

DIALYSIS – TRANSPLANTATION

# Differentiation between chronic rejection and chronic cyclosporine toxicity by analysis of renal cortical mRNA

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## **Differentiation between chronic rejection and chronic cyclosporine toxicity by analysis of renal cortical mRNA.**

**Background.** In kidney transplantation, chronic allograft nephropathy (CAN) is the major cause of graft loss. Causes of CAN include chronic rejection and chronic cyclosporine A (CsA) nephrotoxicity. It is necessary to differentiate between these two entities in order to apply the appropriate therapeutic regimen for the individual patient, but this is hampered by the lack of discriminating functional and morphologic parameters. We investigated whether renal cortical mRNA levels for several matrix proteins can serve as discriminating parameters.

**Methods.** Patients with chronic rejection ( $N = 19$ ) and chronic CsA toxicity ( $N = 17$ ) were selected by clinical and histologic criteria. Protocol biopsies without histologic abnormalities, taken at 6 months after transplantation from patients receiving CsA, were used as controls ( $N = 6$ ). Total RNA was extracted from the renal biopsy tissue, and mRNA levels of transforming growth factor- $\beta$  (TGF- $\beta$ ) and the extracellular matrix (ECM) molecules collagen I $\alpha$ 1, III $\alpha$ 1, IV $\alpha$ 3, decorin, fibronectin, and laminin  $\beta$ 2 were measured by real-time polymerase chain reaction (PCR).

**Results.** In both patient groups, the mean collagen IV $\alpha$ 3 and fibronectin mRNA levels were significantly elevated compared to those in controls, whereas only in CsA toxicity were the laminin  $\beta$ 2 and TGF- $\beta$  mRNA levels significantly increased. The increase of laminin  $\beta$ 2 and TGF- $\beta$  mRNA levels was significantly higher in the CsA toxicity group than in the chronic rejection group ( $P < 0.001$  and  $P = 0.004$ , respectively). Receiver-operating characteristic (ROC) curve analysis showed that with a 15.6-fold increase in laminin  $\beta$ 2 mRNA expression as cut-off point, the presence of CsA toxicity could be predicted with an 87% sensitivity and an 88% specificity.

**Conclusion.** Renal laminin  $\beta$ 2 and TGF- $\beta$  mRNA levels can be used to differentiate between chronic rejection and chronic CsA toxicity in renal transplants. The method of mRNA quantification might be applicable as an additional diagnostic tool in clinical practice.

**Key words:** allograft, diagnosis, medication, TGF- $\beta$ , laminin.

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Over the past decade, renal transplantation has become a very successful treatment modality for end-stage renal disease (ESRD). Due to improvement of immunosuppressive therapy, acute rejection episodes can be treated effectively, and the prevalence of early graft loss has diminished significantly [1]. Long-term graft loss, however, currently forms a major problem in renal transplantation [1–3].

The term chronic allograft nephropathy (CAN) refers to the pathologic changes, including interstitial fibrosis, tubular atrophy, and fibrous intimal thickening, which are found in chronically dysfunctioning kidney transplants [2]. Several risk factors for CAN have been recognized, such as the number and the severity of acute rejection episodes [4], ongoing chronic rejection, and excessive exposure to calcineurin inhibitors such as cyclosporine A (CsA) [2, 5]. Therefore, the lesions that occur in biopsies of patients with CAN may result from either one or a combination of these factors. Paradoxically, changes induced by chronic rejection are clinically and histopathologically hard to distinguish from those caused by the nephrotoxic effects of chronic exposure to CsA, meant to prevent chronic rejection. This makes it difficult to determine the optimal dose of the immunosuppressive regimen, in which the beneficial and nephrotoxic effects of CsA are balanced [6].

Some changes observed in routine light microscopy may help reveal the cause of chronic renal allograft dysfunction. These include peripheral nodular arteriolar hyalinosis, suggestive of chronic CsA toxicity, and transplant vasculopathy (intimal fibrosis, disruption of the lamina elastica in the presence of inflammation), suggestive of chronic rejection [7, 8]. However, these lesions are not decisively present in all patients with either of the diagnoses. Furthermore, due to the fact that peripheral nodular arteriolar hyalinosis and transplant vasculopathy appear focally in the tissue, sampling errors may obscure their presence.

In this study, we describe how differentiation between chronic rejection and chronic CsA toxicity may be

improved with the aid of molecular techniques, based on the results of a quantitative analysis of the renal cortical mRNA levels of several extracellular matrix (ECM) components and the ECM-regulating molecule transforming growth factor- $\beta$  (TGF- $\beta$ ) in two groups of patients suffering from either disease entity.

We focused on several molecules that make up the interstitial compartment of the kidney and are known to accumulate in renal fibrosis, including collagens I and III, and fibronectin, together with the ECM-regulating molecule TGF- $\beta$  and its potential inhibitor decorin. In recent publications, attention has been drawn to expression of collagen IV $\alpha$ 3 and laminin  $\beta$ 2 in the discrimination between chronic rejection and CsA toxicity [9]. These molecules are also the subject of the current study.

## METHODS

### Patient populations

We reviewed all kidney transplant biopsies performed in our center over the past 20 years, taken because of renal function loss beyond 1 year after transplantation. We selected two groups of patients: the chronic rejection group and the chronic CsA toxicity group.

The chronic rejection group ( $N = 19$ ), consisted of patients who received either prednisone and azathioprine ( $N = 6$ ), or prednisone and CsA ( $N = 13$ ). Of the 13 patients using CsA, seven received the Sandimmune formulation and six received the Neoral formulation. These patients, with an initially well-functioning kidney transplant, developed a progressive decline in renal function. A biopsy was taken  $4.8 \pm 3.8$  years after transplantation, in which transplant vasculopathy (intimal fibrosis, intimal inflammation, and disruption of the lamina elastica), transplant glomerulopathy [characterized by double contours of the glomerular basement membrane (GBM)] or both were present as a histopathologic indication of chronic rejection, as defined by the Banff 97 classification [7]. Biopsies with peripheral nodular arteriolar hyalinosis, a lesion suggestive of CsA toxicity, and biopsies with signs of de novo or recurrent native disease were excluded. Patients suffering from diabetes and patients with graft arterial stenosis were excluded.

