, multicenter trial, we investigated whether conversion to sirolimus-based immunosuppression in long-term RTRs would diminish the rate of new cutaneous invasive SCCs.

PATIENTS AND METHODS

Patients studied

RTRs were recruited from five transplantation centers in the Netherlands and 16 in the United Kingdom. Inclusion criteria included: first or second kidney transplantation with ≥ 1 biopsy-confirmed cutaneous invasive SCC, age ≥ 18 years, >12 months post-transplantation, stable graft function with estimated glomerular filtration rate ≥ 20 ml/min/1.73m^2, receiving maintenance calcineurin inhibitor, azathioprine, mycophenolate, and/or steroids for at least 12 weeks before random assignment, and no acute rejection episode within 12 weeks before random assignment.

Exclusion criteria included: metastatic cutaneous SCC, internal malignancies (documented after transplantation), serum creatinine at screening increased >30% above the last value obtained at least 12 weeks earlier, total WBC count < 3,000/μL, platelet count < 75,000/ μL, fasting-riglycerides >3.95 mmol/l, cholesterol >7.8 mmol/l (± statins), transaminases >2 x above normal, planned/present pregnancy, evidence of systemic infection or HIV infection at random assignment, or Fitzpatrick skin type V to VI. The independent ethics committee or institutional review board of each site approved the protocol. Participants provided written informed consent in accordance with the Declaration of Helsinki.

Random assignment

After patient consent, random assignment took place using blinded envelopes containing treatment codes for either continuation of maintenance therapy or conversion to sirolimus. The random assignment (1:1) was stratified by transplantation center, number of biopsy-confirmed SCCs (< 10 v ≥ 10) before random assignment, and recipient age (< 55 v ≥ 55 years). For each of the defined stratification groups, random assignment envelopes with a fixed random assignment order per stratum were available with a number of four per random assignment block.

Procedures

The target blood level of sirolimus was 5 to 10 ng/ml; sirolimus was started the day the purine antagonist (azathioprine or mycophenolate mofetil) and/or calcineurin inhibitor (cyclosporine or tacrolimus) was withdrawn (loading dose, of 8 mg and maintenance dose, of 4 mg). Between days 5 to 7, a sirolimus trough level was measured and the dose adjusted to the defined range. All patients were also treated with at least 5 mg of prednisone daily. The immunosuppressive regimen was not changed in control patients. At regular three monthly intervals, a complete skin inspection was undertaken by a dermatologist, and renal function and adverse events were monitored by a

nephrologist. The dermatologists were blinded for the treatment arm, but patients and their nephrologists were unblinded.
Skin lesions clinically suspected to be invasive SCCs or BCCs were biopsied for histological interpretation by the local dermatopathologists. In RTRs, actinic keratoses and SCC in situ are clinically difficult to discern, and these lesions were not routinely biopsied. Only biopsy-confirmed invasive SCCs, SCCs in situ, and BCCs were included in the study.
Laboratory data were recorded every 3 months. Adverse events were evaluated during the study period, and the reasons for dropout were documented.
The development of cutaneous invasive SCC within 2 years after conversion was the primary end point of this study. Secondary end points included: incidence, severity, and reversibility of biopsy-confirmed acute rejection episodes; patient and graft survival; and renal function 2 years after random assignment.

Statistical analyses

Sample size calculation was based on the risk to develop a subsequent cutaneous invasive SCC, which was reported earlier to be 50% in 2 years.[8,9] In addition, a relative risk reduction of 50% was expected in the patients assigned to be converted to sirolimus compared with patients who would continue their original immunosuppressive regimen, based on results obtained on new skin lesions in de novo patients.[20] To detect a difference in recurrence probability at 2 years of 50% versus 25% at a two-sided alpha level of 5% and a power of 0.9, assuming 25% loss to follow-up, 154 patients would be needed. Hence, we aimed at 80 patients per arm (NCSS Statistical Software; NCSS, Kaysville, Utah, UT).
The occurrence of SCC was assessed with Kaplan-Meier curves and Cox regression to calculate the hazard ratio (HR). The annualized per-patient SCC recurrence rate was modelled using a negative binomial regression model. All RTRs were included in the intention-to-treat (ITT) analyses and observed during the 2-year follow-up period or until death. The same analyses were performed for the first year and stratified for one previous SCC or > one SCC. We also performed per-protocol population (PPP) analyses including the RTRs until they dropped out because of adverse events or withdrew informed consent (Fig 1).
First analyses (Table 1) showed that sex was imbalanced despite random assignment (P=0.003), but this did not affect the other analyses. The unexpected low recruitment rate in two thirds of centers in the United Kingdom, in combination with the fixed order per stratum, resulted in the imbalance of the stratification factors. To analyze the impact of this imbalance on treatment outcome, we performed multivariable analyses and analyses stratified for sex, age at random assignment, immunosuppressive drugs, and country. Finally, adjustments were only made for age and number of invasive SCCs

before inclusion, the predefined characteristics for which we had stratified. For the analyses, we used the PASW Statistics software package (release 17.02; SPSS, Chicago, IL).

RESULTS

We included 103 RTRs in the Netherlands and 52 in the United Kingdom between January 2004 and September 2009. The pathway of patients recruited and outcomes are shown in Figure 1. Demographic and baseline characteristics are presented in Table 1.

Table 1. Patient demographics and baseline clinical characteristics.

Characteristic	Patients converting to sirolimus (n = 74)		Patients continuing original immunosuppression (n=81)	
	No.	%	No.	%
Sex:*				21
Female	32	43	17	79
Male	42	57	64	
Age at random assignment, years				
<55	31	42	23	28
≥55	43	58	58	72
Functioning transplant at random assignment, years				
Mean	19		18	
SD	8		7	
Serum creatinine, µmol/L				
Mean	121		137	
SD	44		48	
Immunosuppressive regimen at random assignment				
- One immunosuppressive drug ± prednisone	63	85	64	79
Aza	42		33	
MMF	10		3	
Cyclosporine	7		21	
Tacrolimus	4		7	
- Two immunosuppressive drugs ± prednisone	11	15	17	21
Calcineurin inhibitor with Aza	9		13	
Calcineurin inhibitor with MMF	2		4	
Skin type (Fitzpatrick I to IV)				
I (very fair skin; Celtic)	9	12	9	11
II	39	53	44	54
III	26	35	25	31
IV (darker skin; Mediterranean)	0	0	3	4
No. of invasive SCCs before random assignment				
1	30	40	23	29
2 to 9	33	45	52	64
≥10	11	15	6	7

Abbreviations: Aza, azathioprine; MMF, mycophenolate mofetil; SCC, squamous cell carcinoma; SD, standard deviation.
*Sex was imbalanced despite random assignment (P = 0.003), but this did not affect the additional analyses.

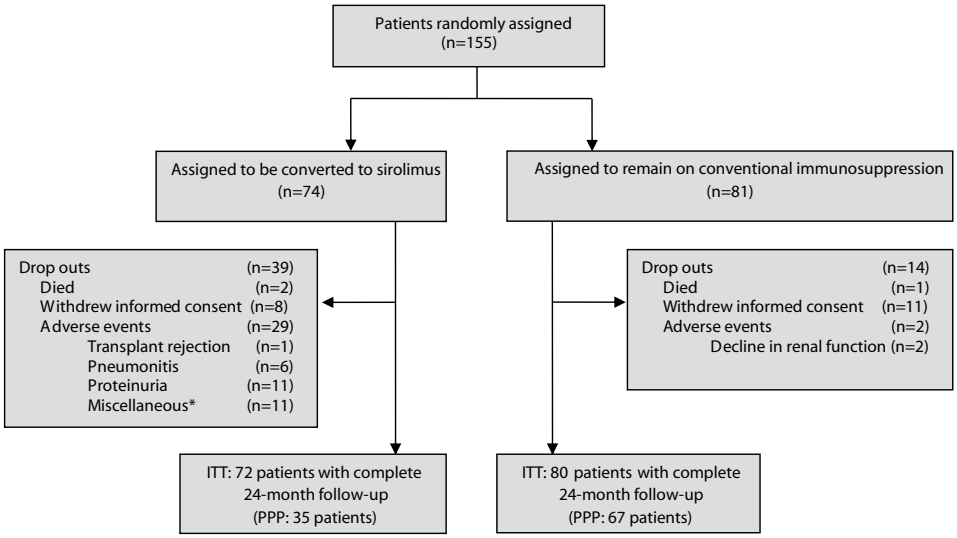

Figure 1. Patient disposition.

ITT, intention to treat; PPP, per-protocol population.

* Slow wound healing (n=1), pulmonary embolus (n=1), pneumonia (n=1), edema (n=2), diarrhea (n=2), fatigue (n=2), 1 skin rash (n=1), dyslipidemia (n=1), and diabetes mellitus (n=1).

Kaplan-Meier analysis of all 155 randomly assigned patients showed separation between the curves, but after 2 years, there was no significant difference for invasive SCC-free survival (P = 0.155; Figs 2A to 2C). In exploratory analyses at 1 year, statistical significance was still present (P = 0.006; Fig 2D). Sirolimus was especially effective during the first year after conversion in RTRs with only one previous SCC. In this subgroup, only one of 30 patients developed a new SCC 9 months after conversion compared with six of 23 patients in the control group (P = 0.015; Fig 2E). Conversion to sirolimus was much less effective in patients with multiple SCCs before inclusion (Figs 2C, 2F).

In multivariable analyses, the HR for SCC recurrence under sirolimus was 0.76 (95% CI, 0.48 to 1.2; P=0.255) after the 2-year follow-up period, representing a statistically nonsignificant 24% reduction in the estimated risk of developing at least one subsequent invasive SCC. In exploratory analyses at 1 year, the HR was 0.50 (95% CI, 0.28 to 0.90; P = 0.021), a significant 50% reduction after the first year of follow-up (Table 2). The ITT and PPP crude and adjusted analyses for invasive and/or in situ SCCs are summarized in the Appendix Table A1. The analyses of in situ SCCs resembled those of invasive SCCs, and the PPP analyses showed a slightly stronger risk reduction than the ITT analyses (Appendix Table A1). A total of 15 patients (20.3%) converted to sirolimus, and 27 (33.3%) of those continuing their original immunosuppression developed ≥ one BCC, which resulted in an HR for BCC recurrence with sirolimus of 0.56 (95% CI, 0.30 to 1.1; P=0.076) and an adjusted HR of 0.67 (95% CI, 0.34 to 1.3; P=0.233).

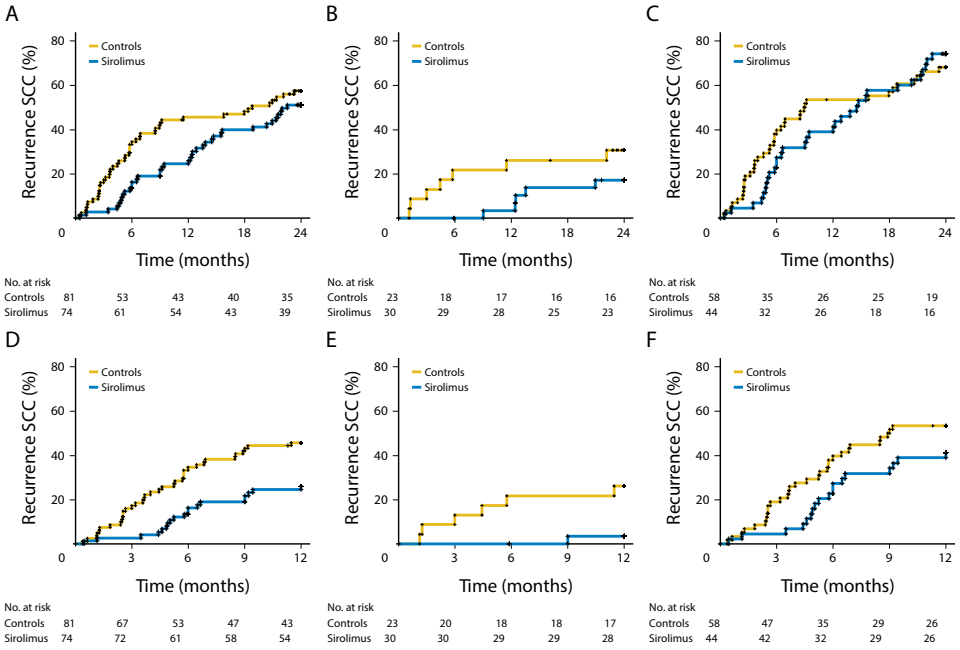

Figure 2. Recurrence of squamous cell carcinoma (SCC) in patients converting to sirolimus and control patients who continued their original immunosuppressive regimen according to the intention to treat analysis. (A) Follow-up until 2 years (primary end point for all patients together; P = 0.155) and (B) stratified for one SCC at inclusion (P = 0.193) or (C) > one SCC at inclusion (P = 0.854). (D) Follow-up until 1 year for all patients together (P = 0.006) and (E) in patients with one SCC (P = 0.015) and (F) > one SCC (P = 0.139).

The annualized per-patient invasive SCC recurrence rate, as shown in Appendix Table A2, was 0.82 in the sirolimus arm compared with 1.38 in controls. The relative risk for developing an invasive SCC was 0.60 (95% CI, 0.35 to 1.02; P = 0.057), and 0.51 (95% CI, 0.32 to 0.82; P = 0.006) when adjusting for number of SSCs at inclusion and age, a 49% reduction in the risk of developing an SCC compared with a non-sirolimus-based regimen. The adjusted and PPP analyses showed stronger risk reductions (Appendix Table A2).

The first sirolimus trough level (± standard deviation [SD]) was 12.0 ± 6.4 ng/mL, measured after a mean (± SD) of 10.6 ± 6.4 days, and was higher in patients who discontinued sirolimus (13.1 ± 6.8 v 10.9 ± 5.6; P=0.149). Mean trough levels (± SD) were 9.4 ± 4.5, 7.8 ± 2.4, and 7.1 ± 1.9 ng/mL at 3, 12, and 24 months, respectively. Within these ranges, sirolimus trough levels did not significantly predict the risk for developing recurrent SCCs and were also not significantly associated with adverse effects or treatment discontinuation (data not shown).

Table 2. Risk for developing ≥ one subsequent SCC.

	Recurrence at 2-year follow-up *					
	Univariate			Multivariable†		
Factor	HR	95% CI	P	HR	95% CI	P
Sirolimus conversion	0.73	0.47 to 1.1	0.157	0.76	0.48 to 1.2	0.255
Age (per 10 years)	0.90	0.73 to 1.1	0.304	0.95	0.77 to 1.2	0.610
Male v female	0.94	0.60 to 1.5	0.781	0.97	0.59 to 1.6	0.886
No. of previous SSCs						
1	1			1		
2 to 9	4.2	2.2 to 7.8	0.000	3.9	2.1 to 7.4	0.000
≥10	7.2	3.3 to 15.7	0.000	7.3	3.3 to 16.1	0.000
	Recurrence at 1-year follow-up‡					
	Univariate			Multivariable†		
Factor	HR	95% CI	P	HR	95% CI	P
Sirolimus conversion	0.47	0.27 to 0.81	0.007	0.50	0.28 to 0.90	0.021
Age (per 10 years)	0.88	0.69 to 1.1	0.333	0.89	0.69 to 1.1	0.339
Male v female	1.5	0.79 to 2.7	0.229	1.4	0.73 to 2.6	0.321
No. of previous SSCs						
1	1			1		
2 to 9	4.1	1.8 to 9.2	0.001	3.6	1.6 to 8.2	0.002
≥10	7.3	2.8 to 18.9	0.000	7.9	3.0 to 20.6	0.000
	Stratified for patients with one and > one SCC‡§					
	Recurrence at 1-year follow-up			Recurrence at 2-year follow-up		
	HR	95% CI	P	HR	95% CI	P
One SCC	0.11	0.01 to 0.94	0.044	0.48	0.15 to 1.5	0.203
> One SCC	0.65	0.36 to 1.2	0.144	0.96	0.60 to 1.5	0.854

Abbreviations: HR, hazard ratio; CI, confidence interval; SCC, squamous cell carcinoma.
* Primary end point.
† Multivariable analyses included sirolimus conversion, age, sex and 3 categories of numbers of squamous cell carcinomas at inclusion of the study.
‡ Exploratory analyses.
§ Univariate analysis.

In the sirolimus arm, 8 RTRs (10.8%) withdrew consent after a median time of 3.5 months (range, 0.99 to 11.8 months) as compared with 11 (13.6%) after 8.4 months (range, 0.03 to 21.2 months) months in controls (Fig 1). As expected in stable RTRs receiving immunosuppression for a mean of 18 years, there were fewer treatment-related adverse events in the patients who continued their original immunosuppression. Two RTRs in

this group had a decline in renal function, which was not treatment related, after 10.6 and 12.8 months, respectively; one died after 16.1 months (cause unknown; Fig 1). In contrast, a total of 29 converted patients (39.1%) had to discontinue sirolimus because of adverse effects after a median time of 5.6 months (range, 0.69 to 18.0 months) or because of death resulting from a cerebrovascular accident after 5.9 months or from metastatic SCC after 6.6 months. One patient developed a borderline rejection with additional signs of chronic allograft nephropathy on a renal biopsy 6 months after conversion. After treatment, serum creatinine stabilized at 300 µmol/L (175 µmol/L at conversion). Other adverse effects are summarized in Table 3.

Table 3. Adverse events not leading to discontinuation of treatment

Adverse event	Patients converting to sirolimus	Patients continuing original immunosuppression
	(No.)	(No.)
Infection		
Respiratory	17	6
Urinary	3	4
Abdominal	2	1
Septicemia	1	1
Skin	14	9
Other	6	0
Other		
Pneumonitis	1	0
Proteinuria	5	0
Skin		
Rash	5	1
Acne	3	0
Diarrhea	7	0
Aphthous stomatitis	4	0
Flu-like symptoms	2	0
Fatigue	1	1
Edema	7	2
Deep venous thrombosis	1	1
New onset diabetes mellitus	0	1
Dyslipidemia (total cholesterol >7.8 mmol/L)	13	3
Thrombocytopenia (< 100,000/µL)	2	3
Leukopenia (< 4000/ µL)	11	5

Among the patients who finished the protocol on therapy in both treatment arms, serum creatinine did not change during the study period (control arm: start, 133 ± 49 µmol/L; end, 135 ± 51 µmol/L; sirolimus arm: start, 115 ± 38 µmol/L; end, 111 ± 37 µmol/L [± SD]). In addition, proteinuria did not change during the study period (control arm: start, 0.3 ± 0.2 g/d; end, 0.4 ± 0.6 g/d; sirolimus arm: start, 0.5 ± 1.4 g/d; end, 0.4 ± 0.4 g/d [[± SD]), although 11 of the included patients stopped using sirolimus mainly because

of proteinuria. Other laboratory investigations (cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, glucose, hemoglobin A1c, albumin, hemoglobin, leukocytes, and platelets) remained stable between start and end of the study.

