ORIGINAL ARTICLE

Tubulointerstitial expression and urinary excretion of connective tissue growth factor 3 months after renal transplantation predict interstitial fibrosis and tubular atrophy at 5 years in a retrospective cohort analysis

Thomas Vanhove[1,2], Hiroshi Kinashi[3,4], Tri Q. Nguyen[3], Christoph Metalidis[1,2], Koen Poesen[5], Maarten Naesens[1,2], Evelyne Lerut[6,7], Roel Goldschmeding[3] & Dirk R. J. Kuypers[1,2]

1 Department of Microbiology and Immunology, KU Leuven – University of Leuven, Leuven, Belgium
2 Department of Nephrology, University Hospitals Leuven, Leuven, Belgium
3 Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
4 Department of Nephrology, Nagoya University Hospital, Nagoya, Japan
5 Clinical Department of Laboratory Medicine, University Hospitals Leuven, Leuven, Belgium
6 Department of Imaging and Pathology, KU Leuven – University of Leuven, Leuven, Belgium
7 Department of Pathology, University Hospitals Leuven, Leuven, Belgium

Correspondence
Dirk R. J. Kuypers MD, PhD, Department of Nephrology and Renal Transplantation, University Hospitals Leuven, Herestraat 49, 3000 Leuven, Belgium.
Tel: +32 16 344 586;
fax: +32 16 344 599;
e-mail: dirk.kuypers@uzleuven.be

SUMMARY

Connective tissue growth factor (CTGF) is an important mediator of renal allograft fibrosis, and urinary CTGF (CTGFu) levels correlate with the development of human allograft interstitial fibrosis. We evaluated the predictive value of CTGF protein expression in 160 kidney transplant recipients with paired protocol biopsies at 3 months and 5 years after transplantation. At month 3 and year 1, CTGFu was measured using ELISA, and biopsies were immunohistochemically stained for CTGF, with semiquantitative scoring of tubulointerstitial CTGF-positive area (CTGFti). Predictors of interstitial fibrosis and tubular atrophy (IF/TA) severity at 5 years were donor age [OR 1.05 (1.02–1.08), $P = 0.001$], female donor [OR 0.40 (0.18–0.90), $P = 0.026$], induction therapy [OR 2.76 (1.10–6.89), $P = 0.030$], and CTGFti >10% at month 3 [OR 2.72 (1.20–6.15), $P = 0.016$]. In subgroups of patients with little histologic damage at 3 months [either ci score 0 ($n = 119$), IF/TA score ≤1 ($n = 123$), or absence of IF/TA, interstitial inflammation, and tubulitis ($n = 45$)], consistent predictors of progression of chronic histologic damage by 5 years were donor age, induction therapy, CTGFti >10%, and CTGFu. These results suggest that, even in patients with favorable histology at 3 months, significant CTGF expression is often present which may predict accelerated accumulation of histologic damage.

Transplant International 2017; 30: 695–705

Key words
connective tissue growth factor, fibrosis, kidney transplant

Received: 3 January 2017; Revision requested: 27 February 2017; Accepted: 23 March 2017; Published online: 9 May 2017

Introduction

Connective tissue growth factor (CTGF; CCN2) is a growth factor that plays a critical role in fibrogenesis in a variety of tissues. In kidney disease, CTGF expression by renal tubular epithelial cells is a key mediator of TGF-β1-dependent interstitial fibrogenesis *in vitro* [1] and in renal allografted mice [2]. CTGF mRNA is

doi:10.1111/tri.12960

overexpressed by interstitial fibroblasts, epithelial, and mesangial cells in proliferative glomerulopathies and tubulointerstitial fibrotic areas in chronic transplant rejection [3]. We have previously demonstrated that CTGFu is higher in the presence of interstitial fibrosis on human renal allograft biopsies [4]. In that study, high levels of CTGFu in renal recipients without fibrosis (ci score = 0) at 3 months after transplantation were also independently predictive of development of moderate-to-severe fibrosis (ci score ≥2) by 2 years.