The chronic CsA toxicity group ( $N = 17$ ), consisted of patients with an initially well-functioning kidney transplant, who developed a progressive decline in renal function only after a switch was made from Sandimmune to Neoral; CsA formulations with a relatively low and a relatively high bioavailability, respectively. Before the switch, immunosuppression was aimed at CsA 24-hour trough levels of 100  $\mu$ g/L by administration of Sandimmune once daily. After the switch, Neoral was administered twice daily, aiming at a 12-hour trough level of 150  $\mu$ g/L. Thereby, the mean daily CsA dose was increased from 3.2 mg/kg to 3.5 mg/kg [10]. Patients who

developed a significant and progressive decrease in renal function after this switch, in the absence of other features that might explain the decline in renal function, were included. Renal biopsies, taken  $7.1 \pm 3.4$  years after transplantation and  $2.5 \pm 1.2$  years after the switch from Sandimmune to Neoral, showed peripheral nodular arteriolar hyalinosis in 16 of the 17 patients, histopathologically supporting the functional selection. Biopsies with histologic features suggestive of chronic rejection or de novo or recurrent glomerulonephritis were excluded. Patients suffering from diabetes and patients with graft arterial stenosis were excluded.

C4d staining was performed on all biopsy samples. None of the patients in the CsA toxicity group showed C4d depositions in their peritubular capillaries, while 26% of the patients in the chronic rejection showed diffuse C4d depositions in the peritubular capillaries.

### Control group

As controls ( $N = 6$ ), we used protocol transplant biopsies taken from patients at 6 months after transplantation with stable graft function at the time of biopsy. Apart from some cases showing signs of minor nonspecific age-related alterations, none of the biopsies showed any signs of rejection or drug toxicity. The glomeruli (at least ten present in the sections for evaluation) did not show any abnormalities. Patients in this control group all used CsA as immunosuppressive medication.

### Clinical data

Clinical data included gender, patient age at time of biopsy, donor age, transplant-origin (cadaveric or living donor), number of acute rejection episodes, delayed graft function, time between transplantation and biopsy, time between switch and biopsy, mean arterial pressure (MAP), number of antihypertensive drugs used, use of angiotensin-converting enzyme (ACE) inhibitors, lowest serum creatinine, serum creatinine at biopsy, proteinuria at time of biopsy, and CsA trough levels. We estimated the best endogenous creatinine clearance and creatinine clearance at biopsy using the Cockcroft-Gault equation [11]. The loss of renal function was defined as the best Cockcroft clearance minus the Cockcroft clearance at the time of biopsy.

### mRNA isolation and cDNA synthesis

Four micrometer cryostat sections of each biopsy were cut, air dried, and stored at  $-20^{\circ}\text{C}$  until use for immunohistochemistry. One section was evaluated to localize the cortex, which was thereafter excised from the biopsy. RNA was subsequently extracted, as described previously [12]. In brief, the tissue was lysed by rigorous mixing after suspension in 500  $\mu$ L TRIzol<sup>®</sup> (Invitrogen Life Technologies, Carlsbad, CA, USA). After adding 100  $\mu$ L

**Table 1.** Primer and probe sequences

Molecule	Forward primer	Reverse primer	TaqMan™ probe
GAPDH	TGG TCA CCA GGG CTG CTT	AGC TTC CCG TTC TCA GCC TT	5'-FAM-TCA ACT ACA TGG TTT ACA TGT TCC AAT ATG ATT CCA CCA A-TAMRA-3'
β <sub>2</sub> m	TGC CGT GTG AAC CAT GTG A	CCA AAT GCG GCA TCT TCA A	5'-TET-TGA TGC TGC TTA CAT GTC TCG ATC CCA CT-TAMRA-3'
HPRT1	TGA CAC TGG CAA AAC AAT GCA	GGT CCT TTT CAC CAG CAA GCT	5'-TET-CTT GAC CAT CTT TGG ATT ATA CTG CCT GAC CA-TAMRA-3'
Collagen Iα1	CCT CAA GGG CTC CAA CGA G	TCA ATC ACT GTC TTG CCC CA	5'-TET-ATG CCT GCA CGA GTC ACA CCG GA-TAMRA-3'
Collagen IIIα1	GAG GAT GGT TGC ACG AAA CA	TGT CAT AGG GTG CAA TAT CTA CAA TAG G	5'-TET-TGA ATA TCG AAC ACG CAA GGC TGT GAG ACT-TAMRA-3'
Collagen IVα3	AAG CCC ACC ACA TGA TTC TGA	GCA GTT GTA GCC AGC CGT ACT	5'-FAM-TCC AAG CAC ACT CCG CAG GCA GT-TAMRA-3'
Decorin	ACA TCC GCA TTG CTG ATA CCA	AGT CCT TTG AGG CTA GCT GCA TC	5'-FAM-TCA CCA GCA TTC CTC AAG GTC TTC CTC C-TAMRA-3'
Fibronectin	GGA GAA TTC AAG TGT GAC CCT CA	TGC CAC TGT TCT CCT ACG TGG	5'-TET-AGG CAA CGT GTT ACG ATG ATG GGA AGA CAT-TAMRA-3'
Laminin β2	GGA TGA GGC TCG GGA CCT	CCC GTC CAA CTG GGC TG	5'-TET-AGG AAT TGG AAG GCA CCT ATG AGG AAA ATG A-TAMRA-3'
TGF-β1	CCC AGC ATC TGC AAA GCT C	GTC AAT GTA CAG CTG CCG CA	5'-TET-ACA CCA ACT ATT GCT TCA GCT CCA CGG A-TAMRA-3'

Abbreviations are: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; β<sub>2</sub>m, β<sub>2</sub>-microglobulin; HPRT1, hypoxanthine phosphoribosyl-transferase 1; TGF-β, transforming growth factor-β; TET, 6-carboxy-4,7,2',7'-tetrachloro-fluorescein; FAM, 6-carboxy fluorescein; TAMRA, 6-carboxy-tetramethyl-rhodamine.

chloroform, the solution was centrifuged at 15,000g for 15 minutes. The RNA was precipitated by addition of 5 μg of glycogen and 250 μL isopropanol. cDNA synthesis was performed using an reverse transcription (RT) kit (Omniscript Reverse Transcriptase) (Qiagen GmbH, Westburg B.V., The Netherlands).