DISCUSSION

Cutaneous invasive SCC is a serious complication of long-term organ transplantation, with significant morbidity, along with mortality rates similar to those seen with post-transplantation lymphoproliferative disease.[4] The primary analysis in this study showed that conversion to sirolimus-based immunosuppression resulted in a nonsignificant 24% reduced risk of a new cutaneous invasive SCC developing within 2 years after conversion. The exploratory analysis at 1 year resulted in a significant 50% risk reduction. There was a 49% reduction in risk of developing multiple invasive SCCs. Comparable risk reductions were observed for SCC in situ and BCC. The effect of conversion to sirolimus was especially eminent in patients with only one previous SCC at the time of conversion. These results are similar to the preliminary findings of a small pilot study from Germany, in which only one of 16 RTRs in the sirolimus conversion group compared with eight of 17 in the control group developed a skin cancer within 12 months of conversion.[26] This study included all forms of nonmelanoma skin cancer and did not require the participant to have had previous skin cancer. A study from Australia included 86 patients with either SCC or BCC.[25] There was a reduction in formation of new SCCs (most of which were SCC in situ) per year from 1.71 in the control group to 0.88 in the sirolimus group in the first year after conversion. The time since transplantation was much shorter (mean, 9.1 years), and skin cancers were mostly SCC in situ, not invasive SCC.[25] A recent study from France included 120 patients with SCC and found a 44% risk reduction of new SCCs at 2 years.[27] Efficacy was restricted to RTRs with only one previous SCC, and the difference remained significant at 2 years. The French study included more patients with only one SCC (55% v our 34%), and analysis occurred earlier after transplantation (12 v 18 years). In the first year after conversion, we observed a comparable benefit for the risk of recurrent SCC. The patient population included in our trial comprised a high-risk group for recurrent SCCs. In contrast to previous studies,[25,27] our 2-year results indicated that conversion to sirolimus did not prevent the occurrence of new SCCs. This conclusion is in line with observations that with time, new SCCs occur earlier and are more often multiple.[8-10] Duration of immunosuppressive therapy influences SCC risk, with compelling evidence for the carcinogenic mechanisms associated with cyclosporine[16-18] and azathioprine.[19] In addition, the impact of withdrawal of the calcineurin inhibitor and/or azathioprine may be more beneficial in patients earlier in their post-transplantation course (ie, those with only one SCC).

In our study the efficacy on skin cancer risk reduction is underestimated by the 42% discontinuation rate of sirolimus because of adverse effects. This is similar to the discontinuation rates of 35% in the Australian study[25] and 25% in the French study[27] and to rates observed in trials designed to investigate the nephron-sparing potential of mTOR inhibitors.[28,29] Several factors contributed to this high discontinuation rate. First, the protocol was designed in 2004 and included a loading dose (8mg) and initial maintenance dose (4mg), resulting in high levels in certain individuals with an increased risk of early toxicity. Subsequent studies, along with clinical practice, abandoned the loading dose and used lower maintenance doses and target levels. In addition, investigators in the early phase of this trial were concerned about the risk of secondary proteinuria and pneumonitis. The combination of high initial sirolimus levels and investigator caution led to early discontinuation of sirolimus in several participants with only mild to moderate proteinuria or other adverse effects who may have responded to dose adjustment and/or the addition of an angiotensin-converting enzyme inhibitor.[30] To prevent high initial sirolimus levels, we now recommend discontinuing the purine antagonist and/or calcineurin inhibitor and commencing sirolimus 2 to 3 mg once daily the next day. Alternatively, gradual conversion over weeks or months with lower initial dosing could result in a more tolerable regimen, but this carries the risk of drug-drug interactions, especially with cyclosporine.

There was no significant change in renal function or increase in proteinuria in the patients who continued sirolimus treatment, suggesting the safety of sirolimus conversion in RTRs many years post-transplantation. There were no deaths related to conversion, but six patients (8%) developed pneumonitis, which resolved with drug cessation (Fig 1); one patient with pneumonitis recovered despite continued treatment with sirolimus (Table 3). This serious complication is rare, but frequency may have been increased in this study as a result of initial higher sirolimus doses, because most cases occurred within a few weeks of sirolimus initiation.[28]

The incidence of skin malignancies in RTRs increases progressively with intensity and duration of immunosuppression and therefore with overall time since transplantation. Prospective cohort studies in transplant recipients have defined risk factors for skin cancer that are clinically robust, allowing reliable identification of patients at highest risk of future skin malignancies who may benefit from early conversion to sirolimus.[8,9] The results of our and other studies examining reduction of skin and nonskin malignancies suggest a benefit in early conversion to sirolimus-based maintenance regimen. Such a proactive rather than reactive policy carries the additional benefit of reducing calcineurin inhibitor-induced progressive loss of renal function, both in RTRs and non-renal organ transplant recipients.[31] In patients with proteinuria and/or already compromised renal allograft function, conversion to an mTOR inhibitor is no longer a valid option.[22]

There are limitations to our study. As experienced by others, recruitment of the patients was unexpectedly difficult. The randomization procedure was implemented correctly, but recruitment of only one or two patients in several centers, in combination with the fixed order per stratum, jeopardized balanced random assignment. In addition, there were differences between the groups regarding the use of azathioprine and/or cyclosporine. Azathioprine in particular has been associated with the more frequent occurrence of SCC and/or may indicate a longer time after transplantation. However, the differences between the groups in the current cohort were not statistically significant, and adjustment had no impact on the results of the analyses.

In conclusion, conversion to low-dose mTOR-inhibition with careful monitoring was not associated with increased risk of transplant dysfunction. However, in our study population, comprising patients with one or more previous SSCs, there was no benefit at 2 years in converting RTRs in terms of SCC-free survival. Conversion in those with only one previous SCC should be carefully balanced against toxicities that can lead to relatively high dropout rates. The benefit afforded by the mTOR-based regimen after conversion in the subgroup of patients with only one previous invasive SCC[27] may suggest that an mTOR inhibitor has the potential to become an effective early immunosuppressive strategy to reduce the risk of cutaneous SCC in RTRs.

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APPENDIX

Table A1. Risk reduction for developing ≥ one subsequent SCC after complete 2-year follow-up.

SCC	Patients converting to sirolimus (n = 74)		Patients continuing original immuno-suppression (n=81)				
	No.	%	No.	%	HR	95% CI	P
Intention to Treat							
Invasive SCC	37	50.0	46	56.8			
- No adjustment					0.73	0.47 to 1.13	0.157
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)					0.65	0.42 to 1.01	0.055
SCC in situ (Bowen's disease)	17	23.0	25	30.9			
- No adjustment					0.70	0.38 to 1.29	0.247
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)					0.51	0.32 to 1.15	0.125
Invasive SCC and SCC in situ (Bowen's disease)	41	55.4	53	65.4			
- No adjustment					0.67	0.45 to 1.01	0.057
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)					0.60	0.39 to 0.91	0.016
Per Protocol							
Invasive SCC	23	31.1	45	55.6			
- No adjustment					0.63	0.38 to 1.04	0.070
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)					0.54	0.32 to 0.91	0.020
SCC in situ (Bowen's disease)	8	10.8	24	29.6			
- No adjustment					0.46	0.21 to 1.03	0.058
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)					0.38	0.17 to 0.86	0.020
Invasive SCC and SCC in situ (Bowen's disease)	26	35.1	51	63.0			
- No adjustment					0.59	0.37 to 0.95	0.028
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)					0.51	0.31 to 0.83	0.006

Abbreviations: HR, hazard ratio; CI, confidence interval; SCC, squamous cell carcinoma.

Table A2. Risk reduction for developing multiple SSCs after complete 2-year follow-up.

SCC	Patients converting to sirolimus (n = 74)	Patients continuing original immunosuppression (n=81)	RR	95% CI	P
	No. of SCCs per year				
Intention to Treat					
Invasive SCC	0.82	1.38			
- No adjustment			0.60	0.35 to 1.02	0.057
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)			0.51	0.32 to 0.82	0.006
SCC in situ (Bowen's disease)	0.30	0.38			
- No adjustment			0.81	0.35 to 1.88	0.623
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)			0.80	0.42 to 1.90	0.770
Invasive SCC and SCC in situ (Bowen's disease)	1.13	1.77			
- No adjustment			0.64	0.37 to 1.09	0.099
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)			0.59	0.37 to 0.97	0.036
Per Protocol					
Invasive SCC	0.62	1.43			
- No adjustment			0.43	0.23 to 0.80	0.007
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)			0.38	0.23 to 0.63	<0.001
SCC in situ (Bowen's disease)	0.17	0.38			
- No adjustment			0.44	0.16 to 1.24	0.122
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)			0.47	0.17 to 1.30	0.148
Invasive SCC and SCC in situ (Bowen's disease)	0.78	1.81			
- No adjustment			0.43	0.24 to 0.77	0.004
- Adjustment for age and No. of SCCs before inclusion (1 to 9 v ≥10)			0.39	0.24 to 0.66	<0.001

Abbreviations: RR, relative risk; CI, confidence interval; SCC, squamous cell carcinoma.

Chapter 6

Urinary excretion of alpha-1-microglobulin and renal outcome in renal transplant recipients

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ABSTRACT

Background

Albuminuria is a marker of glomerular damage and a predictor of renal outcome in patients with kidney disease. However, deterioration of renal function is best correlated with tubulointerstitial injury. Urinary excretion of α_1-microglobulin reflects tubulointerstitial damage and improves prediction of outcome in patients with glomerular diseases. Until now, little is known about the value of α_1-microglobulin for predicting graft outcome in renal transplant patients.

Methods

In a cross sectional pilot study we evaluated α_1-microglobulin in four different categories of transplant recipients (HLA-compatible, never transplant rejection, with transplant rejection and with chronic allograft nephropathy). In a prospective study samples were collected at 3 and 12 months after transplantation of patients who received a renal transplant between 2006 and 2010. Urinary α_1-microglobulin, albumin, and IgG excretion were measured and related to progression of renal dysfunction.

Results

Cross sectional study: 41 patients were studied of which 14 received an HLA-compatible kidney. Urinary α_1-microglobulin excretion exceeded normal values in the majority of patients who had received an HLA compatible kidney transplant and had stable renal function. Urinary α_1-microglobulin excretion was higher in patients with rejection or chronic allograft nephropathy and correlated with eGFR ($r = -0.56$; $P < 0.001$).
Prospective study: 139 patients were studied. Of these, 34 patients had graft rejection within 3 months after transplantation. Median follow-up after renal transplantation was 48 months, eGFR increased by 0.6 ml/min/1.73m^2/yr during follow-up, 10 patients died and 3 patients developed graft failure. Urinary α_1-microglobulin excretion was above normal in 86% of patients at 3 months and in 78% of patients at 12 months after transplantation. Increased urinary α_1-microglobulin excretion at 12 months was associated with the type of donor, gender of the recipient, eGFR, albuminuria, α_1-microglobulin at 3 months and prior rejections. There was no correlation between urinary α_1-microglobulin excretion and the change in eGFR during follow-up.

Conclusions

The majority of patients after kidney transplantation has elevated levels of urinary α_1–microglobulin excretion at 3 and 12 months after transplantation. Urinary α_1-microglobulin does not predict renal outcome. Our data suggest that elevated urinary α_1-microglobulin reflects fibrosis and not active tubular cell injury.

INTRODUCTION

The number of patients requiring renal replacement therapy (RRT) because of end-stage renal disease (ESRD) is still increasing. In 2010, the overall incidence rate of RRT among all registries reporting to the ERA–EDTA Registry was 123 per million population (pmp). The overall prevalence of RRT among all registries reporting to the ERA–EDTA Registry was 741 pmp. Five-year survival rates on RRT are low, at only 46%.[1] Kidney transplantation is the preferred modality for renal replacement therapy, and graft loss due to acute rejection has been greatly reduced over the past few years. However, late graft loss remains an important problem and little progress has been achieved.[2] In the first ten years after transplantation, chronic allograft nephropathy (CAN) is the most prevalent cause of allograft dysfunction, though its pathogenesis remains elusive.[3] Already before clinically apparent kidney dysfunction, histological changes are present.[4] Although any anatomical compartment can be involved in CAN, interstitial accumulation of extracellular matrix in association with progressive tubular atrophy is mostly observed.[5]

Proteinuria is a common finding in kidney diseases. An increased excretion of proteins can result from a loss of glomerular barrier function, a decrease of the tubular protein reabsorption, or a combination of both. In general, glomerular injury is characterized by urinary losses of high molecular weight proteins such as albumin, and IgG. In contrast, tubular disorders are characterized by increased urinary losses of low-molecular weight proteins (LMWPs). Of the LMWPs α_1-microglobulin and β_2-microglobulin, it is known that they are readily filtered by the glomerulus and reabsorbed and catabolized by proximal tubular cells. When the proximal tubular handling of these LMWPs is disturbed, they appear in the urine.[6] LMWPs and IgG are valuable in predicting the progression of renal function decline in patients with proteinuric glomerular diseases such as focal segmental glomerulosclerosis or membranous nephropathy.[7,8] However, little is known about the possible value of measuring these proteins in the transplantation setting.

Therefore, in a pilot experiment we measured α_1-microglobulin and IgG in a cross sectional study. We subsequently measured α_1-microglobulin and IgG in a prospective cohort of kidney transplant patients at 3 and 12 months after kidney transplantation, and correlated the levels with graft loss, and progression of renal dysfunction.

MATERIALS AND METHODS

Population

We analysed the data of adult patients at the Department of Nephrology, Radboud University Nijmegen Medical Centre. All subjects gave informed consent according to Dutch ethical regulations

Cross sectional study:
This study was conducted between February and July 2006. The study included renal transplant recipients with a minimum follow-up of at least one year. We tried to include patients from different categories, i.e. HLA-compatible, never transplant rejection, with transplant rejection and with chronic allograft nephropathy. Urine and serum samples were obtained at one of their regular visits in our outpatient clinic. After a follow-up period of 8 years renal function, graft rejection and graft failure were studied.

Prospective study:
This study included patients who received a renal transplant between 2006 and 2010. Initial immunosuppression consisted of standard triple therapy with prednisone, mycophenolate, and a calcineurin inhibitor. Six months after transplantation mycophenolate was withdrawn in the majority of patients with the exception of patients with a higher rejection risk where triple therapy was continued. During the first year post transplantation, all patients visit our centre frequently according to protocol. Urine and serum samples of patients were obtained at 3 and 12 months post-transplantation. After the first year post transplantation renal function, graft rejection and graft failure was studied prospectively till 2014.

Laboratory Measurements

In the blood samples, we assessed creatinine, albumin, and IgG. In urine samples, we measured creatinine, albumin, α_1-microglobulin, and IgG. The concentrations of serum creatinine, urinary albumin, and urinary creatinine were determined with standard automated techniques. The concentrations of albumin, α_1-microglobulin, and IgG were measured by immunonephelometry on a BNII nephelometer (Behring, Marburg, Germany) using antibodies whose specificity was checked by Ouchterlony double immunodiffusion and immunoelectrophoresis (Dako, Glostrup, Denmark).

Calculations and definitions

Body mass index (BMI) was calculated as the ratio between weight and height squared. Mean arterial pressure was calculated as the diastolic pressure plus one third of the pulse pressure. The glomerular filtration rate (eGFR) was estimated using the abbreviated Modification of Diet in Renal Disease equation.[9] Change in kidney function was defined as eGFR at the last follow-up visit after renal transplantation subtracted by eGFR at 3 or 12 months after renal transplantation, divided by time from 3 or 12 months to last follow-up and expressed in ml/min/1.73m^2/yr. Graft failure was censored for death and defined as return to dialysis or re-transplantation.

Statistical analysis

Parameters between groups were compared using the Kruskal-Wallis test for nonparametric continuous data, independent t-test for parametric data and chi-square test for categorical data. Spearman's bivariate correlation test was used to examine correlations between nonparametric data. All values are given as mean (±SD) or median [interquartile range] where appropriate. All statistics were performed using SPSS software (IBM SPSS statistics 21). $P<0.05$ was considered significant.

RESULTS

Cross sectional study:

Between February and July 2006 we studied 41 renal transplant recipients with a minimum follow-up of at least one year. The characteristics of the patients are presented in table 1.

Patients with chronic allograft nephropathy excreted more albumin and IgG than other patient categories ($P<0.001$), reflecting the glomerular damage in chronic allograft nephropathy.

Urinary α_1-microglobulin excretion was higher in the patients with previous rejection or chronic allograft nephropathy and correlated with eGFR ($r = -0.56$; $P < 0.001$).

It was evident that patients who received an HLA-compatible living related donor kidney also showed more or less proteinuria. Fifty percent of patients who received an HLA-compatible kidney had a urinary α_1-microglobulin excretion exceeding 20 mg/10 mmol creatinine (normal value < 15 mg/10 mmol creatinine). These patients did not differ significantly from patients with urinary α_1-microglobulin excretion below 20 mg/10 mmol creatinine with respect to gender (both groups male:female 2:5), recipient age (48 in low vs 57 years in high urinary α_1-microglobulin; $P=0.08$), mean arterial blood pressure (107 in low vs 103 mmHg in high urinary α_1-microglobulin; $P=0.54$), eGFR (57 in low vs 65 ml/min/1.73m^2 in high urinary α_1-microglobulin; $P=0.48$), years after

transplantation (9.6 in low vs 9.0 years in high urinary α_1-microglobulin; P=0.87), and donor age (38 in low vs 45 years in high urinary α_1-microglobulin; P=0.22). All patients who received an HLA-compatible kidney had a stable renal function in the year before the measurements. Nobody developed transplant rejection in 8 years follow-up. Four patients died with a functional graft within 8 years follow-up. The remaining patients all kept a stable renal function in 8 years follow-up. Even the patient with the highest urinary α_1-microglobulin excretion of 82 mg/10mmol creatinine had a stable renal function 8 years after the measurement took place (serum creatinine 109 µmol/l).