Connective tissue growth factor has also come under interest as a potential therapeutic target. A CTGF antisense oligodeoxynucleotide attenuated the upregulation of CTGF, fibronectin, and α1-collagen genes and decreased the number of renal myofibroblasts in rats with unilateral ureter obstruction [5]. In Lewis-Fischer transplanted rats, injection of small inhibitory RNA against CTGF was associated with a lower incidence of chronic allograft nephropathy and lower serum creatinine after 8 weeks [6]. Finally, in a phase I study in 24 diabetic patients with micro-albuminuria, administration of the monoclonal anti-CTGF antibody FG-3019 resulted in a reduction of the urinary albumin–creatinine ratio from 48 to 20 mg/g [7].

There is relatively little data regarding the expression of CTGF in human renal allografts. In a microarray analysis of consecutive renal protocol biopsies, development of early interstitial fibrosis and tubular atrophy (IF/TA) was predicted by upregulation of 30 unique genes, one of which was CTGF [8]. Another study, however, reported that CTGF mRNA expression in 3-month protocol biopsies in 101 renal recipients did not predict chronic allograft damage index (CADI) at 12 months [9], but longer histologic follow-up was not available. The goals of this study were to evaluate whether early tubulointerstitial CTGF expression predicts progression of interstitial fibrosis and tubular atrophy (IF/TA) as well as functional deterioration over the first 5 years after transplantation.

Materials and methods

Study design

This was a single center, observational, retrospective cohort study. Adult patients who had received a single kidney allograft between March 2004 and May 2009 were included if the immunosuppressive regimen at 3 months consisted of the combination of tacrolimus, mycophenolate, and steroids, and if repeat protocol biopsies at 3 months and 5 years were available. Blood samples for routine biochemistry and morning midstream urine sample were collected on the day of the biopsy. Induction therapy was used at the discretion of the physician in patients deemed at high immunological risk. This study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of the University Hospitals Leuven (S53364; ML7499). All patients provided written informed consent.

Histology and renal function

Biopsies were obtained under real-time ultrasound guidance using a Biopty-Cut® gun with a 16-gauge needle. The tissue cylinder was fixed in buffered formalin and embedded in paraffin. Slides containing 4–10 paraffin sections (2–3 μm) were routinely stained with hematoxylin–eosin, periodic acid-Schiff, and silver methenamine (Jones) for light microscopic examination. The severity of histologic lesions was semiquantitatively scored according to the revised Banff 1997 criteria [10]. For the purpose of this analysis, the sum of ci and ct scores is referred to as IF/TA score; fibrosis with inflammation is defined as ci score >0 and i score >0. All episodes of biopsy-proven acute rejection (BPAR) and subclinical BPAR were treated with high-dose steroids and/or antithymocyte globulin. Borderline rejection was not treated. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula [11]. Delayed graft function (DGF) was defined as the need for dialysis in the first 7 days after transplantation. Post-transplant diabetes mellitus (PTDM) was defined as need for treatment with oral antidiabetic drugs and/or insulin after transplantation.

Quantification of CTGF and α1-microglobulin

Plasma and urines samples were stored at −80 °C within 4 h after collection. CTGFu and plasma CTGF (CTGFp) concentrations were determined with a proprietary sandwich enzyme-linked immunosorbent assay (FibroGen, San Francisco, CA, USA) using two monoclonal antibodies against distinct epitopes on the N-terminal part of human CTGF, detecting full-length CTGF as well as the N-fragment, as previously described [12]. Urinary CTGF was normalized to urinary creatinine and is expressed as pmol/g creatinine. Fractional excretion of CTGF (FeCTGF) was calculated as [(CTGFu × plasma creatinine)/(urinary creatinine × CTGFp]. Urinary α1-microglobulin (A1M) concentration was determined using nephelometry. A1M was selected as a marker

for tubular proteinuria because urine had not been alkalinized, precluding reliable measurement of β2-microglobulin (B2M).