### Real-time polymerase chain reaction (PCR)

For several ECM molecules and TGF-β1, forward and reverse primers (Life Technologies BRL and Biosource International, Nivelles, Belgium) and TaqMan™ probes (Biosource International) were designed, using Primer Express® 1.5 software (PE Applied Biosystems, Foster City, CA, USA). To prevent amplification of genomic DNA, primers or probes were chosen spanning an exon-intron junction. Primers were located near the 3' end of the mRNA. The 5' ends of the Taqman™ probes were 6-carboxy-4,7,2',7'-tetrachloro-fluorescein (TET)-labeled, except those for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), collagen IVα3, and decorin, which were 6-carboxy fluorescein (FAM)-labeled. The quencher dye at the 3' side of the probe was 6-carboxy-tetramethyl-rhodamine (TAMRA). The sequences of the primers and of the TaqMan™ probes are shown in Table 1.

Real-time PCR was performed using the ABI Prism™ 7700 sequence detector and software (PE Applied Biosystems) [13]. Amplification cycles were 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds and at 60°C for 60 seconds. Kinetics of the reactions were determined using a standard curve. We used the ratio of the levels of the investigated molecule and GAPDH, a constitutively expressed gene, to correct for the amount of tissue used for RNA extraction and the efficiency of cDNA synthesis. To confirm the suitability

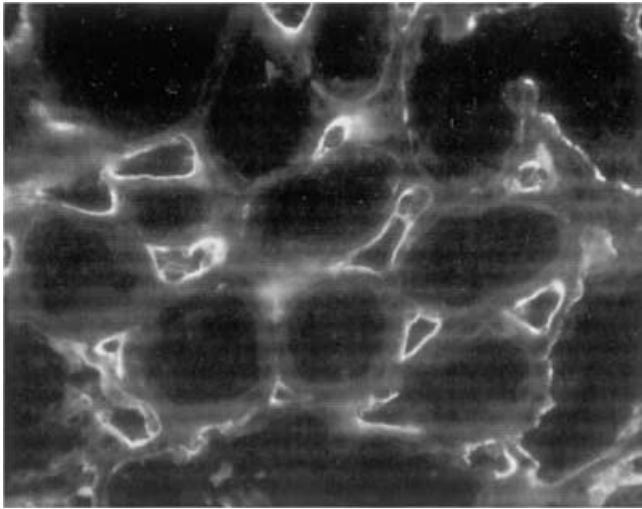
of GAPDH as a housekeeping gene, we tested the correlation between the expression of two additional housekeeping genes [i.e., hypoxanthine phosphoribosyl transferase 1 (HPRT1) and β<sub>2</sub>-microglobulin (β<sub>2</sub>m)], and that of GAPDH in all samples.

### Immunofluorescence for C4d

Immunofluorescence staining for C4d was performed on untreated slides using standard procedures as described before [14, 15]. As the primary antibody, mouse anti-C4d antibody (Quidel, San Diego, CA, USA), diluted to 2 ng/mL in phosphate-buffered saline (PBS) and supplemented with 1% bovine serum albumin (BSA), was used. The secondary antibody was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, MO, USA), diluted 1:200 in BSA/PBS. The staining was evaluated independently by two of the authors (K.K. and M.E.), blinded for the diagnosis of the samples. C4d staining was observed in the peritubular capillaries (PTC) in a circumferential pattern. Sporadically, mesangial and GBM areas of the glomerulus stained positive. In accordance with scoring methods described in the literature on C4d deposition in renal allografts [16, 17], biopsies were scored C4d-positive when more than 25% of the PTC showed an intense and circumferential staining as depicted in Figure 1. In most cases of diffuse positive samples all PTC were affected. In the few cases of discordant scoring, decision was reached by consensus.

### Immunohistochemistry

For evaluation of protein expression in the tissue, immunohistochemistry was performed for laminin β2 and TGF-β. Four micrometer frozen sections were thawed,



**Fig. 1. Immunofluorescence staining for C4d.** In some chronic rejection samples diffuse circumferential staining of peritubular capillaries for C4d was observed (original magnification  $\times 400$ ).

air dried, and incubated for 1 hour with mouse monoclonal anti-laminin  $\beta 2$  antibodies (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) diluted 1:16 in BSA/PBS, or rabbit polyclonal anti-TGF- $\beta$  (Dako, Glostrup, Denmark), diluted 1:400 in BSA/PBS. The anti-TGF- $\beta$  antibodies stain both the active and latent form of TGF- $\beta$ . The slides were washed in PBS, and subsequently incubated with horseradish peroxidase (HRP)-conjugated antimouse Envision and HRP-conjugated antirabbit Envision, respectively (Dako). After 45 minutes of incubation, the slides were washed in PBS and the staining was developed with diaminobenzidine (DAB). The color was enhanced by incubating the slides in 0.5%  $\text{CuSO}_4$  solution for 5 minutes. After counterstaining with hematoxylin, the slides were dehydrated and mounted. For each staining, all biopsy samples were stained in one session.