Table 1. Urinary proteinuria after renal transplantation in different patient categories (cross sectional data).

	HLA-compatible	Never transplant rejection	Transplant rejection	Chronic allograft nephropathy
No. of patients	14	10	9	8
Age (years)	53 ± 10	51 ± 20	42 ± 16	49 ± 12
Gender (male:female)	4:10	6:4	8:1	5:3
Mean arterial pressure (mmHg)	104 ± 13	98 ± 9	103 ± 7	104 ± 7
Amount of antihypertensiva	1.5 ± 1.0	2.4 ± 0.9	2.0 ± 1.0	2.6 ± 1.3
Immunosuppression:				
- Calcineurin inhibitor and steroids	2	6	6	0
- Azathioprine/MMF and steroids	12	4	3	8
Donor:				
- Age (years)	41 ± 11	53 ± 13	43 ± 18	39 ± 19
- Gender (male:female)	5:9	7:3	4:5	4:4
- Type (living:postmortal)	14:0	4:6	5:4	3:5
Serum creatinine (µmol/l)	104 ± 29	152 ± 47	187 ± 72	163 ± 79
eGFR MDRD (ml/min/1.73m^2)	61 ± 19	44 ± 13	41 ± 14	48 ± 26
Albuminuria (mg/10 mmol creatinine)	35 [8-77]	9 [7-66]	164 [37-464]	1700 [397-696]
Urinary α-1-microglobulin (mg/10 mmol creatinine)	17 [8-24]	38 [7-78]	62 [17-96]	68 [10-149]
Urinary IgG (mg/10 mmol creatinine)	4.0 [2.0-9.0]	4.8 [2.0-11.1]	20.4 [7.8-24.8]	92.6 [24.4-82.1]
Time after transplantation (years)	9.9 [3.8-11.6]	4.5 [3.5-10.7]	7.5 [3.1-14.2]	9.8 [6.9-13.9]

Data are depicted as mean±standard deviation or median [interquartile range] where appropriate.

In the patient category without transplant rejection (not HLA compatible and no CAN) urinary α_1-microglobulin was elevated >35 mg/10 mmol creatinine in six patients. None of these six patients kept a stable renal function in 8 years follow-up. In three of these six patients eGFR was already decreasing in the year prior to the measurement. The other four patients in this category had a α_1-microglobulin excretion < 10 mg/10mmol creatinine of which three patients kept a stable renal function during 4 years follow-up. In the fourth patient renal function deteriorated slowly, probably due to calcineurin

inhibitor toxicity. In this patient category without earlier transplant rejection urinary IgG was significantly correlated with albuminuria (r=0.90, P<0.01) and α1-microglobulin excretion (r=0.68, P=0.03). α_1-microglobulin excretion significantly correlated with eGFR (r=-0.86, P<0.01).

In the patient category with prior transplant rejection the two patients with urinary α_1-microglobulin < 10 mg/10mmol creatinine kept a stable renal function during follow-up. Retrospectively five patients with urinary α_1-microglobulin > 50 mg/10mmol creatinine already had albuminuria at the time measurements took place. In all these patients renal function deteriorated during follow-up. In this patient category with prior transplant rejection albuminuria was significantly correlated with urinary IgG (r=0.94, P<0.01) and α1-microglobulin excretion (r=0.79, P=0.04).

In the patient category with chronic allograft nephropathy the three patients with urinary α_1-microglobulin < 15 mg/10mmol creatinine kept a stable renal function during follow-up and in all of the four patients with α_1-microglobulin > 80 mg/10mmol creatinine renal function deteriorated. In this patient category with chronic allograft nephropathy urinary IgG was significantly correlated with albuminuria (r=0.96, P<0.01) and α_1-microglobulin excretion significantly correlated with eGFR (r=-0.81, P=0.02).

Prospective study:

We evaluated 139 patients who received a kidney transplant in the Radboud University Medical Centre between January 2006 and February 2010 and had urine samples available at 3 and 12 months. The characteristics of the patients are presented in table 2. Initial immunosuppression consisted of standard triple therapy with prednisone, mycophenolate and a calcineurin inhibitor. Three months after transplantation 100% of the patients were on a calcineurin inhibitor and one year after transplantation in 93% of patients a calcineurin inhibitor was part of the immune suppressive regimen. Median follow-up after renal transplantation was 48 months. Thirty-six patients suffered rejection, which occurred in the majority (n=34) within 3 months after transplantation. At 3 months patients who had developed graft rejection within 3 months after transplantation did differ significantly from patients without graft rejection with respect to albuminuria (43 [18-146] versus 38 [13-75] mg/10 mmol creatinine; P=0.021) and urinary α_1-microglobulin (55 [22-101] versus 38 [22-59] mg/10 mmol creatinine; P=0.003).

Table 2. Characteristics of patients in the prospective study.

No. of patients (% male)	139 (61)
Age at transplantation (yr)	47 ± 14
BMI 3 months after transplantation (kg/m^2)	25 ± 4
MAP 3 months after transplantation (mmHg)	99 ± 10
Calcineurin inhibitors (%)	
- 3 months	100
- 12 months	93
- Last FU	79
eGFR (ml/min/1.73 m^2)	
- 3 months	53 ± 16
- 12 months	50 ± 15
Albuminuria (mg/10 mmol creatinine)	
- 3 months	38 [14-83]
- 12 months	32 [11-85]
Urine α_1-microglobulin (mg/10 mmol creatinine)	
- 3 months	40 [23-65]
- 12 months	30 [16-50]
Urinary IgG (mg/10 mmol creatinine)	
- 3 months	7.1 [4.2-11.1]
- 12 months	8.4 [4.3-15.8]
Rejection (n (%))	36 (15.9)
- within 3 months after tx	34 (24.5)
- after	2 (1.4)
Time to rejection (months)	0.3 [0.2-0.8]
Graft failure at max FU (n (%))	3 (2.2)
- recurrence (n (%))	1 (0.7)
- transplant rejection (n (%))	1 (0.7)
- unknown (n (%))	1 (0.7)
Death with functioning graft (n (%))	10 (7.1)
Time to patient or graft failure (months)	40 [29-47]
Change eGFR 3-12 months (ml/min/1.73m^2)	-2.9 [-8.7-3.4]
Change eGFR 3 months-last FU(ml/min/1.73m^2/yr)	-0.2 [-3.3-2.3]
Change eGFR 12 months-last FU(ml/min/1.73m^2/yr)	0.6 [-2.0-3.5]
Follow-up (months)	48 [36-48]

Data are depicted as mean±standard deviation or median [interquartile range] where appropriate.
Abbreviations: BMI, body mass index; MAP, mean arterial pressure; FU, follow-up.

Urinary α_1-microglobulin excretion was above 15 mg/10 mmol creatinine in 86% of patients at three months and in 78% of patients at twelve months after transplantation. Increased urinary α_1-microglobulin at twelve months was associated with the type of donor, gender of the recipient, eGFR, albuminuria, α_1-microglobulin at 3 months and prior rejections (Table 3). Median change in eGFR between 12 months and end of follow-up was 0.6 ml/min/1.73m^2/yr, indicating that renal function remained relatively stable during follow-up.

Table 3. Urinary α_1-microglobulin per quartile at 12 months after renal transplantation

	α_1-microglobulin ratio (mg/10 mmol creatinine) at 12 months				
	2.7-15.6 (n=35)	15.6-29.4 (n=35)	29.4-50.0 (n=34)	50.0-311.9 (n=35)	P
Postmortal donor (n (%))	12 (34)	15 (43)	8 (24)	20 (57)	0.03
Gender (male:female)	17:18	18:17	24:10	26:9	0.06
Recipient age at transplantation (yr)	47 ± 14	45 ± 13	45 ± 14	49 ± 14	0.36
Calcineurin inhibitors (%)					
- 3 months	100	100	100	100	
- 12 months	100	94	91	86	0.13
- Last follow-up	63	49	62	43	0.25
Smoking during follow-up (n (%))	1 (3)	2 (6)	3 (9)	9 (26)	0.04
eGFR (ml/min/1.73 m^2)					
- 3 months	58 ± 16	50 ± 16	47 ± 12	43 ± 13	<0.01
- 12 months	60 ± 15	50 ± 15	47 ± 12	44 ± 1	<0.01
- Last follow-up	61 ± 18	53 ± 15	49 ± 16	46 ± 16	<0.01
Albuminuria (mg/10 mmol creatinine)					
- 3 months	28 [10-51]	39 [12-81]	47 [27-115]	43 [16-94]	0.22
- 12 months	15 [5-41]	28 [9-89]	34 [17-117]	60 [17-177]	0.08
α_1-microglobulin (mg/10 mmol creatinine)					
- 3 months	17 [12-38]	28 [23-45]	46 [34-63]	79 [53-135]	<0.01
- 12 months	10 [6-14]	22 [21-28]	38 [33-43]	75 [55-113]	<0.01
- Delta 3-12 months	-9[-27- - 2]	-5 [-27-0]	-6 [-26-2]	-1 [-35-29]	0.35
Rejection (n (%)	6 (17)	7 (20)	8 (24)	15 (43)	0.06
- < 3 months after transplantation	6 (17)	6 (17)	8 (24)	14 (40)	0.09
- > 3 months after transplantation	0 (0)	1 (3)	0 (0)	1 (3)	0.57
Graft failure at max follow-up (n (%))	1 (3)	0 (0)	0 (0)	2 (6)	0.30
Death with functioning graft (n (%))	2 (6)	0 (0)	5 (15)	3 (9)	0.12
Change eGFR					
- 3 -12 months (ml/min/1.73m^2)	-2.9 [-9.1-3.2]	-3.9 [-10.0- -1.4]	-2.1 [-6.9-3.8]	-2.6 [-8.4-4.5]	0.97
- 3 months-last follow-up (ml/min/1.73m^2/yr)	-0.9 [-3.6-2.8]	-1.0 [-3.3-2.1]	-0.3 [-3.3-2.3]	0.9 [-3.0-2.9]	0.94
- 12 months-last follow-up (ml/min/1.73m^2/yr)	1.0 [-1.4-6.0]	0.2 [-2.3-3.6]	0.3 [-3.0-2.5]	0.4 [-0.9-4.0]	0.63
Follow-up (months)	36 [36-48]	48 [36-60]	36 [27-48]	48 [36-48]	0.10

Data are depicted as mean ± standard deviation or median [interquartile range] where appropriate.

Three patients (2%) developed graft failure and 10 patients (7%) died with a functioning graft. Median time to patient or graft failure was 40 months. There was no association between urinary α_1-microglobulin and change in eGFR during follow-up. Because only three patients developed graft failure nothing can be concluded about the predictive value of α_1-microglobulin on graft failure.

In a subgroup of 68 patients with α_1-microglobulin > 30mg/10mmol creatinine at 12 months after transplantation renal function deteriorated in 31 patients and improved in 37 patients. Patients with renal function deterioration did differ significantly from patients with renal function improvement with respect to albuminuria (77 [21-321] versus 35 [16-68] mg/10 mmol creatinine; P=0.019).

DISCUSSION

In the cross sectional study micro-albuminuria was noted in most patients. As expected, patients with chronic allograft nephropathy and to a lesser extent patient with earlier transplant rejection showed higher levels of urinary albumin and IgG, reflecting glomerular damage. This observation seems to be very reliable because urinary IgG was very well correlated with albuminuria in these patient categories.

Not unexpectedly, these patients also excreted larger amounts of α_1-microglobulin, compatible with the known tubulointerstitial scarring that is observed in biopsies of patients with chronic allograft nephropathy. Of note, renal function deteriorated in most patients, with the exception of the 5 patients with normal α_1-microglobulin excretion. Our hypothesis that urine α_1-microglobulin excretion could be a predictor of outcome was further supported by the observation that in patients without a previous rejection increased α_1-microglobulin excretion predicted an unfavourable outcome. However, increased α_1-microglobulin excretion can also reflect earlier acquired damage, because in some patients eGFR was already decreasing prior to the measurement.

Surprisingly, 50% of patients who received an HLA-compatible living related kidney also showed urinary α_1-microglobulin excretion exceeding 20 mg/10 mmol creatinine. In this category, even in patients with the highest values of urinary α_1-microglobulin, renal function did not deteriorate during follow-up. These findings suggest that higher values of urinary α_1-microglobulin can sometimes be accepted as "normal" after renal transplantation and do not necessarily reflect active, progressive tubulointerstitial injury.

In view of these somewhat contrasting results, and the limitations of a cross sectional study, we planned a prospective study to evaluate the potential of urinary α_1-microglobulin and IgG for predicting outcome after kidney transplantation. Most importantly, we found no significant correlation between α_1-microglobulin at 3 or 12 months and future graft failure or change in eGFR during follow-up. This apparent lack of predictive power might relate at least in part to limitations of the study, in particular the small number of events (only three graft failures) and the relatively short follow-up period with relatively stable renal function.

Although we included quite a large amount of patients, our study may be criticized because we collected urine samples at three and twelve months after transplantation in only 30% of all patients transplanted between 2006-2010. The study was done during routine patient care. This caused missing data due to patient and physician incompliance. This might have caused some selection bias. Patients with graft failure within one year after transplantation were also not studied, because we only included patients where we collected urine at 3 and 12 months after transplantation. However we intended to investigate the long-term value of α_1-microglobulin on renal function, future transplant rejection and graft failure and did not want to find a marker for already existing renal damage. Unfortunately, we were not able to draw any conclusion on the prognostic value of α_1-microglobulin on graft failure because of the very limited events that took place in the relatively short follow-up. Renal function remained relatively stable during follow-up with a small improvement in median eGFR between 12 months and end of follow-up of 0.6 ml/min/1.73m^2/yr. Our study might have been underpowered to proof an existing association between urinary α_1-microglobulin and change in eGFR during follow-up, because in only 19 patients (14%) eGFR decreased more than 3 ml/min/1.73m^2/yr.

Urinary α_1-microglobulin excretion was above normal in 86% of patients at three months and in 78% of patients at twelve months after transplantation. Increased urinary α_1-microglobulin at twelve months was associated with the type of donor, gender of the recipient, eGFR, albuminuria and prior rejections. This suggests that in transplanted patients an elevated urinary α_1-microglobulin reflects fibrosis and not active tubular cell injury. How can we explain that urinary α_1-microglobulin do predict renal failure in glomerular diseases, especially membranous nephropathy, but don't predict renal failure in transplanted patients? We propose that increased α_1-microglobulin excretion in glomerular diseases is more a reflection of ongoing tubulointerstitial inflammation instead of fibrosis.

Whether elevated urinary α_1-microglobulin in transplanted patients might have implications for progression and outcome after longer observation periods will have to be explored in future studies with extended follow-up.

In conclusion the majority of patients after kidney transplantation has elevated levels of urinary α_1-microglobulin at 3 and 12 months after transplantation. In the short term, urinary α_1-microglobulin and IgG do not predict renal outcome in this relatively limited exploratory study. Our data suggest that elevated urinary α_1-microglobulin reflects fibrosis and not active tubular cell injury. However, the value of α_1-microglobulin and IgG in the setting of renal transplantation warrants a larger study involving more events and longer follow-up.

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Chapter 7

Urinary biomarkers after donor nephrectomy

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ABSTRACT

Introduction. As the beginning of living-donor kidney transplantation, physicians have expressed concern about the possibility that unilateral nephrectomy can be harmful to a healthy individual. To investigate whether the elevated intra-abdominal pressure (IAP) during laparoscopic donor nephrectomy causes early damage to the remaining kidney, we evaluated urine biomarkers after laparoscopic donor nephrectomy.

Methods. We measured albumin and alpha-1-microglobulin (α-1-MGB) in urine samples collected during and after open and laparoscopic donor nephrectomy and laparoscopic cholecystectomy and colectomy. Additionally, kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were measured in urine samples collected during and after laparoscopic donor nephrectomy and colectomy. The same biomarkers were studied in patients randomly assigned to standard or low IAP during laparoscopic donor nephrectomy.

Results. We observed a peak in urinary albumin excretion during all procedures. Urine α-1-MGB rose in the postoperative period with a peak on the third postoperative day after donor nephrectomy. Urine α-1-MGB did not increase after laparoscopic cholecystectomy and colectomy. After laparoscopic nephrectomy, we observed slight increases in urine KIM-1 during surgery and in urine NGAL at day 2-3 after the procedure. After laparoscopic colectomy, both KIM-1 and NGAL were increased in the postoperative period. There were no differences between the high- and low-pressure procedure.

Conclusion. Elevated urinary α-1-MGB suggests kidney damage after donor nephrectomy, occurring irrespective of IAP during the laparoscopic procedure.

INTRODUCTION

Kidney transplantation remains the treatment of choice for patients with end-stage renal disease (ESRD). However, the gap between the demand and supply of deceased donor kidneys continues to grow. Living-donor programs have gradually become an attractive strategy to expand the donor pool for kidney transplantation. Grafts from living-related donors display superior function and longer survival than those obtained from deceased donors.[1,2] Living-donor surgery has changed radically in the past decade as laparoscopic techniques have supplanted open nephrectomy. In a meta-analysis, laparoscopic donor nephrectomy was found to be associated with reduced analgetic consumption, shorter hospital stay, and faster return to normal physical functioning as compared to the open technique.[3] As the beginning of living-donor kidney transplantation, physicians have expressed concern about the possibility that unilateral nephrectomy can be harmful to a healthy individual although survival and the risk of ESRD in carefully screened kidney donors appear to be similar to those in the general population.[4] To monitor renal problems after donor nephrectomy, we closely monitored the urine of the donor after donor nephrectomy.

We observed increased levels of urinary alpha-1-microglobulin (α-1-MGB), a marker of renal tubular dysfunction, in several patients after laparoscopic donor nephrectomy. This suggested that the procedure may cause some harm to the remaining kidney. It is well known that the kidney is especially vulnerable to the effects of intra-abdominal pressure (IAP) due to its anatomical position and blood supply. It is now also accepted that the adverse effects of elevated IAP on renal function can occur at lower levels of IAP, long before development of overt abdominal compartment syndrome.[5] To investigate whether the IAP during laparoscopic donor nephrectomy causes early damage to the remaining kidney, we evaluated urine biomarkers after laparoscopic donor nephrectomy in two studies.