CTGF immunohistochemistry and analysis

Renal biopsies processed for light microscopy were stained for CTGF in a single batch, as previously described [13]. Tubulointerstitial CTGF-positive surface area (CTGFti) in the entire cortical area was assessed by a single researcher (HK) blinded to clinical information and semiquantitavely categorized as absent (<1%), minimal (1–10%), moderate (11–25%), or extensive (>25%).

Statistical analysis

Data are reported as mean ± SD, unless stated otherwise. Normality was tested using the Shapiro–Wilk test. Urinary and plasma CTGF and A1M were not normally distributed and log transformed for analyses. Proteinuria was categorized as <0.3, 0.3–1, and >1 g/g creatinine for analysis. Undetectable concentrations of A1M were set at half the lowest observed concentration prior to log transformation. Differences in continuous variables between both time points were assessed using paired-samples *t*-test, differences in categorical variables using Mcnemar's test, differences in histologic score distribution using the Friedman test, and correlations using chi-square test (Pearson's *r*). Differences in a continuous variable between categories of a categorical variable were assessed using ANOVA. Ordinal multivariable regression models were constructed using the SPSS GENLIN procedure to assess which variables predicted ci and ct scores at 5 years. All clinical and histologic variables presented in Tables 1 and 2 were considered as potential predictors of histologic and renal functional outcomes. These variables were simultaneously entered into a multivariable regression analysis (independent of significance in univariable regression) with backward conditional retention. All variables with $P < 0.157$ are reported in the final model [14]. Ci and ct scores at 3 months were always included as predictors in the respective models, regardless of significance. Similarly, the outcomes of IF/TA score ≥2 and chronicity score ≥3 at 5 years were assessed using binary logistic regression with backward conditional inclusion of predictors. Logistic regression models were validated using leave-one-out cross-validation. When, in case of (quasi-) complete separation of data for a particular predictor variable, estimates could not be calculated, Firth's penalized maximum likelihood estimation was used, utilizing the Firth binary logistic regression extension via the SPSS R-plugin (R logistf package) [15]. Resulting odds ratio confidence intervals for these variables were still typically very wide; the exact value of the odds ratios should not be overinterpreted. Intercepts were included in all models but are not reported. A two-sided *P*-value <0.05 was considered statistically significant. All analyses were performed using IBM SPSS Statistics version 22 (IBM, New York, NY, USA). Figures were generated using GRAPHPAD PRISM version 6 (San Diego, CA, USA).

Results

Demographics and evolution of histology

The flowchart of patient selection is presented in Fig. 1. Repeat protocol biopsies were available for 171 patients. Eleven patients were excluded because insufficient renal tissue was available for CTGF staining. According to Banff criteria, one, two, and three biopsies were inadequate for assessment of glomerular/vascular pathology at 3, 12 and 60 months, respectively. Demographics of the final 160 patients as well as the entire cohort assessed for eligibility are presented in Table 1. Patients in the study cohort had lower donor age, less delayed graft function, higher HLA mismatch, and were less likely to have undergone a previous transplant or induction therapy. Evolution of renal function, proteinuria, and selected histologic parameters are presented in Table 2. Evolution of all individual Banff scores is presented in Table S1, available online. Donor-specific antibody (DSA) screening was not systematically performed during most of the study period. DSA was present in 0/9, 0/12, and 3/99 of patients who were screened at 3, 12 and 60 months, respectively.

CTGF staining and CTGFu

In the tubulointerstitial compartment, CTGF staining was mainly positive in the cytoplasm of proximal (and, to a lesser degree, distal) tubular epithelial cells (Fig. 2). Staining was less pronounced at the tubular brush border and in interstitial cells. Table 2 shows CTGFti, urinary and plasma CTGF, and FeCTGF at 3 and 12 months as well as A1M values at 3 months. As CTGF is known to be upregulated in the context of inflammatory renal pathology [3], we first examined the cross-sectional relationship between CTGF staining and morphological evidence of inflammation at 3 months. CTGFti >10% was present in 21 of the 24 patients with *i* score >0 ($P = 0.182$) and in 18 of 21 patients with

Table 1. Demographics of study cohort versus all renal recipients assessed for eligibility (transplanted March 2004—May 2009 and treated with tacrolimus–MMF–steroids).