### Digital image analysis

To quantify the amount of staining for TGF- $\beta$  and laminin  $\beta 2$ , images of the cortical part of the biopsies were taken at a  $200\times$  magnification using a Zeiss microscope equipped with a Sony DXC-950P 3 CCD color camera (Sony Corporation, Tokyo, Japan) and further analyzed using KS-400 image analysis software (version 3.0 for Windows) (Carl Zeiss Vision GmbH, Oberkochen, Germany). The cortical area stained was defined as the amount of staining within the color spectrum specific for the enhanced DAB staining, and above a fixed intensity threshold, as described previously [18]. Recording and analysis of the images were performed with fixed settings.

### Statistical analysis

Statistical analysis was performed using SPSS 10.0.7 for Windows software. We used log transformed ( $^{10}\log$ ) mRNA levels for analysis. A one-way analysis of vari-

**Table 2.** Clinical characteristics of the patient groups and the control group<sup>a</sup>

Group	Chronic rejection	Chronic cyclosporine A toxicity	Controls
Number	19	17	6
Gender <i>female</i>	10 (53%)	4 (24%)	2 (33%)
Number of patients treated with cyclosporine A	13 (68%)	17 (100%)	6 (100%)
Patient age <i>years</i> $\pm$ <i>SD</i>	44 $\pm$ 14	49 $\pm$ 13	45 $\pm$ 6
Donor age <i>years</i> $\pm$ <i>SD</i>	35 $\pm$ 16	44 $\pm$ 10	47 $\pm$ 17
Living-donor kidney transplants %	5	35 <sup>c</sup>	
Number of acute rejection episodes	0.8 $\pm$ 1.0	0.5 $\pm$ 0.6	
Delayed graft function %	21	18	
Time between transplantation and biopsy <i>years</i> $\pm$ <i>SD</i>	4.8 $\pm$ 3.8	7.1 $\pm$ 3.4	
Time between switch and biopsy <i>years</i> $\pm$ <i>SD</i>		2.5 $\pm$ 1.2	
Mean arterial pressure	108 $\pm$ 10	106 $\pm$ 8	
Number of antihypertensive drugs used (0/1/2/ $\geq$ 3)	2/2/8/7	2/5/5/5	
Use of angiotensin-converting enzyme inhibitors %	21	29	
Best serum creatinine level $\mu\text{mol/L}$ $\pm$ <i>SD</i>	113 $\pm$ 27	124 $\pm$ 25	
Serum creatinine at time of biopsy $\mu\text{mol/L}$ $\pm$ <i>SD</i>	238 $\pm$ 83	201 $\pm$ 45	118 $\pm$ 25
Best creatinine clearance <i>mL/min</i> $\pm$ <i>SD</i>	74 $\pm$ 22 <sup>c</sup>	61 $\pm$ 11	
Creatinine clearance at time of biopsy <i>mL/min</i> $\pm$ <i>SD</i>	36 $\pm$ 14	40 $\pm$ 15	68 $\pm$ 14
Loss of renal function $\mu\text{mol/L}$ $\pm$ <i>SD</i> <sup>b</sup>	38 $\pm$ 16 <sup>d</sup>	21 $\pm$ 13	
Proteinuria at time of biopsy <i>g/24 hours</i> $\pm$ <i>SD</i>	2.9 $\pm$ 2.6	1.3 $\pm$ 1.5	
Cyclosporine A trough levels at time of biopsy $\mu\text{g/L}$ $\pm$ <i>SD</i>	110 $\pm$ 31	114 $\pm$ 30	

<sup>a</sup>Creatinine clearance was estimated by the Cockcroft-Gault equation.

<sup>b</sup>Loss of renal function was defined as the difference between the best creatinine clearance and the creatinine clearance at the time of biopsy.

<sup>c</sup> $P < 0.05$ ; <sup>d</sup> $P < 0.01$ .

ance (ANOVA) with a Bonferroni post hoc correction was used for comparison of differences between groups. Using a receiver-operating characteristic (ROC) curve, we determined the cut-off point of mRNA levels with the best combination of sensitivity and specificity that predicted the presence of CsA toxicity. Correlations between the mRNA data and the clinical characteristics of the patient groups were calculated using Pearson's correlation test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Patient data

The clinical characteristics of the patients and the controls are listed in Table 2. Renal function at time of biopsy, donor age, patient age, number of acute rejection episodes, delayed graft function, the time interval between transplantation and biopsy, MAP, number of antihypertensive drugs used, and use of ACE inhibitors did not differ significantly between patient groups. There

**Table 3.** Log transformed mRNA levels<sup>a</sup>

	Total chronic rejection (Ia) <sup>b</sup>	Chronic rejection using cyclosporine A (Ib) <sup>b,c</sup>	Chronic cyclosporine A toxicity (II) <sup>b</sup>	Controls (III)
Collagen Iα1	0.3 ± 0.2	0.2 ± 0.3	0.2 ± 0.3	0.8 ± 0.2
Collagen IIIα1	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	0.3 ± 0.1
Collagen IVα3	0.1 ± 0.2 <sup>d</sup>	0.1 ± 0.2 <sup>d</sup>	0.5 ± 0.1 <sup>d</sup>	-1.9 ± 0.3
Decorin	0.0 ± 0.2	-0.2 ± 0.2	-0.1 ± 0.2	-0.4 ± 0.2
Fibronectin	-0.1 ± 0.2 <sup>d</sup>	-0.3 ± 0.2 <sup>d</sup>	-0.1 ± 0.2 <sup>d</sup>	-1.6 ± 0.2
Laminin β2	0.6 ± 0.1 <sup>f</sup>	0.5 ± 0.2 <sup>‡</sup>	1.1 ± 0.1 <sup>d</sup>	-0.1 ± 0.3
Transforming growth factor-β	0.8 ± 0.1 <sup>e</sup>	0.8 ± 0.2 <sup>e</sup>	1.3 ± 0.1 <sup>d</sup>	0.5 ± 0.2
Ratio transforming growth factor-β/decorin	0.8 ± 0.2 <sup>e</sup>	0.8 ± 0.2 <sup>e</sup>	1.4 ± 0.1	0.9 ± 0.2

<sup>a</sup>All values are mean ± SEM.