PATIENTS AND METHODS

Study 1

Patients

Between January 2010 and July 2011, we included 10 donors who underwent a laparoscopic donor nephrectomy at the Radboud University Medical Centre. For comparison, we studied nine patients who underwent a laparoscopic cholecystectomy in the Isala Zwolle, and 12 donors who underwent an open donor nephrectomy in the University Medical Centre Utrecht. Inclusion criteria were as follows: age>18 years and estimated GFR>60 ml/min/1.73m^2 (abbreviated MDRD formula). Exclusion criteria were

as follows: blood pressure>160/90 mmHg and/or use of antihypertensive medication and/or the presence of (micro) albuminuria before kidney donation.
Between July and September 2014, we additionally included eight patients who underwent a laparoscopic colectomy as a second control group, because of the short duration of laparoscopic cholecystectomy in comparison with laparoscopic nephrectomy.

Procedure

In all patients, a urinary catheter was inserted directly preoperatively. In laparoscopic procedures, IAP during the period of pneumoperitoneum was maintained at 12-14 mmHg. A combination of remifentanil, fentanyl, sufentanil, propofol, and rocuronium was used for anesthesia.
Urine samples were obtained preoperative, at start operation (after insertion of the urinary catheter), directly postoperative (all urine produced during the procedure), on the first, second, and third postoperative day and finally at about 4-6 weeks postoperatively.

Outcome measures

Serum creatinine was measured at baseline (day 0) and postoperative at day 1, 2, 3, and at about 4-6 weeks postoperatively. Urine samples were centrifuged and urine supernatants were stored in 1 ml aliquots at -80 ^{o}C until further use. Creatinine, albumin and α-1-MGB were measured in all collected urine samples. After analysis of all data, frozen-stored urine samples of the laparoscopic procedures were used for measurement of kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL).
Patients provided informed consent. The local ethical committee waived the requirement for formal approval of this study.

Study 2

Patients

The second study was performed using stored urine samples obtained from 20 donors who participated in the recent Leopard study.[6] In summary, this randomized, double-blinded study included 20 donors undergoing a laparoscopic donor nephrectomy between September 2011 and January 2012 at the Radboud University Medical Center. This study investigated the effect of low-pressure pneumoperitoneum (7 mmHg) versus normal pressure pneumoperitoneum (14 mmHg) on donors' comfort [6]. All Dutch speaking individuals who were medically suitable for donation were eligible. Exclusion criteria were a history of kidney or adrenal gland surgery at the side of donation.

Potential participants were informed about the study at the outpatient clinic. Informed consent was mandatory for inclusion. Approval for this study was given by the Central Committee on Research involving Human Subjects of the Radboud University Medical Centre Nijmegen and has therefore been performed in accordance with the ethical standards laid down in an appropriate version of the 2000 Declaration of Helsinki as well as the Declaration of Istanbul 2008.

Procedure

Randomization using a single block was used to create 20 sealed envelopes numbered from 1 to 20, containing instructions for the setting of the carbon dioxide inflator, at, respectively 7 mmHg for the experimental and 14 mmHg for the control arm. Prior to the operation a nurse who was not involved in the operation installed the pneumoperitoneum pressure. All other personnel were blinded for the allocation of treatment.

General anesthesia was induced using a standardized protocol, which included remifentanil, propofol, and rocuronium. Further details concerning the randomization, operation, and anesthesia procedures were described earlier.[6]

Urine samples were taken preoperatively (day -1), directly postoperatively (urine produced during the procedure from insertion of a urinary catheter till closure), the first, second, and third postoperative day, and finally 1 week postoperative. All samples were centrifuged and urine supernatants were stored in 1 ml aliquots at -80 ºC until further use. No urine samples were obtained directly preoperatively (after insertion of a urinary catheter) in this study.

Outcome measures

Donor serum creatinine was measured at baseline (day -1) and postoperative at day 1, 2, and 7. Urinary output and saline infusion were also registered carefully in the periods from first incision to insufflation, from insufflation to desufflation (pneumoperitoneum phase), and from desufflation to closure.

Creatinine, albumin, α-1-MGB, KIM-1, and NGAL were measured in all collected urine samples. All outcome assessments were performed on coded samples by investigators who were blinded for the treatment group to which each sample belonged.

Statistical analysis

Parameters between groups were compared using independent t-test or ANOVA for parametric continuous data. Mann-Whitney U-test or Kruskal-Wallis test was used for nonparametric continuous data. Chi-square test was used for categorical data. Parameters within groups were compared using related-samples Wilcoxon's signed-rank

test. Categorical variables are presented as numbers and continuous variables as mean values (± standard deviation) or median values [range]. P-values <0.05 were considered significant. Data on urinary biomarkers are presented in figures as median values. To test significance of changes between groups, a quadratic mixed model analysis was used. All statistics were performed using SPSS software, version 20.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Study 1

Patients' characteristics

Patients' characteristics and outcome measures are presented in Table 1. The average time of elevated IAP was 34 ± 19 min in laparoscopic cholecystectomy compared with 99 ± 19 min in laparoscopic donor nephrectomy ($p < 0.001$). Most baseline characteristics of patients in the laparoscopic colectomy group, including the average time of elevated IAP of 86 ± 35 min, were comparable with the characteristics of the patients in the laparoscopic donor nephrectomy group, with the exception of older age ($p = 0.026$) and higher ASA classification ($p = 0.023$) in the colectomy group.

Urinary biomarkers

Urinary biomarkers are presented in Table 1 and Figs 1a, 2a,3a and 4a. Increased levels of urine albumin were observed in the urine samples collected during the procedure in the open donor nephrectomy, laparoscopic donor nephrectomy as well as in laparoscopic cholecystectomy and colectomy group (Fig. 1a). Albuminuria disappeared rapidly after the procedure.

Urine α-1-MGB followed a different time course. We observed a significant increase of urine α-1-MGB after laparoscopic donor nephrectomy, with a peak on the third postoperative day (Fig. 2a). A similar although more variable pattern was observed in donors after open nephrectomy. In contrast, in patients who underwent a laparoscopic cholecystectomy or colectomy urine α-1-MGB was not significantly elevated in the postoperative period.

In view of these discordances, we analysed the recently identified urinary biomarkers KIM-1 and NGAL in the stored urine samples of patients in the laparoscopic nephrectomy and laparoscopic colectomy group. Remarkably, after laparoscopic nephrectomy, urinary excretion of KIM-1 increased slightly in the perioperative period, in parallel with the albuminuria. In contrast, urine KIM-1 increased more after laparoscopic colectomy and exceeded normal values in the postoperative period. After laparoscopic nephrectomy urine NGAL followed the pattern of α-1-MGB with a peak in excretion on

the 2nd or 3rd postoperative day. Similarly, variable increases in urine NGAL were noted after colectomy (Figs 3a and 4a).
There was no relationship between the duration of IAP during laparoscopic donor nephrectomy and the amount of urinary α-1-MGB excretion in the postoperative period. Urinary α-1-MGB excretion did not correlate with postoperative rise in serum creatinine after laparoscopic donor nephrectomy. There was no correlation between urine α-1-MGB and urine NGAL.

Study 2

Main outcome data have been reported.[6]

Patients' characteristics

Patients' characteristics and outcome measures are presented in Table 2. Operation time and time of elevated IAP were significantly longer in patients operated with low-pressure pneumoperitoneum compared with normal pressure pneumoperitoneum. Patients operated with low-pressure pneumoperitoneum showed a higher urine output during the pneumoperitoneum phase compared with normal pressure pneumoperitoneum ($p = 0.041$).

Urinary biomarkers

Urinary biomarkers are presented in Table 2 and Figs 1b, 2b, 3b and 4b. The data closely resembled the results of study 1. We observed a significant increase in urinary albumin excretion during the procedure, whereas urine α-1-MGB was not increased during surgery, but rose significantly in the postoperative period with a peak on the third postoperative day (Figs 1b and 2b). Urine KIM-1 paralleled albumin, whereas NGAL followed the same pattern as α-1-MGB.
There were no significant differences between the high- and low-pressure procedure (Figs 1b, 2b, 3b and 4b).

Table 1. Baseline characteristics and outcome measures study 1.

	Laparoscopic cholecystectomy (n=9)	Laparoscopic colectomy (n=8)	Laparoscopic nephrectomy (n=10)	Open Nephrectomy (n=12)	P-value (between groups)
Age (years)	46.6 (16.4)	65.7 (8.9)	55.6 (8.1)	47.5 (11.3)	0.005
Male gender	3	4	6	3	ns
BMI (kg/m^2)	26.4 (3.7)	28.3 (5.5)	25.3 (3.3)	26.7 (2.5)	ns
Serum creatinine (µmol/l)	66 (12)	78 (14)	69 (10)	78 (9)*	ns
ASA classification†					
I (healthy)	9	4	10	12	<0.001
II or III	0	4	0	0	
Operation length (min)					
Skin-to-skin time	61 (18)	132 (57)	136 (22)	179 (26)	<0.001
Pneumoperitoneum	34 (19)	86 (35)	99 (19)	-	<0.001
Mean arterial pressure					
During operation	75 (11)	77 (4)	79 (11)	71 (6)	ns
Urine output (ml/h)					
During operation	121 (81)	101 (94)	55 (29)	56 (52)	ns
Infusion volume (ml/h)					
During operation	1098 (387)	459 (318)	485 (224)	776 (185)	<0.001
Serum creatinine (µmol/l)					
4-6 weeks postoperatively	75 (17)	n.d.	106 (18)	113 (17)	<0.001
Albuminuria (mg/mmol creatinine) ‡					
Preoperative	n.d.	0.5 [0.2-8.8]	0.9 [0.4-2.1]	0.3 [0.2-5.0]	ns
Start operation	0.7 [0.0-2.9]	1.3 [0.4-14.0]	1.4 [0.3-4.2]	0.5 [0.2-21.6]	ns
During operation	3.0 [0.2-31.2]#	9.2 [2.3-18.6]	10.7 [5.5-39.9]#	3.4 [0.9-25.0]#	ns
First postoperative day	0.6 [0.3-6.3]	2.0 [1.0-14.1]	2.6 [0.5-7.9]#	1.6 [0.4-6.8]	0.041
Second postoperative day	n.d.	3.1 [1.2-5.2]	4.5 [0.7-13.5]#	1.9 [0.3-8.4]	ns
Third postoperative day	0.7 [0.3-1.3]	1.4 [0.6-5.8]	2.1 [0.6-22.4]	2.2 [0.9-13.0]	0.013
4-6 weeks postoperative	0.6 [0.3-2.0]	0.9 [0.4-2.0]	0.9 [0.5-2.8]	0.7 [0.2-3.5]	ns

Table 1. Baseline characteristics and outcome measures study 1. *(Continued)*

	Laparoscopic cholecystectomy (n=9)	Laparoscopic colectomy (n=8)	Laparoscopic nephrectomy (n=10)	Open Nephrectomy (n=12)	P-value (between groups)
Urinary α-1-MGB (mg/mmol) §					
Preoperative	n.d.	0.3 [0.2-3.1]	0.7 [0.3-1.6]	0.6 [0.1-2.5]	ns
Start operation	0.6 [0.4-2.6]	1.1 [0.1-2.3]	0.3 [0.1-1.0]	0.3 [0.1-6.3]	ns
During operation	0.6 [0.2-1.4]	0.9 [0.1-2.6]	0.4 [0.1-0.9]	0.8 [0.2-8.6]	ns
First postoperative day	0.3 [0.2-1.0]	0.7 [0.2-3.3]	7.6 [3.5-12.1]#	5.6 [1.9-16.4]#	<0.001
Second postoperative day	n.d.	1.9 [0.4-10.6]	10.5 [5.3-19.1]#	7.2 [1.7-25.5]#	0.003
Third postoperative day	0.4 [0.2-1.2]	2.1 [0.1-9.4]	11.2 [3.1-13.9]#	7.2 [3.0-21.6]#	<0.001
4-6 weeks postoperative	0.4 [0.2-0.9]	0.4 [0.3-2.3]	2.3 [1.2-5.9]#	2.9 [0.9-17.5]	0.001
KIM-1 (µg/mmol creatinine) ¶					
Preoperative	n.d.	0.16 [0.08-0.33]	0.02 [0.00-0.09]	n.d.	<0.001
Start operation	n.d.	0.03 [0.00-0.38]	0.08 [0.00-0.15]	n.d.	ns
During operation	n.d.	0.23 [0.04-0.73]#	0.08 [0.00-0.28]	n.d.	ns
First postoperative day	n.d.	0.24 [0.07-1.51]#	0.03 [0.00-0.20]	n.d.	0.003
Second postoperative day	n.d.	0.86 [0.02-4.93]#	0.05 [0.00-0.17]	n.d.	0.001
Third postoperative day	n.d.	0.23 [0.00-0.44]#	0.04 [0.00-0.17]	n.d.	0.012
4-6 weeks postoperative	n.d.	0.10 [0.03-0.31]	0.00 [0.00-0.05]	n.d.	0.005
NGAL (µg/mmol creatinine) ¶					
Preoperative	n.d.	0.7 [0.0-3.5]	0.9 [0.0-3.2]	n.d.	ns
Start operation	n.d.	0.5 [0.0-2.1]	0.3 [0.0-3.3]	n.d.	ns
During operation	n.d.	0.2 [0.0-12.0]	0.0 [0.0-3.3]	n.d.	ns
First postperative day	n.d.	0.0 [0.0-2.6]	1.5 [0.0-4.9]#	n.d.	ns
Second postoperative day	n.d.	5.2 [0.0-19.4]#	2.6 [0.0-18.2]#	n.d.	ns
Third postoperative day	n.d.	1.6 [0.0-27.1]#	1.8 [0.8-7.3]#	n.d.	ns
4-6 weeks postoperative	n.d.	1.7 [0.0-2.3]	0.7 [0.0-20.5]	n.d.	ns

Categorical variables are presented as numbers and continuous variables as mean values (standard deviation) or median values [range]. n.d., not determined; KIM-1, kidney injury molecule 1; α-1-MGB, alpha-1-microglobulin; NGAL, neutrophil gelatinase-associated lipocalin.

* Serum creatinine was imbalanced between groups due to differences in determination method (Jaffe method in open nephrectomy group).

†American Society of Anesthesiologists classification.

In some cases, the urine sample for a particular time point could not be collected due to insufficient urine production during surgery, or because it was impossible to collect a urine sample on a specific postoperative day: ‡ 24 of 225 samples missing; § 29 of 225 samples missing; ¶ 4 of 108 samples missing.

# p< 0.05 compared with start operation.

Table 2. Baseline characteristics and outcome measures study 2.

	Standard pressure (n=10)	Low-pressure (n=10)	P-value
Age (years)	50.7 (8.9)	51.6 (10.2)	ns
Male gender	3	7	ns
BMI (kg/m^2)	24.9 (2.5)	25.7 (3.9)	ns
Serum creatinine (µmol/l)	64 (13)	70 (14)	ns
ASA classification*			
I (healthy)	10	10	ns
II or III	0	0	
Operation length (min)			
Skin-to-skin time	111 (19)	149 (86)	0.003
Pneumoperitoneum	86 (16)	126 (27)	0.001
Mean arterial pressure			
Pneumoperitoneum phase	73 (8)	76 (9)	ns
Urine output (ml/h)			
Pneumoperitoneum phase	11 (20)	23 (35)	0.041
Infusion volume (ml/h)			
Pneumoperitoneum phase	746 (253)	693 (134)	ns
Donor eGFR (ml/min/1.73 m^2)			
Postoperative week 1	56.3 (10.6)	56.0 (10.4)	ns
Albuminuria (mg/mmol creatinine)†			
Preoperative	1.0 [0.3-2.4]	1.4 [0.2-4.7]	ns
During operation	13.6 [8.2-95]#	17.3 [10.0-43.0]#	ns
First postoperative day	1.4 [0.4-5.8]	2.0 [0.2-35.4]	ns
Second postoperative day	2.4 [0.7-8.5]	3.1 [0.8-11.2]#	ns
Third postoperative day	1.5 [0.5-4.0]	3.1 [1.0-11.9]	ns
One week postoperative	1.8 [0.3-21.3]	1.4 [0.5-4.9]	ns

Table 2. Baseline characteristics and outcome measures study 2. *(Continued)*

	Standard pressure (n=10)	Low-pressure (n=10)	P-value
Urinary α-1-MGB (mg/mmol) †			
Preoperative	0.1 [0.0-0.5]	0.3 [0.1-0.6]	ns
During operation	0.1 [0.1-0.4]	0.1 [0.0-0.2]	ns
First postoperative day	2.3 [0.1-12.3]#	2.8 [0.1-9.3]	ns
Second postoperative day	3.1 [0.3-23.7]#	2.1 [0.5-10.1]#	ns
Third postoperative day	4.7 [0.7-8.2]#	8.0 [0.5-31.5]#	ns
One week postoperative	0.7 [0.1-5.4]#	0.4 [0.1-6.7]	ns
KIM-1 (µg/mmol creatinine)‡			
Preoperative	0.02 [0.00-0.07]	0.00 [0.00-0.12]	ns
During operation	0.26 [0.02-0.52]#	0.20 [0.08-0.41]#	ns
First postoperative day	0.04 [0.00-0.28]	0.04 [0.00-0.21]	ns
Second postoperative day	0.01 [0.00-0.10]	0.09 [0.00-0.12]#	ns
Third postoperative day	0.04 [0.00-0.08]	0.04 [0.00-0.13]#	ns
One week postoperative	0.00 [0.00-0.31]	0.03 [0.00-0.06]	ns
NGAL (µg/mmol creatinine)‡			
Preoperative	1.0 [0.0-12.0]	1.5 [0.0-5.7]	ns
During operation	0.6 [0.0-1.7]	0.4 [0.1-2.2]	ns
First postoperative day	1.6 [0.5-10.1]	1.6 [0.0-8.2]	ns
Second postoperative day	2.0 [0.5-10.7]	3.1 [0.7-11.4]	ns
Third postoperative day	3.2 [0.6-7.0]	3.5 [0.7-27.1]	ns
One week postoperative	2.5 [0.0-5.7]	0.7 [0.0-5.0]	ns

Categorical variables are presented as numbers and continuous variables as mean values (standard deviation) or median values [range]. KIM-1, kidney injury molecule 1; α-1-MGB, alpha-1-microglobulin; NGAL, neutrophil gelatinase-associated lipocalin.
*American Society of Anesthesiologists classification.
In some cases, the urine sample for a particular time point could not be collected due to insufficient urine production during surgery, or because it was impossible to collect a urine sample on a specific day postoperative: † 11 of 60 samples missing; ‡ 8 of 60 samples missing.
# $p < 0.05$ compared with preoperative.

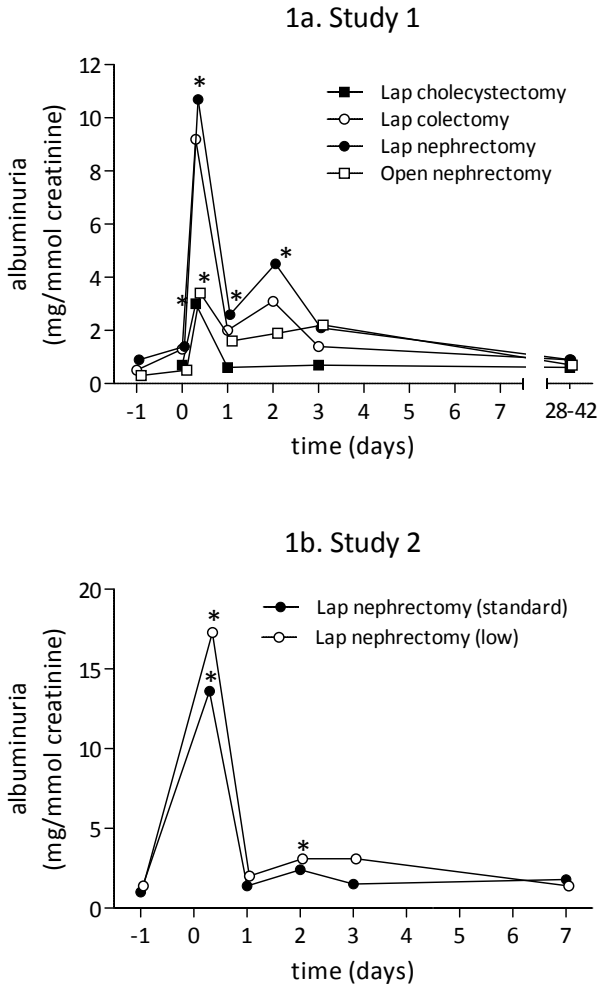

Figure 1. Albuminuria during the perioperative period (mg/mmol creatinine; median)

* $p < 0.05$ versus baseline.
(1a) study 1: laparoscopic cholecystectomy, laparoscopic colectomy, open donor nephrectomy and laparoscopic donor nephrectomy.
(1b) study 2: laparoscopic donor nephrectomy with standard pressure pneumoperitoneum (14 mmHg) and laparoscopic donor nephrectomy with low-pressure pneumoperitoneum (7mmHg).

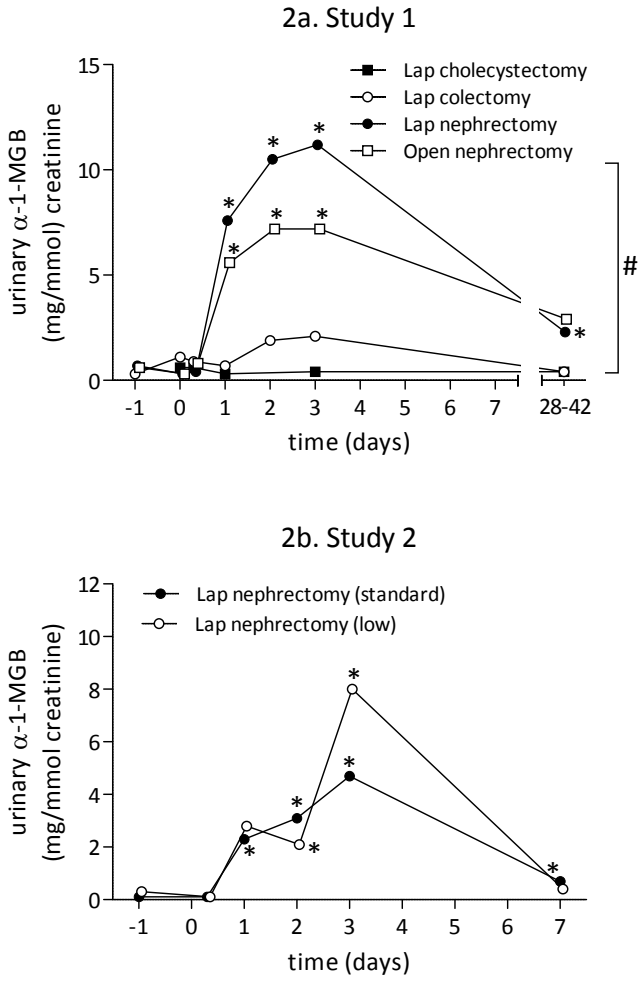

Figure 2. Urinary excretion of α-1-microglobulin (α-1-MGB**)** (mg/mmol creatinine; median)

* $p < 0.05$ versus baseline. # $p < 0.05$ laparoscopic nephrectomy versus laparoscopic cholecystectomy and laparoscopic colectomy in quadratic mixed model analysis.
(2a) study 1: laparoscopic cholecystectomy, laparoscopic colectomy, open donor nephrectomy and laparoscopic donor nephrectomy.
(2b) study 2: laparoscopic donor nephrectomy with standard pressure pneumoperitoneum (14 mmHg) and laparoscopic donor nephrectomy with low-pressure pneumoperitoneum (7mmHg).

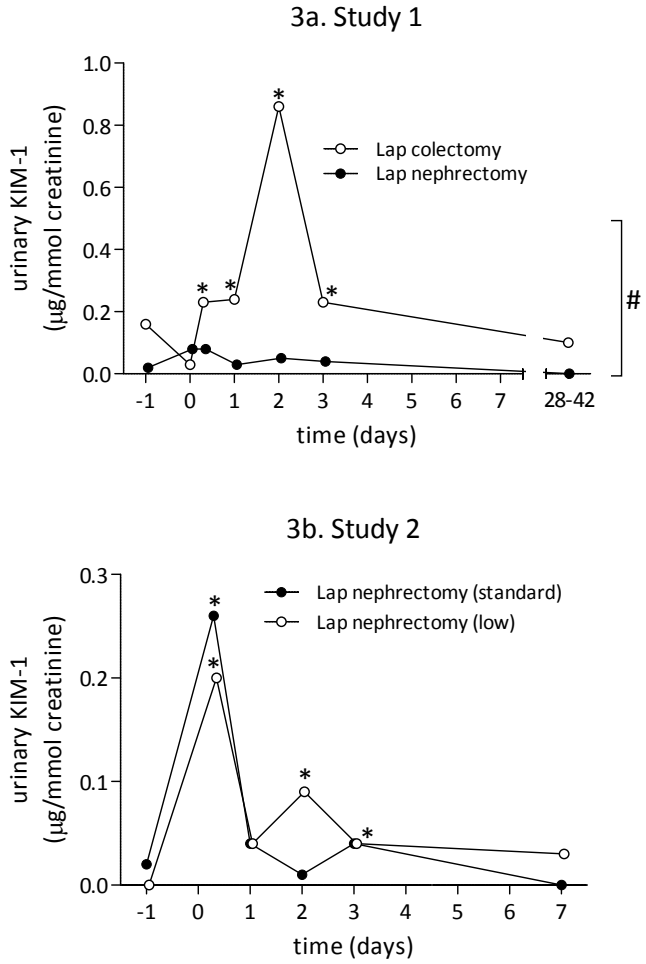

Figure 3. Urinary excretion of kidney injury molecule 1 (KIM-1) (µg/mmol creatinine; median)

* $p < 0.05$ versus baseline. # $p < 0.05$ laparoscopic nephrectomy versus laparoscopic colectomy in quadratic mixed model analysis.

(3a) study 1: laparoscopic donor nephrectomy and laparoscopic colectomy

(3b) study 2: laparoscopic donor nephrectomy with standard pressure pneumoperitoneum (14 mmHg) and laparoscopic donor nephrectomy with low-pressure pneumoperitoneum (7mmHg).

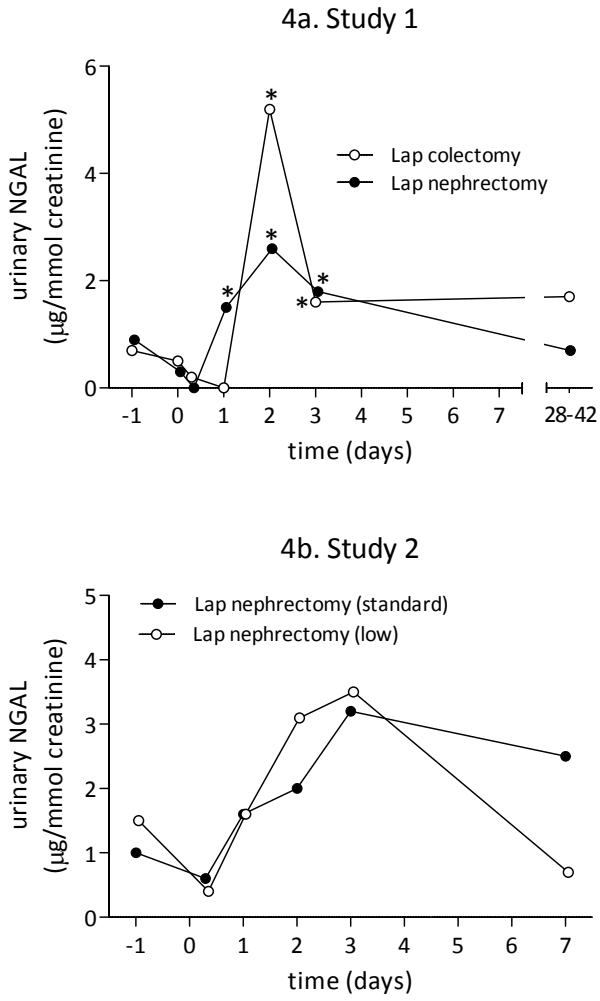

Figure 4. Urinary excretion of neutrophil gelatinase-associated lipocalin (NGAL) (µg/mmol creatinine; median)

* $p < 0.05$ versus baseline.
(4a) study 1: laparoscopic donor nephrectomy and laparoscopic colectomy
(4b) study 2: laparoscopic donor nephrectomy with standard pressure pneumoperitoneum (14 mmHg) and laparoscopic donor nephrectomy with low-pressure pneumoperitoneum (7mmHg).

DISCUSSION

Our study illustrates the effect of laparoscopic donor nephrectomy on urinary biomarker excretion. A short-lasting increase of albumin and KIM-1 excretion was noted during the procedure. In contrast, there was a slow rise in urine α-1-MGB and NGAL, which peaked on day 2 or 3, and in some patients remained elevated at 4-6 weeks after the procedure. The latter finding is suggestive for the occurrence of subtle tubular damage after donor nephrectomy, a finding that warrants further study. Of note, there was no evidence of acute kidney injury in any patient.

Serum creatinine and e-GFR are imprecise and insensitive markers of kidney injury. Therefore, urine biomarkers are advocated to allow an early diagnosis of acute kidney injury. For many years, albumin and low molecular weight proteins such as α-1-MGB have been used as markers of kidney injury. Urine albumin reflects glomerular injury, although increased urine albumin excretion can be observed in patients with severe tubular injury. The excretion of urine low molecular weight proteins reflects tubular injury.

The observation that urinary albumin excretion but not α-1-MGB excretion was elevated during surgery indicates that during surgery glomerular permeability is altered. The rapid normalization suggests that this is a reversible process, either the result of the anesthesia or hemodynamic changes that occur during surgery.

In addition to glomerular changes, there was also the suggestion of tubular injury after open and laparoscopic donor nephrectomy, as indicated by a peak in α-1-MGB excretion on the third postoperative day. Remarkably α-1-MGB excretion was still elevated 4-6 weeks postoperative. To support these findings, we performed additional studies. We analyzed novel biomarkers of kidney injury. In addition, we studied a control group of patients who underwent a laparoscopic procedure either cholecystectomy or colectomy.

The novel biomarkers included KIM-1 and NGAL. KIM-1 is a type 1 transmembrane protein, not detectable in normal kidney tissue, but is expressed at high levels in kidneys with dedifferentiated proximal tubule epithelial cells after ischemic or toxic injury. NGAL is synthesized mostly in the distal nephron of the kidney in response to nephrotoxic and/or ischemic injury. However, in response to renal injury, NGAL is also systemically produced. After glomerular filtration, most of this NGAL is endocytosed by the proximal tubule epithelia, and little is secreted in the urine. NGAL is an early predictor of acute kidney injury after cardiac surgery, contrast-induced nephropathy, and in critically ill patients.[7] After laparoscopic nephrectomy, we observed a slight increase of urinary excretion of KIM-1 in the immediate perioperative period, in parallel with albuminuria. In contrast, urine NGAL followed the pattern of α-1-MGB after laparoscopic nephrectomy

with a peak in excretion on the 2nd or 3rd postoperative day. It has been shown that anesthesia can be associated with increased urinary excretion of biomarkers. Patients undergoing breast surgery, anesthetized with ketorolac and sevoflurane, developed increased urinary excretion of α-1-MGB in the first postoperative day.[8] Another study after elective surgery showed elevated excretion of total protein, β-2-microglobulin, and N-acetyl-β-d-glucosaminidase after anesthesia with sevoflurane.[9]

It is unclear whether the anesthetics used in our studies could be responsible for the increased excretion of the biomarkers that we have observed. To further evaluate this, we have performed additional studies in patients who underwent laparoscopic cholecystectomy and colectomy. The laparoscopic cholecystectomy patients showed a slight increase in albuminuria during the operative procedure. There was no increase in urinary α-1-MGB. However, the laparoscopic cholecystectomy group might not be a good control group because the laparoscopic procedure was of significantly shorter duration. Therefore, we performed additional studies in patients who underwent laparoscopic colectomy. These patients also showed the perioperative increase in albuminuria. However, the patterns of urinary excretion of the tubular biomarkers were clearly different from that observed after laparoscopic nephrectomy. Specifically, after laparoscopic colectomy, there was no increase in urinary α-1-MGB excretion, and there were clear increases in urinary KIM-1 and NGAL occurring in the 2nd and 3rd day after the procedure. Taken all together, our data indicate that the increase in urinary α-1-MGB excretion is specific for nephrectomy. The typical and unique pattern of increased urinary α-1-MGB after nephrectomy is supported by the absence of a correlation between the excretion of α-1-MGB and the excretion of urine NGAL. Clearly, the increased urinary α-1-MGB excretion thus cannot be attributed to the anesthesia, nor to the elevated intra-abdominal pressure.

This points to a specific injury after donor nephrectomy, situated in the proximal tubules. We can only hypothesize that an alteration in blood supply, possibly mediated by renal reflexes, to the remaining kidney might be an explanation for the damage observed. Another possible explanation might be that nephrectomy causes hypertrophy and dedifferentiation of tubular cells in the remaining kidney. These dedifferentiated tubular cells may have more limited reabsorption capacity resulting in an increased urinary excretion of otherwise reabsorbed low molecular weight proteins. Our study also illustrates that the new biomarkers of kidney injury respond differently to the various operative procedures. More detailed studies are needed to find explanations for these specific expression patterns.

In adult patients undergoing elective cardiac surgery, Koyner et al. measured urinary biomarkers, such as KIM-1 and NGAL.[10] They demonstrated that the 6-h postoperative urinary NGAL best detected early stage 3 acute kidney injury. They also demonstrated

that preoperative KIM-1 was predictive for the development of stage 1 and stage 3 acute kidney injury. Potential sources of this preoperative elevation include exposure to radiocontrast, hypotensive events, or pre-existing chronic kidney disease. The population of healthy kidney donors included in the present study differs from patients undergoing cardiac surgery or critically ill patients in which most biomarker studies were performed. Therefore, profiles which accurately detect cardiac surgery-related acute kidney injury or acute kidney injury in septic patients are not necessarily useful in other patient groups such as kidney donors.

Limitations of our study are mainly related to its design as a pilot. Without a power calculation, the conclusions are preliminary and should be confirmed in a larger trial.

Urinary biomarkers are very sensitive for true histological tubular damage, and in research conditions, these high-sensitivity markers accurately predict potential nephrotoxicity. However, in clinical practice, we need to define what degree of subclinical damage as detected by biomarkers is clinically relevant, in terms of complications and long-term renal function. Therefore, studies linking acute tubular damage as assessed by biomarkers to long-term outcomes are needed.

CONCLUSION

Elevated α-1-MGB suggests kidney damage after open and laparoscopic donor nephrectomy. This occurs irrespective of intra-abdominal pressure during the laparoscopic procedure. Further studies are required to assess the mechanism underlying biomarker elevation after donor nephrectomy.

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Chapter 8

Summary and discussion

SUMMARY

Kidney transplantation is the preferred treatment option for most patients with end stage renal disease. A major drawback of kidney transplantation is the need for lifelong immunosuppressive therapy to prevent rejection episodes. The development of potent immunosuppressive drugs, especially calcineurin inhibitors (CNI) reduced the rate of acute rejections and improved short-term results of kidney transplantation. However, long-term results have not been improved to the same extent. Nephrotoxicity is a major side effect of CNI, and contributes to graft function loss. The use of immunosuppressive therapy after kidney transplantation is also responsible for the increased incidence of cancer, particularly skin cancer. The immunosuppressive drug sirolimus has been reported to be less nephrotoxic and may offer an opportunity to avoid exposure to CNI and thus prevent the associated nephrotoxicity. Sirolimus has also important anti-neoplastic properties, and may prove to be a drug that can be safely continued to prevent allograft rejection in transplant recipients who have developed a malignancy. Another possibility to improve the long-term results of kidney transplantation is to develop biomarkers that predict late allograft loss, thus allowing to identify and treat patients at highest risk in an early stage. A completely different problem with kidney transplantation is the shortage of organs and the long waiting time. Transplantation with a kidney of a living-donor may circumvent this problem, but the question is whether this procedure is safe for the donor, particularly in light of the now broadly used laparoscopic nephrectomy.