<sup>b</sup> $P < 0.05$ , I or II vs. III.

<sup>c</sup>Mean log transformed mRNA levels of the chronic rejection group after omission of patients using azathioprine as immunosuppression.

<sup>d</sup> $P < 0.01$ , I or II vs. III.

<sup>e</sup> $P < 0.05$ , I vs. II.

<sup>f</sup> $P < 0.01$ , I vs. II.

was significantly greater loss of renal function in the chronic rejection group than in the chronic CsA toxicity group ( $38 \pm 16$  mL/min and  $21 \pm 13$  mL/min, respectively) ( $P < 0.01$ ). There were significantly more patients with a living-donor kidney transplant in the CsA toxicity group, compared with the chronic rejection group (35% and 5%, respectively) ( $P = 0.02$ ).

### Cortical mRNA levels

GAPDH mRNA levels did not differ between groups (data not shown). Within the chronic rejection group, mean GAPDH expression did not significantly differ between CsA-using and non-CsA-using patients. There was a significant correlation between GAPDH mRNA and mRNA of the two other housekeeping molecules measured ( $r = 0.74$  and  $r = 0.92$  for  $\beta_2m$  and HPRT1, respectively) ( $P < 0.001$ ). Additionally, we tested comparisons between the chronic rejection and the CsA toxicity group for all transcripts using  $\beta_2m$  as the housekeeping molecule. This yielded the same results as when GAPDH was used. These findings support the suitability of GAPDH as a housekeeping molecule in the experiments.

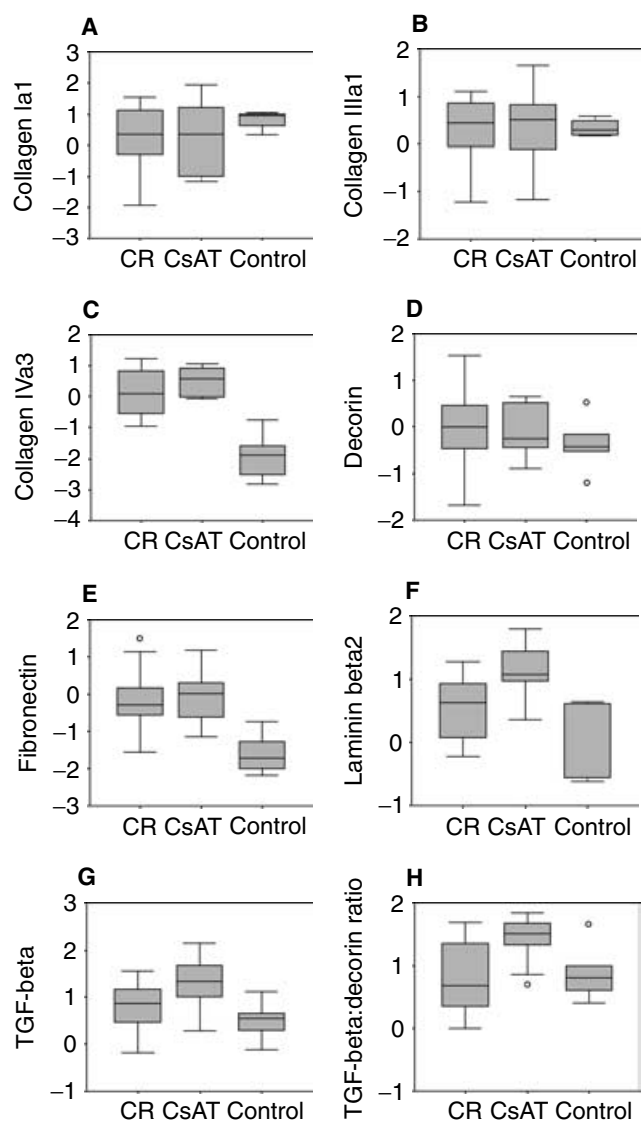
The mean log transformed mRNA levels of collagen Iα1, collagen IIIα1, collagen IVα3, decorin, fibronectin, laminin β2, and TGF-β are shown in Table 3 and depicted in Figure 2. The mRNA levels of collagen IVα3 and fibronectin were higher in both patient groups compared to controls. The mRNA levels of laminin β2 and TGF-β were higher in the CsA toxicity groups compared to controls (Table 3) (Fig. 2F and H). The renal mRNA levels of collagen Iα1, IIIα1, and IVα3, decorin, and fibronectin were not significantly different between patients with chronic rejection and patients with CsA toxicity (Table 3) (Fig. 2A to E). The renal mRNA levels of laminin β2, TGF-β, and the ratio of TGF-β to decorin were significantly higher in patients with CsA toxicity than in patients with chronic rejection (Table 3) (Fig. 2F and G). After omitting patients who did not use CsA

from the chronic rejection group, comparative analyses between groups yielded comparable results (Table 3). In addition, there were no differences in mRNA expression of all molecules analyzed between chronic rejection patients who used CsA and those who did not use CsA as immunosuppression (collagen Iα1,  $P = 0.44$ ; collagen IIIα1,  $P = 0.51$ ; collagen IVα3,  $P = 0.57$ ; decorin,  $P = 0.07$ ; fibronectin,  $P = 0.10$ ; laminin β2,  $P = 0.52$ ; TGF-β,  $P = 0.53$ ). There were no significant differences between mRNA expression levels in cadaveric transplants and living-donor transplants within each patient group.