In 2005 we started a prospective randomized controlled trial that was aimed to investigate whether the nephrotoxicity that occurs under the current standard immunosuppressive treatment regimen with tacrolimus, low-dose steroids and mycophenolate mofetil (MMF) can be decreased by using a regimen with sirolimus, daclizumab, low-dose steroids and MMF without an increased incidence of acute rejections. As described in **chapter 2**, this study was prematurely ended because of an unacceptable high rate of acute rejections. After a mean follow-up of 15 weeks, seven of 10 patients developed an acute rejection in the sirolimus group compared with three of 20 patients in the tacrolimus group ($P < 0.01$). Three of the seven rejections in the sirolimus group occurred between 8 and 15 weeks after transplantation, at the time the prednisolone dose was reduced to below 10 mg/day. In all these cases the trough sirolimus level appeared to be below the target range at the time of rejection, which certainly could have contributed to the high rejection rate. Still, the data suggested that the treatment regimen was not suitable. We conducted a pilot study in 9 patients, to determine if a comparable CNI free treatment protocol with sirolimus could be suitable if higher doses of steroids where

used. In these 9 patients no acute rejection occurred. However, in this pilot study most patients experienced serious adverse events, such as delayed wound healing, infection, pulmonary embolus, proteinuria and diarrhoea. Based on this experience we concluded that at this time point there is no place for a sirolimus-based CNI free regimen in the direct post-transplant period.

Studies have reported a significant increase of proteinuria in kidney transplant recipients who were switched from a CNI to sirolimus. This has (partly) been attributed to the renal hemodynamic effects of CNI withdrawal. In **chapter 3** we have evaluated the evolution of proteinuria in renal transplant recipients who underwent conversion from azathioprine to sirolimus. In our centre 25 patients were included in a randomized study that evaluated the efficacy and safety of conversion to sirolimus in stable renal transplant recipients (RTR) with a cutaneous squamous cell carcinoma (SCC). After a mean follow-up of 360 days, mean proteinuria increased from 0.37±0.34 to 1.81±1.73 g/24h after conversion to sirolimus ($P<0.005$). In the control group with patients who continued their current immunosuppressive treatment there was no change in proteinuria. A significant increase of proteinuria was observed in all seven patients with proteinuria before conversion, whereas proteinuria remained absent in all patients without previous proteinuria. Two of the patients with proteinuria were converted from cyclosporine and five patients were converted from azathioprine to sirolimus. Sirolimus was discontinued in five patients with proteinuria, and in all of them proteinuria declined to baseline values. This study demonstrates that conversion from azathioprine to sirolimus after kidney transplantation is associated with an increase in proteinuria that is often reversible. The mechanism of this effect is unclear, but must be related to a direct effect of sirolimus.

Proteinuria can be the consequence of tubular and glomerular injury, and sirolimus thus could induce proteinuria by several mechanisms. Based on the findings in the above mentioned study, we performed experimental studies to examine the effect of sirolimus on proteinuria in a mouse model of focal segmental glomerulosclerosis (FSGS) as described in **chapter 4**. Heterozygous Thy-1.1 transgenic mice express the Thy-1.1 antigen on the podocytes. These mice slowly and spontaneously develop albuminuria and focal glomerulosclerosis over a period of 26 weeks. Importantly, the process of FSGS development can be accelerated. Injection of anti-Thy-1.1 monoclonal antibodies in Thy-1.1 transgenic mice induces an acute albuminuria, which is followed by a rapid development of FSGS within 3 weeks after injection of the monoclonal antibody. Use of this mouse model allowed us to investigate the effect of sirolimus both in non-proteinuric mice (experiment 1), as well as in mice made proteinuric before

start of sirolimus (experiment 2). We did not observe any increase in proteinuria after administration of sirolimus: neither in experiment 1 nor in experiment 2. We also did not detect differences in the occurrence of glomerulosclerosis. Thus, we were unable to replicate the finding of increased proteinuria that was observed in patients treated with sirolimus. Of note, we even observed some reduction of proteinuria in transgenic mice treated with sirolimus. Thus, our data might suggest a dose dependent protective effect of sirolimus on proteinuria in Thy-1.1 transgenic mice. The mechanisms responsible for this protective effect were not further evaluated.

In light of the significant morbidity and mortality of cutaneous invasive squamous cell carcinomas (SCCs) in renal transplant recipients we performed a prospective, multicentre study to examine if conversion to sirolimus-based immunosuppression can reduce the recurrence rate of these skin cancers. In **chapter 5** data are presented of a two-year randomized controlled trial in which 155 RTRs in the Netherlands and the United Kingdom with at least one biopsy-confirmed SCC were stratified according to age and number of previous SCCs and randomly assigned to conversion to sirolimus (n=74) or continuation of their original immunosuppressive therapy (n=81). Development of a new SCC within 2 years after random assignment was the primary end point. After 2 years of follow-up, there was no significant reduction of new SCCs in the sirolimus group, with a hazard ratio (HR) in multivariable analysis of 0.76 (95% CI, 0.48 to 1.2; P=0.255). Unfortunately, analysis of the efficacy of sirolimus was confounded by high 53% drop-out rate in the sirolimus group. When analyzing the data after the first year, there was a significant 50% risk reduction, with an HR of 0.50 (95% CI, 0.28 to 0.90; P=0.021). Sirolimus was most effective in patients with only one previous SCC with an HR of 0.11 (95% CI, 0.01 to 0.94; P = 0.044). The tumor burden of SCC was reduced during the 2-year follow-up period in those receiving sirolimus (0.82 v 1.38 per year, relative risk 0.51; 95% CI, 0.32 to 0.82; P=0.006) if adjusted for the number of previous SCCs and age. In retrospect the combination of high initial sirolimus levels and investigator caution led to early discontinuation of sirolimus in several participants with mild to moderate proteinuria and other adverse effects, who may have responded to dose adjustment and/or the addition of an angiotensin-converting enzyme inhibitor. There was no significant change in renal function or increase in proteinuria in the patients who continued sirolimus treatment, suggesting the safety of sirolimus conversion in RTRs many years post-transplantation. In conclusion, conversion to low-dose mTOR-inhibition with careful monitoring was not associated with increased risk of transplant dysfunction. However, in our study population, comprising patients with one or more previous SSCs, there was no benefit after two years in terms of SCC-free survival. Conversion in those with only one previous SCC should be carefully balanced

against toxicities that can lead to relatively high dropout rates. The benefit afforded by the mTOR-based regimen after conversion in the subgroup of patients with only one previous invasive SCC may suggest that an mTOR inhibitor has the potential to become an effective early immunosuppressive strategy to reduce the risk of cutaneous SCC in RTRs.

In **chapter 6** we investigated if graft function deterioration and graft loss can be predicted by measuring urinary protein markers. Albuminuria is a marker of glomerular damage and a predictor of renal outcome in patients with kidney disease. However, deterioration of renal function is best correlated with tubulointerstitial injury. Urinary excretion of α_1-microglobulin reflects tubulointerstitial damage and improves prediction of outcome in patients with glomerular diseases. However, little is known about the possible value of α_1-microglobulin for predicting graft outcome in RTRs. Therefore, in a cross sectional pilot study we evaluated urinary α_1-microglobulin excretion in four different categories of RTRs (HLA-compatible, never transplant rejection, with transplant rejection and with chronic allograft nephropathy). We subsequently measured urinary α_1-microglobulin excretion in a prospective cohort of RTRs three and 12 months after transplantation, and correlated the levels with graft loss, and progression of renal dysfunction. In the cross sectional study 41 patients were studied of which 14 received an HLA-compatible kidney. Urinary α_1-microglobulin excretion exceeded normal values in the majority of patients who had received an HLA compatible kidney. In this category, even in patients with the highest values of urinary α_1-microglobulin, renal function did not deteriorate during follow-up. These findings suggest that higher values of urinary α_1-microglobulin can sometimes be accepted as "normal" after renal transplantation and do not necessarily reflect active, progressive tubulointerstitial injury. Urinary α_1-microglobulin excretion was higher in the patients with rejection or chronic allograft nephropathy and correlated with eGFR ($r = -0.56$; $P < 0.001$). In the prospective study 139 patients were evaluated. Of these, 34 patients had graft rejection within 3 months after transplantation. Median follow-up after renal transplantation was 48 months, eGFR increased by 0.6 ml/min/1.73m^2/yr during follow-up, 10 patients died and 3 patients developed graft failure. Urinary α_1-microglobulin excretion was above normal in 86% of patients at three months and in 78% of patients at twelve months after transplantation. Increased urinary α_1-microglobulin excretion at twelve months was associated with the type of donor, gender of the recipient, eGFR, albuminuria, α_1-microglobulin at 3 months and prior rejections. Unfortunately, we were not able to draw any conclusion on the prognostic value of α_1-microglobulin on graft failure because of the limited number of events during follow-up. There was no correlation between urinary α_1-microglobulin excretion and the change in eGFR during follow-up. Our study might have been

underpowered to proof an association between urinary α_1-microglobulin and change in eGFR during follow-up, because in only 19 patients (14%) eGFR decreased more than 3 ml/min/1.73m^2/yr. In conclusion, the majority of patients after kidney transplantation has elevated levels of urinary α_1-microglobulin at 3 and 12 months after transplantation. In the short term, urinary α_1-microglobulin does not predict renal outcome in this relatively limited exploratory study. Our data suggest that in RTRs elevated urinary α_1-microglobulin reflects fibrosis and not active tubular cell injury. However, the value of α_1-microglobulin in the setting of renal transplantation warrants a larger study involving more events and longer follow-up.

As the beginning of living-donor kidney transplantation, physicians have expressed concern about the possibility that unilateral nephrectomy can be harmful to a healthy individual. In **chapter 7** we investigated whether the elevated intra-abdominal pressure during laparoscopic donor nephrectomy causes early damage to the remaining kidney by evaluation of sensitive urine biomarkers after laparoscopic donor nephrectomy. We measured albumin and α_1-microglobulin in urine samples collected during and after open and laparoscopic donor nephrectomy, laparoscopic cholecystectomy, and laparoscopic colectomy. Additionally, kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were measured in frozen urine samples collected during and after laparoscopic donor nephrectomy and laparoscopic colectomy. The same biomarkers were studied in patients randomly assigned to standard (14 mmHg) or low (7 mmHg) intra-abdominal pressure during laparoscopic donor nephrectomy. We observed a peak in urinary albumin excretion during all procedures. Urine α_1-microglobulin was not increased during surgery, but rose in the postoperative period with a peak on the third postoperative day after donor nephrectomy. Urine α_1-microglobulin did not significantly increase after laparoscopic cholecystectomy and colectomy. In the laparoscopic nephrectomy group we observed slight increases in urine KIM-1 during surgery and in urine NGAL at day 2-3 after the procedure. After laparoscopic colectomy, both KIM-1 and NGAL were increased in the postoperative period. The observation that urinary albumin excretion but not α_1-microglobulin excretion was elevated during surgery indicates that during surgery glomerular permeability is altered. The rapid normalization suggests that this is a reversible process, either the result of the anesthesia, or hemodynamic changes that occur during surgery. In addition to glomerular changes, there was also the suggestion of tubular injury after open and laparoscopic donor nephrectomy, as indicated by a peak in α_1-microglobulin excretion on the third postoperative day. This occurred irrespective of intra-abdominal pressure during the laparoscopic procedure. Remarkably α_1-microglobulin excretion was still elevated 4-6 weeks postoperative. Our data indicates that the increase in

urinary α_1-microglobulin excretion is specific for nephrectomy. The typical and unique pattern of increased urinary α_1-microglobulin after nephrectomy is supported by the absence of a correlation between the excretion of α_1-microglobulin and the excretion of urine NGAL. Clearly, the increased urinary α_1-microglobulin excretion thus cannot be attributed to the anesthesia, nor to the elevated intra-abdominal pressure. Further studies are required to assess the mechanism underlying biomarker elevation after donor nephrectomy. Our study also illustrates that the new biomarkers of kidney injury respond differently to the various operative procedures. More detailed studies are needed to find explanations for these specific expression patterns.

DISCUSSION

We performed several studies to investigate whether an immunosuppressive regimen with sirolimus can improve long-term results after kidney transplantation. Calcineurin inhibitors (CNI) have become the cornerstone of immunosuppressive therapy after kidney transplantation. However these agents are nephrotoxic and often contribute to kidney allograft dysfunction. Therefore, we studied the efficacy and safety of a CNI free regimen in which sirolimus replaced the CNI. Unfortunately, this trial had to be stopped because of an unacceptably high rejection rate. Meanwhile large trials also have shown that induction therapy with a CNI free regimen containing sirolimus after transplantation is associated with an increased risk of acute rejections.[1] Moreover, several studies have shown that sirolimus-containing regimens used after kidney transplantation attenuate recovery from delayed graft function when compared to other immunosuppressive regimens.[2,3] Finally, overall the use of sirolimus in the immediate post-transplant period was associated with an increased risk of impaired wound healing and an increased incidence of lymphoceles, often resulting in withdrawal of sirolimus treatment. Based on the above findings it is clear that there is currently no place for a CNI free regimen with sirolimus-based immunosuppression immediately after kidney transplantation. However, it has been suggested that mTOR inhibitors can be safely used and replace CNI when conversion is started 3 months after transplantation.[4] A recent review concluded that the use of sirolimus in combination with tacrolimus minimization therapy enables better preservation of kidney function, without increased risk of graft rejection.[5] Future studies have to proof that the early withdrawal of CNI and replacement with sirolimus improves graft survival or decreases long-term side effects. It has been suggested that mTOR inhibitors could be used in the immediate post-transplant period, if used in low dosages, combined with low dose CNI. We will have to wait the results of ongoing studies.

Squamous cell carcinomas (SCCs) are a frequent complication after kidney transplantation. Although our study failed to show a benefit in terms of SCC-free survival at 2 years, subgroup analysis suggested a benefit in patients who received sirolimus after the first manifestation of a SCC. A similar conclusion was drawn by Euvrard *et al.*, who also performed a randomized controlled trial evaluating the efficacy of an mTOR-based regimen in preventing the development of new skin carcinomas. These investigators also showed a benefit of mTOR treatment in the subgroup of patients with only one previous invasive SCC.[6] Thus, both studies suggest that treatment with an mTOR inhibitor may reduce the risk of cutaneous SCC in renal transplant recipients. However, most benefit is gained in patients who were switched early, thus after the first manifestation of SCC. Based on these findings it can be hypothesized that the mammalian target of rapamycin (mTOR) inhibitor may be most effective when given early after transplantation in patients with high risks for SCCs. However, the very early use of sirolimus is associated with a higher rejection risk. Future studies should demonstrate whether conversion of a CNI to an mTOR inhibitor 3 to 6 months after transplantation results in equal graft survival, and a lower malignancy rate.

Although mTOR inhibitors might reduce the risk of nephrotoxicity and malignancy, their use was associated with many adverse events. One of the notable side effects was the occurrence of proteinuria. Our study clearly demonstrated that proteinuria also was observed in patients who started sirolimus as replacement for azathioprine. Sirolimus-induced proteinuria therefore cannot be attributed to the hemodynamic effects of CNI withdrawal. Proteinuria was mostly observed in patients who were treated with sirolimus because of chronic allograft nephropathy and proteinuria.[7]

We recommend that all patients who start sirolimus therapy should be closely monitored for proteinuria. Risks of conversion are highest in patients with pre-existing proteinuria (> 800 mg/day), lower eGFR (< 40 ml/min), or chronic allograft injury.[8] Strategies for managing mild proteinuria include lowering blood pressure with angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers, dietary sodium and protein restriction, controlling obesity and lipids (with statins), and smoking cessation.[8,9] If proteinuria increases, drug withdrawal may be necessary to reduce the risk of renal failure. Proteinuria generally decreases within a few months after mTOR inhibitor discontinuation, and the majority of patients show no long-term residual kidney damage.[9]

The first reports of sirolimus induced proteinuria stimulated a search for the development of an animal model. However, we were unable to document increased

proteinuria in a mouse model for focal segmental glomerulosclerosis. Our studies even suggested some protective effects of sirolimus in this mouse model. A review of the literature data also disclosed contrasting findings, with some studies reporting an increase in proteinuria, however in the majority of studies a decrease of proteinuria was reported. The reasons for these seemingly discrepant findings have been elucidated by detailed studies of the role of the mTOR pathway in podocytes. Podocyte development and podocyte maintenance are dependent on a tight regulation of the mTORC1 and mTORC2 complexes. Both overactivation and complete inhibition of mTORC activity may cause podocyte dysfunction, glomerulosclerosis and proteinuria. The delicate balance likely explains the observed divergent effects of sirolimus. The effects of sirolimus on proteinuria are probably influenced by factors such as the nature and presence of pre-existing renal damage, timing of administration of the drug, dosage of sirolimus used, and prior exposure to a CNI. More detailed studies of the mTOR pathway in podocytes, and development of more specific inhibitors are needed to develop better treatment strategies.

Altogether, our studies indicate that at this moment there is no place for a CNI free regimen in the immediate post-transplant period. mTOR inhibitors such as sirolimus or everolimus should be considered in selected patients such as patients with posttransplant malignancies and patients with CNI nephrotoxicity. In each patient the risk and benefits of sirolimus therapy should be weight carefully on a case-by-case basis.