### Immunohistochemistry

We performed immunohistochemical stainings to evaluate the tissue expression of laminin β2 and TGF-β at the protein level (Fig. 3). In biopsies of controls, laminin β2 staining was observed in the GBM and in cortical vessels (Fig. 3A). In chronic rejection as well as in CsA toxicity, sporadic expression of laminin β2 was seen in the tubular basement membrane (Fig. 3B and C). TGF-β staining was sporadically observed in glomeruli and tubuli of controls (Fig. 3D). In sections of biopsies from patients suffering from chronic rejection or CsA toxicity, some tubuli showed very intense staining for TGF-β (Fig. 3E and F). There was no relation between this sporadic intensive staining and mRNA expression levels or clinical parameters.

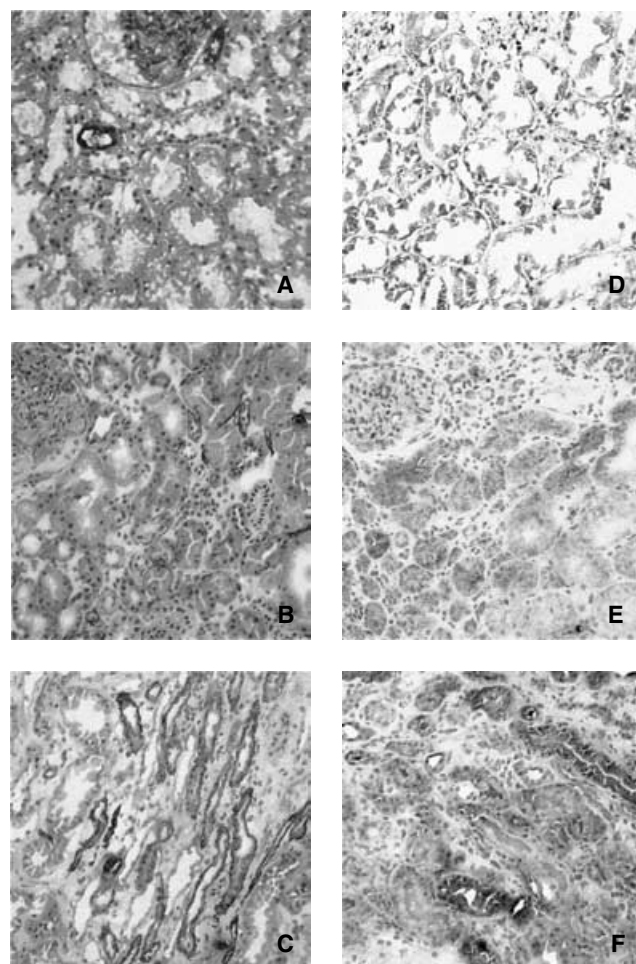
In both patient groups, the amount of laminin β2 staining was increased compared to controls. The mean cortical area percentage was slightly higher in the CsA toxicity group than in the chronic rejection group ( $5.7 \pm 1.5$  and  $5.3 \pm 1.0$ , mean ± SEM) (Fig. 4A). The differences between groups were not significant. The amount of TGF-β staining was higher in patient groups than in controls. The mean cortical area percentage was higher in the CsA toxicity group than in the chronic rejection group ( $15.8 \pm 3.6$  and  $13.4 \pm 2.2$ , mean ± SEM) (Fig. 4B), though this difference did not reach statistical significance.



**Fig. 2.** Box and whisker plots of the log transformed mRNA levels. The boxes contain 50% of the values. The upper and lower border indicate the 25th and the 75th percentile, respectively. The upper and lower whisker indicate the highest and lowest value, respectively. The black line in the box indicates the median and the (○) indicates an outlier. (A) Collagen Ia1. (B) Collagen IIIa1. (C) Collagen IVa3 [ $P < 0.001$  chronic rejection (CR) and cyclosporine A (CsA) toxicity (CsAT) vs. controls]. (D) Decorin. (E) Fibronectin ( $P < 0.001$  chronic rejection and CsA toxicity vs. controls). (F) Laminin  $\beta 2$  ( $P < 0.001$  CsA toxicity vs. controls) ( $P = 0.002$  chronic rejection vs. CsA toxicity). (G) Transforming growth factor- $\beta$  (TGF- $\beta$ ) ( $P = 0.004$  CsA toxicity vs. controls) ( $P = 0.020$  chronic rejection vs. CsA toxicity). (H) TGF- $\beta$ /decorin ratio ( $P = 0.014$  chronic rejection vs. CsA toxicity).

### ROC curve analysis

ROC curve analysis (Fig. 5) showed that a 15.6-fold increase of laminin  $\beta 2$  mRNA levels compared to controls indicates the presence of CsA toxicity with an 87% sensitivity and an 88% specificity. The area under the curve was 0.90 ( $P < 0.001$ ). Similarly, a ninefold increase of TGF- $\beta$  mRNA levels predicts the presence of chronic CsA tox-



**Fig. 3.** Immunohistochemical stainings for laminin  $\beta 2$  (A to C) and transforming growth factor- $\beta$  (TGF- $\beta$ ) (D to F). In control tissue laminin  $\beta 2$  staining was observed in the glomerular basement membrane and in cortical vessels (A). In chronic rejection (B) and cyclosporine A (CsA) toxicity (C), expression of laminin  $\beta 2$  was observed in the tubular basement membrane. TGF- $\beta$  staining was sporadically observed in glomeruli and tubuli of controls (D). In chronic rejection (E) and CsA toxicity (F), some tubuli showed a very intense staining for TGF- $\beta$ .

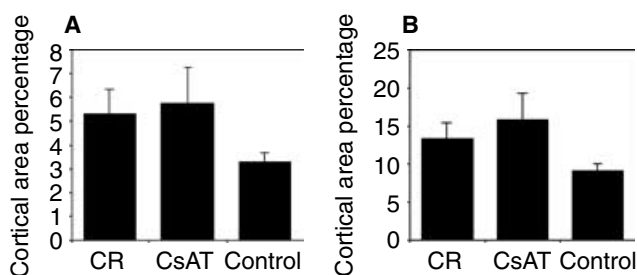
icity with a 60% sensitivity and an 88% specificity (area under the curve 0.76) ( $P < 0.05$ ).

### Correlations

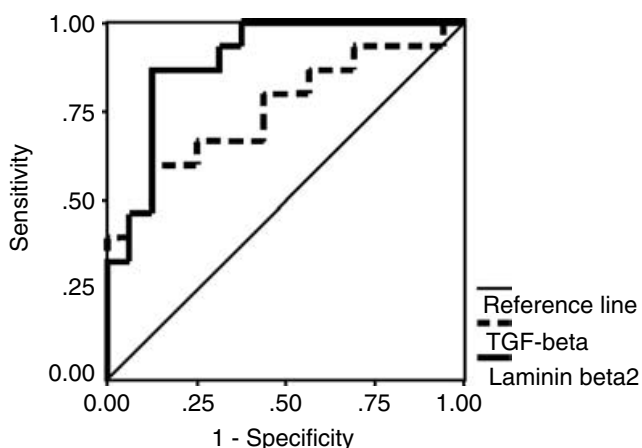
No correlations were found between mRNA levels and age, donor age, number of acute rejection episodes, delayed graft function, time between transplantation and biopsy, the time between switch and biopsy, loss of renal function, MAP, and use of ACE inhibitors. Only in the chronic rejection group, TGF- $\beta$  mRNA levels and the ratio of TGF- $\beta$  to decorin mRNA levels correlated significantly with laminin  $\beta 2$  mRNA levels ( $r = 0.56$  and  $r = 0.68$ , respectively) ( $P < 0.05$ ).

### DISCUSSION

This study was designed to enhance the discrimination between chronic rejection and chronic CsA toxicity as a



**Fig. 4. Immunohistochemical staining for laminin  $\beta 2$  and transforming growth factor- $\beta$  (TGF- $\beta$ ).** Bars represent mean cortical area percentage  $\pm$  SEM. (A) The amount of laminin  $\beta 2$  staining is slightly higher in the cyclosporine A toxicity (CsAT) group compared to the chronic rejection (CR) group, but the difference between groups does not reach statistical significance. (B) TGF- $\beta$  staining is increased in the CsA toxicity group compared to the chronic rejection group. There are no significant differences between groups.



**Fig. 5. Receiver-operating characteristic (ROC) curve of laminin  $\beta 2$  and transforming growth factor- $\beta$  (TGF- $\beta$ ).** The fraction of true positive results (sensitivity) and false positive results (1-specificity) for laminin  $\beta 2$  (solid line) and TGF- $\beta$  (dashed line) mRNA levels. The area under the curve indicates the accuracy of the test: 0.5 is the value expected by chance (diagonal line), 1.0 represents the ideal predictor. The area under the curves for laminin  $\beta 2$  and TGF- $\beta$  are 0.896 and 0.758, respectively. Using a 15.6-fold increase in laminin  $\beta 2$  mRNA levels to those of normal controls as cut-off point, the sensitivity is 87% and the specificity 88% for the prediction of the presence of chronic cyclosporine A toxicity.

cause of CAN. With current clinical and histologic parameters this distinction is difficult to make because of the similarities in clinical presentation and aspecificity of the lesions in needle biopsies of patients with CAN. We show that quantification of mRNA levels of laminin  $\beta 2$  and TGF- $\beta$  can be used to distinguish chronic rejection from CsA toxicity, which will help in fine-tuning the immunosuppressive regimen. In this way, the beneficial effects of CsA are not abrogated by its nephrotoxic side effects [3].

In our center, we had the opportunity to define on functional criteria a patient group that suffered from chronic CsA toxicity, comprising patients who developed a progressive decline in renal function after a change in

immunosuppressive medication was made leading to a higher CsA exposure [10]. The appropriateness of these selection criteria was supported by histopathologic findings: peripheral nodular arteriolar hyalinosis, regarded as a finding suggestive of CsA toxicity, was present in 94% of these patients, and C4d deposition in PTCs, a feature frequently seen in chronic rejection [19], was absent in all patients of the CsA toxicity group. An additional selection criterion for the CsA toxicity group and a further proof of CsA toxicity might have been the recovery of renal function after stopping or reducing CsA administration. Unfortunately, these data were not available. In the chronic rejection group, 13 out of 19 patients also received CsA. However, since the CsA formulation in the majority of these patients had a relatively low bioavailability and administration was applied only once-daily, this group is less likely to have suffered from CsA toxicity. In addition, patients in this group had transplant vasculopathy or glomerulopathy, histologic features suggestive of chronic rejection, but they did not show histologic features consistent with CsA toxicity. Finally, although there was some heterogeneity in immunosuppressive medication in the chronic rejection group, no differences were found in mRNA data between patients using CsA and those who did not use CsA as immunosuppression, and analysis with only the group of chronic rejection patients using CsA as immunosuppression yielded the same results as the chronic rejection group as a whole. A striking difference in patient characteristics was that 35% of the CsA toxicity group, but only 5% of the transplants in the chronic rejection group were living donor transplants. This might support the opinion that injuries inflicted to cadaveric allografts before or during transplantation elicit a predisposition for the development of chronic rejection, as has been suggested before [2, 20].

The ECM is a meshwork of proteins, in which remodeling continuously takes place by means of protein synthesis and degradation. Accumulation of ECM proteins reflects an imbalance of this dynamic process, resulting from an increase in protein synthesis, a decrease in protein degradation, or a combination of both. The synthesis of ECM components is enhanced by several profibrotic cytokines, including TGF- $\beta$ . During chronic renal allograft dysfunction, this cytokine has been shown to be up-regulated at the mRNA and protein level in the grafts, sera, and peripheral blood mononuclear cells of patients taking CsA-based immunosuppression [21, 22]. TGF- $\beta$  mRNA levels are up-regulated in cultured murine proximal tubular epithelial cells and fibroblasts after exposure to CsA [23]. Moreover, in rats receiving CsA, administration of anti-TGF- $\beta 1$  antibodies reduces the extent of histologic damage reminiscent of CsA toxicity [24]. Decorin, a low-molecular-weight proteoglycan, can bind and inactivate TGF- $\beta$ , thereby preventing its prosclerotic action [25]. The TGF- $\beta$ /decorin mRNA ratio may therefore be

a better indicator of TGF- $\beta$  activity than TGF- $\beta$  mRNA levels alone. We found a significant increase of TGF- $\beta$  mRNA levels and of the TGF- $\beta$ /decorin ratio in CsA toxicity, supporting the notion that CsA has a stimulatory effect on TGF- $\beta$  expression.