Although the introduction of CNI has reduced the number of acute rejection episodes, long-term allograft survival has not improved at a similar rate. In many patients kidney allograft function deteriorates slowly but persistently, a process often called chronic allograft nephropathy. Many processes are involved in chronic allograft nephropathy including humoral rejection, hypertension, smoking, dyslipidemia, nephrotoxicity, recurrence of the original disease, and atherosclerosis. Clinically, this process is often difficult to detect. Most patients present with a slowly increasing serum creatinine. However at the time point that there is a significant increase of serum creatinine much damage already has occurred, and a kidney biopsy often discloses irreversible damage with interstitial fibrosis and tubular atrophy. Early recognition of this continuous process of kidney damage would enable an early intervention, and multitargeted therapy. Based on findings in patients with glomerular diseases we hypothesized that measurement of the urinary excretion of the low molecular weight protein α_1-microglobulin might help to predict late allograft failure. Our studies clearly showed that urinary α_1-microglobulin is not a suitable parameter in kidney transplant recipients. In many patients increased levels of urinary α_1-microglobulin were observed, even in patients with stable renal

function over a period of many years after measurement. These results suggested that increased urinary α_1-microglobulin is not a reflection of active tubular injury, but more a reflection of established fibrosis and tubular atrophy. We cannot exclude that a repeated measurement of urinary α_1-microglobulin, which would allow to calculate a change in its excretion might be better suited to predict future allograft failure. Alternatively, we should consider evaluating other tubular biomarkers. In recent years new kidney biomarkers have been studied, especially in the setting of acute kidney injury. It would be useful to evaluate these biomarkers such as KIM-1, NGAL, interleukin-18 in the setting of renal allograft nephropathy.

We evaluated changes in the excretion of several urinary biomarkers after kidney donation. We specifically questioned if a higher abdominal pressure could induce subtle kidney injury. Notably, we did not observe differences in biomarker excretion between laparoscopic procedures with lower or higher abdominal pressure. However, we observed an increased urinary excretion of α_1-microglobulin occurring in the first days after the open and laparoscopic donor nephrectomy. Moreover, levels had not normalized 4-6 weeks after the nephrectomy. We do not know if the increased urinary α_1-microglobulin excretion is the consequence of limited tubular damage, or a physiological response to hyperfiltration that occurs after nephrectomy. Clearly, long-term kidney injury is not the rule after kidney donation. Large studies have documented the overall good outcome of living kidney donors. Still, we suggest that more studies are needed to explain our observations. If the increased excretion of α_1-microglobulin is the consequence of hyperfiltration, then there is no reason for concern. Alternatively, it might well be that the procedure causes very limited injury. This should have no consequences for the long-term outcome. However, if some tubular injury is present in the immediate postoperative period, it may be important to limit the use of nephrotoxic agents or nephrotoxic procedures (contrast CT scans) in this period. We have not changed our current transplantation policy. However, the data have triggered new study proposals. Specifically, we aim at evaluating long-term α_1-microglobulin excretion in kidney donors, at studying α_1-microglobulin after partial nephrectomy, and at measuring other kidney injury markers.

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Chapter 9

Samenvatting

SAMENVATTING

Er bestaan drie vormen van nierfunctievervangende therapie: hemodialyse, peritoneale dialyse en niertransplantatie. Niertransplantatie is de behandeling van voorkeur voor de meeste patiënten met eindstadium nierfalen, omdat dit een langere levensverwachting en een betere kwaliteit van leven geeft. Daarnaast is niertransplantatie ook minder kostbaar dan chronische dialysebehandeling. Omdat een transplantaatnier door het afweersysteem van de ontvanger herkend wordt als lichaamsvreemd kan er een afstotingsreactie ontstaan. Een belangrijk nadeel van niertransplantatie is dan ook de noodzaak tot levenslange behandeling met medicijnen die de afweer verzwakken (immunosuppressieve middelen) om een afstoting van het transplantaat te voorkomen. De ontwikkeling van krachtige immunosuppressieve geneesmiddelen, met name calcineurineremmers, hebben het aantal acute afstotingsreacties verminderd en daarmee de korte termijn resultaten van niertransplantatie enorm verbeterd. De lange termijn resultaten zijn echter niet in dezelfde mate verbeterd. Nefrotoxiciteit is een belangrijke bijwerking van calcineurineremmers en draagt daardoor bij aan het optreden van transplantaatverlies op de lange termijn. Het gebruik van immunosuppressieve geneesmiddelen na niertransplantatie is ook verantwoordelijk voor een verhoogde kans op het krijgen van kwaadaardige ziekten, met name huidkanker. Van het immunosuppressieve medicijn sirolimus is beschreven dat het minder nefrotoxisch is. Dit middel biedt daarmee een mogelijkheid om blootstelling aan de nefrotoxische calcineurineremmers te vermijden. Sirolimus heeft ook belangrijke antineoplastische eigenschappen. Theoretisch zou het dus een geneesmiddel kunnen zijn dat veilig gecontinueerd kan worden om transplantaatafstoting te voorkomen bij ontvangers van een niertransplantaat, die een kwaadaardige ziekte hebben ontwikkeld. Een andere manier om de lange termijn resultaten van niertransplantatie te verbeteren is het ontwikkelen van biomarkers die later transplantaatverlies kunnen voorspellen. Dit zou het mogelijk kunnen maken om patiënten met een hoog risico op transplantaatverlies in een eerder stadium op te sporen en te behandelen. Een heel ander probleem is het tekort aan donororganen en de daarmee samenhangende lange wachttijd tot een donororgaan beschikbaar is. Transplantatie met een nier van een levende donor kan dit probleem omzeilen. De vraag is echter of deze procedure veilig is voor de donor, vooral in het licht van de nu algemeen uitgevoerde laparoscopische donornefrectomie.

In 2005 is een prospectieve gerandomiseerde gecontroleerde studie gestart waarmee we wilden onderzoeken of de nefrotoxiciteit die optreedt onder het standaard immunosuppressieve behandelingsschema met tacrolimus, lage dosis steroïden en mycofenolaat mofetil (MMF) kan worden verminderd door een regime bestaande uit sirolimus, daclizumab, lage dosis steroïden en MMF zonder dat de incidentie van acute

afstotingen toeneemt. Zoals beschreven in **hoofdstuk 2**, werd deze studie voortijdig beëindigd vanwege een onaanvaardbaar hoog percentage acute afstotingsreacties. Na een gemiddelde follow-up van 15 weken, ontwikkelden 7 van de 10 patiënten een acute afstoting in de sirolimusgroep vergeleken met 3 van de 20 patiënten in de tacrolimusgroep ($p < 0,01$). Bij 3 van de 7 patiënten in de sirolimusgroep die een acute afstoting ontwikkelden trad die afstoting op tussen 8 en 15 weken na de transplantatie, op het moment dat de prednisolondosis was gereduceerd naar minder dan 10 mg/dag. Ten tijde van de afstoting was bij deze 3 patiënten de sirolimusspiegel lager dan het beoogde streefniveau. Dit kan zeker hebben bijgedragen aan het hoge afstotingspercentage. Desondanks suggereren de uitkomsten dat het onderzochte regime geen geschikt behandelingsschema is. Vervolgens is een pilotstudie uitgevoerd met 9 patiënten om na te gaan of een vergelijkbaar calcineurineremmervrij behandelschema met sirolimus wel geschikt is als hogere doses steroïden worden gebruikt. Bij deze 9 patiënten traden geen acute afstotingen op. In deze pilotstudie ontwikkelden patiënten echter wel veel ernstige bijwerkingen zoals vertraagde wondgenezing, infecties, longembolie, proteïnurie en diarree. Op basis van deze ervaringen hebben wij geconcludeerd dat er op dit moment geen plaats is voor een op sirolimus gebaseerd calcineurineremmervrij immunosuppressief regime in de eerste periode na niertransplantatie.

In diverse studies wordt melding gemaakt van een significante toename van eiwit in de urine, ook wel proteïnurie genoemd, bij niertransplantatiepatiënten waarbij de calcineurineremmer werd vervangen door sirolimus. Dit is (deels) toegeschreven aan de renale hemodynamische effecten die optreden bij het stoppen van de calcineurineremmer. In **hoofdstuk 3** is de ontwikkeling van proteïnurie geëvalueerd bij niertransplantaatontvangers die behandeld werden met een calcineurineremmervrij regime, waarbij de azathioprine werd vervangen door sirolimus. In ons centrum werden 25 patiënten geïncludeerd in een gerandomiseerde studie, die de werkzaamheid en veiligheid onderzocht van conversie naar sirolimus in stabiele niertransplantaatontvangers met een cutaan plaveiselcelcarcinoom (PCC). Na een gemiddelde follow-up van 360 dagen steeg de proteïnurie na conversie naar sirolimus van gemiddeld 0,37±0,34 naar 1,81±1,73 g/24h ($p < 0,005$). In de controlegroep van patiënten waarin de immunosuppressieve behandeling niet aangepast werd trad geen verandering op in proteïnurie. Er werd een significante stijging in proteïnurie waargenomen bij alle zeven patiënten met proteïnurie voorafgaand aan de conversie, terwijl proteïnurie afwezig bleef bij alle patiënten die tevoren geen proteïnurie hadden. Twee van de patiënten met proteïnurie waren geconverteerd van ciclosporine en 5 waren geconverteerd van azathioprine naar sirolimus. Bij vijf patiënten die proteïnurie ontwikkelden werd de sirolimus gestaakt, waarna bij alle vijf de proteïnurie weer naar

de uitgangswaarde daalde. Deze studie toont aan dat conversie van azathioprine naar sirolimus na niertransplantatie is geassocieerd met een toename van proteïnurie die vaak omkeerbaar is. Het mechanisme van dit effect blijft onopgehelderd, maar moet worden toegeschreven aan een direct effect van sirolimus.

Proteïnurie kan het gevolg zijn van tubulaire en glomerulaire schade en sirolimus kan dus proteïnurie veroorzaken via verschillende mechanismen. Gebaseerd op de bevindingen in de hierboven genoemde studie, zijn experimentele studies uitgevoerd in een muismodel van focale segmentale glomerulosclerose (FSGS) om het effect van sirolimus op proteïnurie te onderzoeken. Dit staat beschreven in **hoofdstuk 4**. Heterozygote Thy-1.1 transgene muizen brengen het Thy-1.1 antigeen tot expressie op de podocyten. Deze muizen ontwikkelen spontaan langzaam progressieve albuminurie en focale glomerulosclerose binnen een periode van 26 weken. Dit proces van FSGS ontwikkeling kan worden versneld. Injectie van anti-Thy-1.1 monoclonale antilichamen in Thy-1.1 transgene muizen induceert een acute albuminurie, die wordt gevolgd door een snelle ontwikkeling van FSGS binnen 3 weken na de injectie van het monoclonale antilichaam. Het gebruik van dit muismodel gaf ons de mogelijkheid om het effect te onderzoeken van sirolimus bij zowel niet proteïnurische muizen (experiment 1) als bij muizen die al proteïnurie hadden vóór de start van sirolimus (experiment 2). We zagen in beide experimenten geen toename van proteïnurie optreden na toediening van sirolimus. We konden ook geen verschillen detecteren in het ontstaan van glomerulosclerose. In dit diermodel waren we dus niet in staat de toename in proteïnurie, die werd waargenomen bij patiënten behandeld met sirolimus, te repliceren. We zagen juist zelfs een afname in proteïnurie bij de transgene muizen die met sirolimus behandeld werden. Onze gegevens suggereren dus een dosis afhankelijk beschermend effect van sirolimus op het ontstaan van proteïnurie bij Thy-1.1 transgene muizen. De mechanismen die verantwoordelijk zijn voor dit beschermend effect werden niet verder geëvalueerd.

In het licht van de significante morbiditeit en mortaliteit die cutane invasieve PCC's veroorzaken bij niertransplantatiepatiënten is een prospectieve, multicenterstudie uitgevoerd om na te gaan of conversie naar een op sirolimus gebaseerd immunosuppressief regime het risico op recidief huidtumoren kan verminderen. In **hoofdstuk 5** worden de resultaten gepresenteerd van een tweejarige gerandomiseerde gecontroleerde studie waarin 155 niertransplantatieontvangers in Nederland en Engeland met tenminste één biopsie bevestigd PCC werden geïncludeerd. Deze patiënten werden gestratificeerd naar leeftijd en het aantal PCC's tot aan inclusie en daarna gerandomiseerd in 2 groepen, te weten conversie naar sirolimus (n = 74) of voortzetting van hun oorspronkelijke immunosuppressieve therapie (n = 81). De

ontwikkeling van een nieuw PCC binnen 2 jaar na randomisatie was het primaire eindpunt. Na 2 jaar follow-up trad er geen significante vermindering op van nieuw ontstane PCC's in de sirolimusgroep met een hazard ratio (HR) in de multivariabele analyse van 0,76 (95% CI, 0,48-1,2; p = 0,255). Helaas werd de beoordeling van de effectiviteit van sirolimus bemoeilijkt door het hoge percentage uitvallers in de sirolimusgroep (53%). Bij analyse van de data na het eerste jaar was er wel een significante risicovermindering op het ontstaan van een nieuw PCC, met een HR van 0,50 (95% CI, 0,28-0,90; p = 0,021). Sirolimus bleek het meest effectief bij patiënten die slechts één PCC hadden gehad voorafgaand aan inclusie met een relatief risico van 0,11 (95% CI, 0,01-0,94; p = 0,044). Het totale aantal tumoren dat patiënten per jaar ontwikkelden tijdens de follow-up periode van 2 jaar was lager in de sirolimusgroep (0,82 v 1,38 per jaar, HR 0,51; 95% CI, 0,32-0,82; p = 0,006) als gecorrigeerd werd voor het aantal eerdere PCC's en leeftijd. In de sirolimusgroep traden veel bijwerkingen op, die bij 29 patiënten leidden tot het voortijdig staken van sirolimus. Terugkijkend heeft de combinatie van hoge initiële sirolimusspiegels en voorzichtigheid van de onderzoeker geleid tot vroegtijdig staken van sirolimus bij verschillende patiënten die een milde tot matige proteïnurie en andere bijwerkingen ontwikkelden, waar dit mogelijk ook zou hebben gereageerd op dosisaanpassing en/of de toevoeging van een angiotensine converterende enzymremmer. Er trad geen significante verandering in nierfunctie of toename van proteïnurie op bij de patiënten die de sirolimustherapie continueerden. Dit suggereert de veiligheid van conversie naar sirolimus bij niertransplantaatontvangers vele jaren na transplantatie. Concluderend kan worden gesteld dat conversie naar lage dosis mTOR-remming niet geassocieerd is met een verhoogd risico op transplantaatdysfunctie wanneer dit onder strikte controle plaatsvindt. In onze studiepopulatie, bestaande uit patiënten met één of meer eerdere PCC's, was er na twee jaar geen aantoonbaar voordeel in termen van PCC vrije overleving. Het mogelijk gunstige effect van conversie naar sirolimus op de ontwikkeling van nieuwe PCC's bij patiënten met slechts één eerder ontwikkeld PCC moet zorgvuldig worden afgewogen tegen de toxiciteit van sirolimus. De voordelen van conversie naar een op mTOR gebaseerd regime in de subgroep van patiënten met slechts één eerder ontwikkeld invasief PCC suggereert dat een mTOR-remmer de potentie heeft om een effectieve vroege immunosuppressieve strategie te worden ter reductie van cutane PCC bij niertransplantaatontvangers.

In **hoofdstuk 6** is onderzocht of met het meten van eiwitten in de urine transplantaatfunctieverslechtering en transplantaatverlies kan worden voorspeld. Albuminurie is een marker voor glomerulaire schade en een voorspeller van nierfunctieverslechtering bij patiënten met nierziekten. Verslechtering van de nierfunctie is echter het beste gecorreleerd met tubulo-interstitiële schade. Excretie in

de urine van α1-microglobuline weerspiegelt tubulo-interstitiële schade en verbetert de uitkomstvoorspelling bij patiënten met glomerulaire ziekten. Er is echter weinig bekend over de mogelijke waarde van α1-microglobuline bij het voorspellen van transplantaatoverleving bij patiënten met een niertransplantaat. Daarom hebben we in een cross-sectionele pilotstudie de urine α1-microglobuline-uitscheiding geëvalueerd in vier verschillende categorieën van patiënten met een niertransplantaat (HLA-compatibel, geen eerdere transplantaatafstoting, met eerdere transplantaatafstoting en chronische transplantaatnefropathie). Vervolgens maten we de urine α1-microglobuline-uitscheiding in een prospectief cohort van niertransplantaatontvangers op 3 en 12 maanden na de transplantatie. We correleerden de waarden met transplantaatverlies en achteruitgang van nierfunctie. In de cross-sectionele studie werden 41 patiënten bestudeerd, waarvan 14 een HLA-compatibele nier ontvingen. De urine α1-microglobuline-uitscheiding was hoger dan normaal in de meerderheid van de patiënten die een HLA-compatibele nier had ontvangen. Zelfs bij patiënten in deze categorie met de hoogste urine α1-microglobuline-excretie verslechterde de nierfunctie niet tijdens follow-up. Deze bevindingen suggereren dat hogere waarden van α1-microglobuline in de urine soms geaccepteerd kunnen worden als "normaal" na niertransplantatie en niet noodzakelijkerwijs overeenkomen met actieve, progressieve tubulo-interstitiële schade. De urine α1-microglobuline-uitscheiding was hoger in de patiënten met een eerdere afstoting of een chronische transplantaatnefropathie en correleerde met de eGFR ($r = 0,56$; $p < 0,001$). In de prospectieve studie werden 139 patiënten geëvalueerd. Van deze patiënten ontwikkelden 34 patiënten een transplantaatafstoting binnen 3 maanden na de transplantatie. De mediane follow-up na niertransplantatie bedroeg 48 maanden, de eGFR steeg met 0,6 ml/min/1,73m^2/jaar gedurende de follow-up, 10 patiënten overleden en 3 patiënten ontwikkelden transplantaatfalen. De urine α1-microglobuline-uitscheiding was drie maanden na transplantatie bij 86% van de patiënten hoger dan de normaalwaarde en twaalf maanden na transplantatie bij 78% van de patiënten. De verhoogde urine α1-microglobuline-uitscheiding op twaalf maanden was geassocieerd met het type donor, het geslacht van de ontvanger, de eGFR, albuminurie, α1-microglobuline-uitscheiding op 3 maanden en voorafgaande afstotingen. Helaas konden we niets concluderen over de prognostische waarde van α1-microglobuline op transplantaatfalen vanwege het beperkte aantal falende transplantaten gedurende de follow-up. Er was geen correlatie tussen de urine α1-microglobuline-excretie en de verandering in eGFR tijdens follow-up. Onze studie kan te weinig power hebben gehad om een associatie aan te tonen tussen urine α1-microglobuline en de verandering in eGFR tijdens de follow-up, omdat bij slechts 19 patiënten (14%) de eGFR meer dan 3 ml/min/1,73m^2/jaar achteruitging. Concluderend heeft de meerderheid van de patiënten 3 en 12 maanden na de transplantatie een verhoogde urine α1-microglobuline-uitscheiding. Op de korte termijn voorspelt de urine α1-microglobuline-uitscheiding de

renale uitkomst niet in deze relatief kleine verkennende studie. De data suggereren dat een verhoogd urine α1-microglobuline meer een weerspiegeling is van fibrose dan van actieve tubuluscelschade. Om de waarde van α1-microglobuline na niertransplantatie beter te kunnen bepalen is een grotere studie nodig met een langere follow-up, waarbij meer transplantaatfalen zal optreden.