Laminin  $\beta$ 2 is a normal component of the renal vasculature and the GBM [26]. We observed an increase in laminin  $\beta$ 2 mRNA expression in the CsA toxicity group compared to controls, the expression in the CsA toxicity group being also significantly higher than in the chronic rejection group. Laminin  $\beta$ 2 mRNA expression was also higher in the CsA toxicity group when comparing it to the chronic rejection group with only those patients using CsA included. Only sparse information about factors stimulating laminin  $\beta$ 2 expression is available. The results of our study suggest a direct or indirect stimulatory effect of CsA on laminin  $\beta$ 2 expression, yet the underlying mechanism is unclear.

We performed immunohistochemistry combined with digital image analysis for laminin  $\beta$ 2 and TGF- $\beta$  to evaluate the expression of these molecules at the protein level. The pattern seen at the protein level resembled that at the mRNA level (i.e., there was a tendency toward a higher expression of laminin  $\beta$ 2 and TGF- $\beta$  in the CsA toxicity group than in the chronic rejection group). However, differences in protein staining between groups did not reach statistical significance. Furthermore, a correlation between mRNA expression levels and protein deposition was absent. The observation that the extent of protein accumulation does not strictly coincide with mRNA levels has been described before [27]. Additionally, in vivo accumulation of protein is not only determined by synthesis, but also by degradation of ECM products. Although we did not evaluate the mechanism of laminin  $\beta$ 2 degradation, the notion that laminin  $\beta$ 2 mRNA levels were increased in the CsA toxicity group compared to the chronic rejection group, while protein levels were not significantly different, might suggest that there is an increased degradation of laminin  $\beta$ 2 in CsA toxicity. This might be due to the microvascular damage exerted by CsA [8].

Collagen I $\alpha$ 1 and collagen III $\alpha$ 1 are components of the renal interstitium that are normally present in relatively small amounts [28]. Accumulation of these molecules has been reported in a variety of chronic human kidney diseases [29] and CAN [30, 31]. There was no difference between the chronic rejection group and the CsA toxicity group in collagens I and III mRNA levels. We observed no differences in the mRNA levels between the patient groups and the control group. This might be explained by the possibility that the accumulation of collagens I and III in CAN is a result of an early increase in collagen synthesis that might have taken place before the overt damage seen in the tissues. Furthermore, it might be that the accumulation of collagens I and III in patients with

CAN is due to an impaired degradation of these proteins, as has been suggested before [32].

Collagen IV $\alpha$ 3 is a component of both the GBM and the distal tubular basement membrane (TBM) [26]. In a study by Abrass et al [9], de novo expression of collagen IV $\alpha$ 3 protein was reported in the proximal TBM in chronic rejection, but not in CsA toxicity. In our study, we did not observe significant differences in collagen IV $\alpha$ 3 mRNA expression levels between the two groups. Since we used total cortical tissue for mRNA analysis, it is possible that subtle differences of collagen IV $\alpha$ 3 mRNA expression in the proximal tubulus epithelium between patient groups remained undetected. When we focused only on the chronic rejection group, the collagen IV $\alpha$ 3 mRNA expression was higher in the C4d+ chronic rejection group than in the C4d- chronic rejection group (data not shown). C4d recently has gained much interest as a marker of humoral rejection, but there is only sparse information about the relation between C4d and accumulation of ECM [33]. Future studies would be needed to decipher whether the relation found in our study is of pathogenic significance.

In both the chronic rejection and the chronic CsA toxicity group, we observed a significant increase in renal cortical fibronectin mRNA levels in comparison to controls. There were no differences in the mRNA levels of fibronectin between the chronic rejection and the CsA toxicity group. This is in line with previous studies showing an increase in fibronectin mRNA levels in a rat model of CsA toxicity [34], and in allograft rejection, both at the mRNA and the protein level [35, 36].

One of the advantages of analyzing mRNA profiles may be that alterations in mRNA levels precede the development or aggravation of tissue damage [37]. This holds promise for an earlier recognition of an unfavorable course after kidney transplantation [38, 39]. Furthermore, quantitative mRNA analysis can be performed rapidly, and requires only small amounts of renal tissue. In the future, diagnostic approaches using molecular analysis simultaneously with conventional strategies are likely to be implemented in clinical practice [40]. We had the opportunity to compare merely on the basis of functional variables two patient groups that represent the extremes of a spectrum of causes leading to the development of CAN. Therefore, we studied the presence of markers that could discriminate between these two highly selected patient groups. An obvious prerequisite for implementation in clinical practice is to test the use of these markers in larger nonselected patient groups.

## CONCLUSION

We measured human renal cortical mRNA expression levels of ECM components in two well-defined groups of patients suffering from either chronic rejection or chronic



CsA toxicity. In both patient groups, the mean mRNA levels for collagen IV $\alpha$ 3 and fibronectin were significantly elevated compared to those in controls, whereas in CsA toxicity the laminin  $\beta$ 2 and TGF- $\beta$  mRNA levels were also significantly increased. Most important, we showed that laminin  $\beta$ 2 and TGF- $\beta$  mRNA levels are significantly higher in patients with CsA toxicity than in patients with chronic rejection, and that measurement of these expression levels may help differentiate chronic CsA toxicity from chronic rejection.

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