Sinds het begin van de uitvoering van levende-donorniertransplantaties hebben artsen hun bezorgdheid geuit over de mogelijkheid dat een eenzijdige nefrectomie schadelijk kan zijn voor een gezond persoon. In **hoofdstuk 7** is onderzocht of de verhoogde intra-abdominale druk tijdens een laparoscopische donornefrectomie vroege schade veroorzaakt aan de resterende nier door evaluatie van gevoelige urinebiomarkers na laparoscopische donornefrectomie. Albumine en α1-microglobuline werden gemeten in urinemonsters, die verzameld werden tijdens en na een open en laparoscopische donornefrectomie, laparoscopische cholecystectomie en laparoscopische colectomie. Bovendien werd uit ingevroren urinemonsters, die tijdens en na de laparoscopische donornefrectomie en laparoscopische colectomie waren verzameld, aanvullend kidney injury molecule-1 (KIM-1) en neutrophil gelatinase-associated lipocalin (NGAL) gemeten. Dezelfde biomarkers werden bestudeerd bij patiënten die gerandomiseerd werden in een groep standaard (14 mmHg) of lage (7 mmHg) intra-abdominale druk tijdens laparoscopische donornefrectomie. We stelden een piek in de urine albumine-uitscheiding vast tijdens alle procedures. Urine α1-microglobuline was niet verhoogd tijdens de operatie, maar steeg in de postoperatieve periode met een piek op de derde postoperatieve dag na donornefrectomie. Urine α1-microglobuline steeg niet significant na laparoscopische cholecystectomie en colectomie. In de laparoscopische nefrectomiegroep zagen we gedurende de operatie een geringe stijging optreden van KIM-1 in de urine en 2-3 dagen na de procedure in de uitscheiding van NGAL in de urine. Na laparoscopische colectomie steeg zowel KIM-1 als NGAL in de postoperatieve periode. De observatie dat de urine albumine-uitscheiding maar niet de α1-microglobuline-uitscheiding steeg tijdens de operatie geeft aan dat tijdens de operatie de glomerulaire permeabiliteit verandert. De snelle normalisatie suggereert dat dit een omkeerbaar proces is, ofwel het resultaat van de narcose, ofwel ten gevolge van hemodynamische veranderingen die zich voordoen tijdens de operatie. Een onverwachte bevinding was dat de excretie van de tubulaire schademarker KIM-1 ook verhoogd was tijdens de operatie. Naast glomerulaire veranderingen waren er ook aanwijzingen voor tubulaire schade na de open en laparoscopische donornefrectomie, die zich uitte in een piek in de α1-microglobuline-uitscheiding op de derde postoperatieve dag. Dit trad onafhankelijk van de intra-abdominale druk tijdens de laparoscopische procedure op. Opmerkelijk was dat de α1-microglobuline-uitscheiding 6 weken postoperatief nog

steeds verhoogd was. Onze data maken duidelijk dat de verhoogde α1-microglobuline-uitscheiding specifiek optreedt bij een nefrectomie. Dit typische en unieke patroon van een verhoogde urine α1-microglobuline-uitscheiding na nefrectomie wordt ondersteund door het ontbreken van een correlatie tussen de uitscheiding van α1-microglobuline en de uitscheiding van urine NGAL. Het is duidelijk dat de verhoogde excretie van α1-microglobuline dus niet is toe te schrijven aan de narcose of aan de verhoogde intra-abdominale druk. Toekomstige studies zijn nodig om de mechanismen te ontrafelen die ten grondslag liggen aan de biomarkerstijging na donornefrectomie. Onze studie toont ook aan dat de nieuwe biomarkers voor nierschade anders op de verschillende operatieve procedures reageren. Meer gedetailleerde studies zijn nodig om de verklaringen voor deze specifieke expressiepatronen te vinden.

DISCUSSIE

We voerden verschillende studies uit om te onderzoeken of een immunosuppressief regime met sirolimus de lange termijn resultaten na niertransplantatie kan verbeteren. Calcineurineremmers zijn de hoeksteen van de immunosuppressieve therapie na niertransplantatie geworden. Deze calcineurineremmers zijn echter nefrotoxisch en dragen vaak bij aan het disfunctioneren van het niertransplantaat. Daarom bestudeerden wij de werkzaamheid en veiligheid van een calcineurineremmervrij regime waarbij de calcineurineremmer wordt vervangen door sirolimus. Helaas moest deze studie gestaakt worden vanwege een onaanvaardbaar hoog aantal acute transplantaatafstotingen. Inmiddels hebben grote studies ook aangetoond dat inductietherapie met een sirolimus bevattend, calcineurineremmervrij, regime na transplantatie geassocieerd is met een verhoogd risico op acute afstotingen. Bovendien is in verschillende studies aangetoond dat bij sirolimus bevattende regimes direct na niertransplantatie het herstel van een vertraagd op gang komend niertransplantaat langer duurt in vergelijking met andere immunosuppressieve regimes. Tenslotte is het gebruik van sirolimus in de periode onmiddellijk na de transplantatie geassocieerd met een verhoogd risico op slechte wondgenezing en een verhoogde incidentie van lymfocèles, vaak resulterend in het moeten beëindigen van de sirolimusbehandeling. Op basis van bovenstaande bevindingen is het duidelijk geworden dat er momenteel geen plaats is voor een op sirolimus gebaseerd calcineurineremmervrij immunosuppressief regime in de periode onmiddellijk na niertransplantatie. Er is echter gesuggereerd dat mTOR-remmers veilig kunnen worden gebruikt en wel de calcineurineremmer kunnen vervangen als conversie pas 3 maanden na transplantatie wordt gestart. Een recente review concludeerde dat het mogelijk moet zijn met het gebruik van sirolimus in combinatie met een geminimaliseerde dosering tacrolimus de nierfunctie beter te

behouden, zonder verhoogd risico op transplantaatafstoting. Toekomstige studies moeten uitwijzen of vervroegde onttrekking van de calcineurineremmer en vervanging door sirolimus de transplantaatoverleving verbetert en of deze conversie het aantal bijwerkingen op de lange termijn vermindert. Er is gesuggereerd dat mTOR-remmers ook gebruikt kunnen worden in de periode onmiddellijk na de transplantatie, als deze in lage doseringen gegeven worden, gecombineerd met lage dosis calcineurineremmer. We zullen de resultaten van lopende studies moeten afwachten om hierover meer duidelijkheid te krijgen.

PCC's treden frequent op als complicatie na niertransplantatie. Hoewel onze studie niet aan kon tonen dat er een voordeel optrad in termen van een PCC-vrije overleving na 2 jaar, suggereerde een subgroepanalyse wel een voordeel bij patiënten die al na de eerste manifestatie van een PCC sirolimus kregen. Een soortgelijke conclusie werd getrokken door de groep van Euvrard, die ook een gerandomiseerd gecontroleerd onderzoek uitvoerden waarbij de effectiviteit werd bekeken van een op mTOR gebaseerd regime bij het voorkomen van de ontwikkeling van nieuwe huidcarcinomen. Deze onderzoekers stelden ook een voordeel van mTOR-behandeling vast in de subgroep van patiënten met slechts één eerdere manifestatie van een invasief PCC. Beide studies suggereren dus dat de behandeling met een mTOR-remmer het risico kan verminderen op het ontwikkelen van cutane PCC's bij niertransplantatie-ontvangers. Het grootste voordeel lijkt te kunnen worden bereikt wanneer patiënten vroeg worden geconverteerd, dus al na de eerste manifestatie van een PCC. Op basis van deze bevindingen kan worden gesteld dat een mTOR-remmer het meest effectief is wanneer deze vroeg na transplantatie gegeven wordt bij patiënten met een hoog risico op PCC's. Dit gebruik van sirolimus vroeg na transplantatie is echter geassocieerd met een hoger risico op afstotingen. Toekomstige studies moeten uitwijzen of conversie van een calcineurineremmer naar een mTOR-remmer 3 tot 6 maanden na de transplantatie resulteert in dezelfde transplantaatoverleving en een lager percentage maligniteiten.

Hoewel mTOR-remmers mogelijk het risico op nefrotoxiciteit en het ontwikkelen van een maligniteit kunnen verminderen, was het gebruik hiervan geassocieerd met veel bijwerkingen. Een van de bekende bijwerkingen was het optreden van proteïnurie. Onze studie heeft duidelijk gemaakt dat proteïnurie ook werd waargenomen bij patiënten die van azathioprine naar sirolimus werden geconverteerd. Sirolimus geïnduceerde proteïnurie kan daarom niet worden toegeschreven aan de hemodynamische effecten van calcineurineremmeronttrekking. Proteïnurie werd vooral waargenomen bij patiënten die werden behandeld met sirolimus vanwege chronisch transplantaatfalen met proteïnurie.

Het is raadzaam alle patiënten die met sirolimustherapie starten strikt te controleren op het ontstaan van proteïnurie. De risico's van conversie zijn het grootst bij patiënten met al bestaande proteïnurie (> 800 mg / dag), een lagere eGFR (< 40 ml / min), of een chronisch transplantaatfalen. Strategieën voor het behandelen van milde proteïnurie omvatten verlaging van de bloeddruk met angiotensine converterende enzymremmers of angiotensine II receptorblokkers, een natrium- en eiwitbeperkt dieet, bestrijding van overgewicht, het corrigeren van stoornissen in de vetstofwisseling (met statines) en stoppen met roken. Als de proteïnurie toeneemt, kan het nodig zijn om de mTOR-remmer te staken om het risico op nierfalen te verminderen. De proteïnurie vermindert dan over het algemeen binnen een paar maanden na het staken van de mTOR-remmer en levert dan in de meerderheid van de patiënten op de lange termijn geen nierschade op.

De eerste verslagleggingen over sirolimus geïnduceerde proteïnurie stimuleerden een zoektocht naar de ontwikkeling van een diermodel. Wij waren echter niet in staat om het ontstaan van proteïnurie te reproduceren in een muismodel voor focaal segmentale glomerulosclerose. Onze studies suggereerden zelfs een beschermend effect van sirolimus in dit muismodel. Een overzicht van de in de literatuur gerapporteerde data bevat ook contrasterende bevindingen, met een aantal studies die een toename van proteïnurie vermeldden, maar waarbij de meeste studies een daling van proteïnurie rapporteerden. De redenen voor deze schijnbare discrepanties zijn toegelicht in gedetailleerde studies naar de rol van mTOR in podocyten. De ontwikkeling en de overleving van de podocyt zijn afhankelijk van een strakke regulering van de mTORC1- en mTORC2-complexen. Zowel overactivatie als volledige remming van mTORC activiteit kan proteïnurie, glomerulosclerose en podocytdysfunctie veroorzaken. Het delicate evenwicht verklaart waarschijnlijk de waargenomen uiteenlopende effecten van sirolimus. De effecten van sirolimus op proteïnurie worden waarschijnlijk beïnvloed door factoren zoals de aard en aanwezigheid van al bestaande nierschade, het tijdstip van toediening en de dosering van sirolimus, evenals de voorafgaande blootstelling aan een calcineurineremmer. Meer gedetailleerde studies naar de effecten van mTOR op podocyten en de ontwikkeling van meer specifieke remmers zijn nodig om betere behandelingsstrategieën te ontwikkelen.

Het geheel beschouwend tonen onze studies aan dat er op dit moment geen plaats is voor een calcineurineremmervrij regime in de periode onmiddellijk na de transplantatie. Het gebruik van mTOR-remmers zoals sirolimus of everolimus moet worden overwogen in geselecteerde patiënten, zoals patiënten met posttransplantatiemaligniteiten en patiënten met calcineurineremmernefrotoxiciteit. Bij elke patiënt zullen de risico's en voordelen van sirolimustherapie voor die specifieke patiënt zorgvuldig moeten worden afgewogen.

Hoewel de invoering van de calcineurineremmer het aantal acute afstotingsepisoden heeft verminderd is de transplantaatoverleving op de lange termijn niet in vergelijkbare mate verbeterd. Bij veel patiënten verslechtert de transplantaatnierfunctie langzaam maar zeker welk proces vaak chronisch transplantaatfalen wordt genoemd. Veel processen zijn betrokken bij chronisch transplantaatfalen inclusief humorale rejectie, hoge bloeddruk, roken, dyslipidemie, nefrotoxiciteit, terugkeer van de oorspronkelijke nierziekte en atherosclerose. Klinisch is dit proces vaak moeilijk op te sporen. De meeste patiënten presenteren zich met een langzaam oplopend serumkreatinine. Op het moment dat het serumkreatinine duidelijk gestegen is, is er al veel schade aanwezig. Een nierbiopsie laat dan vaak onomkeerbare schade met interstitiële fibrose en tubulaire atrofie zien. Vroege herkenning van dit voortgaande proces van nierschade zou een vroege interventie en een op meerdere aspecten gerichte therapie mogelijk maken. Gebaseerd op bevindingen bij patiënten met glomerulaire ziekten veronderstelden wij dat het meten van de excretie in de urine van het kleinmoleculaire eiwit α1-microglobuline kan helpen bij het voorspellen van laat optredend transplantaatfalen. Uit onze studies is duidelijk gebleken dat de uitscheiding van α1-microglobuline in de urine hiervoor geen geschikte parameter is bij niertransplantaatontvangers. Bij veel patiënten werd een verhoogde waarde van α1-microglobuline in de urine waargenomen, zelfs bij patiënten met een nog jarenlang stabiele nierfunctie na de meting. Deze resultaten suggereerden dat een verhoogde excretie van α1-microglobuline niet een weerspiegeling is van actieve tubulaire schade, maar meer een weerspiegeling is van ontstane fibrose en tubulaire atrofie. We kunnen niet uitsluiten dat het herhaald meten van α1-microglobuline in de urine, waarmee een verandering in de uitscheiding kan worden berekend, wellicht beter geschikt is om toekomstig transplantaatfalen te voorspellen. Anderzijds moeten we het evalueren van andere tubulaire biomarkers overwegen. In de afgelopen jaren zijn nieuwe renale biomarkers bestudeerd, met name bij acuut nierletsel. Het zou zinvol zijn om de rol van deze biomarkers, zoals KIM-1, NGAL en interleukine-18, na te gaan bij het voorspellen van niertransplantaatfalen.

We onderzochten veranderingen in de uitscheiding van verschillende urinebiomarkers na nierdonatie. Wij vroegen ons specifiek af of een hogere intra-abdominale druk subtiel nierletsel kan induceren. We hebben geen verschillen waargenomen in de uitscheiding van biomarkers tussen laparoscopische procedures met lage en hoge abdominale druk. We detecteerden echter wel een verhoogde excretie van α1-microglobuline in de urine in de eerste dagen na de open en laparoscopische donornefrectomie. Bovendien waren deze waarden 4-6 weken na de nefrectomie nog niet genormaliseerd. We weten niet of de verhoogde uitscheiding van α1-microglobuline het gevolg is van beperkte tubulaire schade, of dat dit een fysiologische reactie is op hyperfiltratie die na de

nefrectomie ontstaat. Duidelijk is dat nierschade ook op de lange termijn niet de regel is na nierdonatie. Grote studies hebben gedocumenteerd dat de uitkomst van levende nierdonoren over het algemeen goed is. Toch stellen wij voor dat er meer onderzoek nodig is om onze bevindingen te verklaren. Als de verhoogde uitscheiding van α1-microglobuline het gevolg is van hyperfiltratie, is er geen reden tot bezorgdheid. Anderzijds zou het goed kunnen zijn dat de procedure toch geringe schade veroorzaakt. Dit hoeft op de lange termijn geen gevolgen te hebben. Als er echter in de onmiddellijke postoperatieve periode enige tubulaire schade aanwezig is, kan het mogelijk belangrijk zijn om het gebruik van nefrotoxische middelen of nefrotoxische procedures (contrast CT-scans) in deze periode te beperken. We hebben ons huidige transplantatiebeleid niet gewijzigd. De data hebben echter geleid tot nieuwe studievoorstellen. Deze zijn specifiek gericht op het onderzoeken van de α1-microglobuline-excretie op de langere termijn na nierdonatie, op het bestuderen van de α1-microglobuline-excretie na een gedeeltelijke nefrectomie en op het meten van andere nierschademarkers.

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Curriculum Vitae

Judith Hoogendijk-van den Akker werd op 17 mei 1972 geboren te Delft. In 1990 werd het VWO-diploma behaald aan het Christelijk Lyceum te Veenendaal. In datzelfde jaar begon zij met de studie biomedische gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen, waar in 1994 de doctorale fase werd afgesloten in de richting klinische epidemiologie. Eveneens aan de Katholieke Universiteit Nijmegen werd in 1996 de doctorale fase van de studie geneeskunde afgesloten (cum laude). Na haar co-assistentschappen werd in 1998 het artsexamen behaald. Aansluitend was zij 1 jaar werkzaam als AGNIO op de afdeling interne geneeskunde van het Rijnstate Ziekenhuis te Arnhem. In 1999 startte zij met de opleiding tot internist in het Rijnstate Ziekenhuis te Arnhem (opleider dr. R. van Leusen). In 2003 werd de opleiding voortgezet in het Universitair Medisch Centrum St. Radboud te Nijmegen (opleider prof. dr. J.W.M. van der Meer). De registratie als internist volgde op 1-11-2005, waarna de opleiding tot nefroloog werd afgerond op 1-11-2006 (opleider prof. dr. J.H.M. Berden). Vanaf november 2006 is zij als internist-nefroloog werkzaam in de Isala te Zwolle.
Judith is getrouwd met Stefan Hoogendijk met wie zij twee kinderen heeft, Thijs (2001) en Lianne (2005).