Characteristic	Study cohort (n = 160)	Entire cohort (n = 489)	P value*
Donor characteristics			
Age (years)	42.8 ± 14.8	45.7 ± 15.3	0.003
Gender (male)	96 (60%)	272 (55.6%)	0.087
Donor type			
Living	14 (8.8%)	32 (6.5%)	0.112†
DBD	119 (74.4%)	390 (79.8%)	
DCD	27 (16.9%)	67 (13.7%)	
ECD	26 (16.3%)	104 (21.3%)	0.059
Cold ischemia time (hours)	13.9 ± 6.2	14.5 ± 5.7	0.739
Anastomosis time (minutes)	33.7 ± 8.0	34.4 ± 8.4	0.244
Recipient characteristics			
Age (years)	51.4 ± 12.2	52.9 ± 13.6	0.098
Gender (male)	96 (60%)	288 (58.9%)	0.729
PRA > 20%	3 (1.9%)‡	5 (1.0%)§	0.800
Diabetes mellitus pretransplant	24 (15%)	91 (18.6%)	0.054
Transplant characteristics			
Repeat transplant	16 (10%)	80 (16.4%)	0.016
Number of HLA mismatches	2.5 ± 1.3	2.4 ± 1.3	0.040
Induction therapy	56 (35%)	201 (41.1%)	0.041
Basiliximab	50 (31.3%)	174 (35.6%)	
ATG	5 (1.0%)	21 (4.3%)	
Alefacept	0	4 (0.8%)	
Rituximab	0	1 (0.2%)	
Daclizumab	0	1 (0.2%)	
Delayed graft function	15 (9.4%)	79 (16.2%)	0.004

ATG, antithymocyte globulin; DBD, donation after brain death; DCD, donation after cardiac death; ECD, extended criteria donor; PRA, panel reactive antibody. HLA mismatch is the sum of broad antigen mismatches.

*Comparison between study cohort (n = 160) and patients assessed for eligibility but not included in primary analysis (n = 329).

†For difference in distribution (Living–DBD–DCD).

PRA information only available for ‡110 patients and §251 patients.

borderline or subclinical acute rejection at month 3 (P = 0.049). Additionally, CTGFti was >10% in 88% of extended criteria donor (ECD) kidneys, versus 64% of kidneys from living donors, 62% of standard criteria donors (SCD) and 59% of deceased of cardiac death (DCD) kidneys (P = 0.011 for ECD versus other groups). In multivariable logistic regression, independent predictors of moderate-to-extensive CTGF staining were ECD kidney and i score >0 (P < 0.05 for both).

In cross-sectional analysis at 3 months, CTGFu correlated with proteinuria (r = 0.375, P < 0.001) and CTGFp (r = 0.196, P = 0.013), but not with A1M, eGFR, FeCTGF, or CTGFti. In multivariable analysis, independent predictors of CTGFu were proteinuria and CTGFp [B = 3.320 (1.825–4.816), R^2 0.136, P < 0.0001 and B = 0.484 (0.131–0.837), R^2 0.034, P = 0.007, respectively], together explaining 17% of variability in CTGFu. There was a significant interaction between proteinuria and CTGFp, the predictive model for CTGFu [B = 1.631 (0.901–2.361), R^2 for full model including interaction term 0.228, P < 0.0001], indicating that the increase in CTGFu observed with increasing proteinuria became more strongly pronounced when CTGFp was high.

Connective tissue growth factor staining did not correlate with plasma CTGF (CTGFp) (P = 0.776) or FeCTGF (P = 0.290). CTGFp is not further discussed, as it was not predictive of any outcome parameter (data not shown). At the 12-month time point, CTGFu correlated weakly with CTGFp (r = 0.178, P = 0.032) and eGFR (r = −0.211, P = 0.011), but not with proteinuria, CTGFti, or FeCTGF.

Table 2. Patient characteristics over time.

Characteristic	Month 3	Year 1	Year 5	*P* value
Clinical characteristics				
eGFR (ml/min/1.73 m²)	50.3 ± 15.0	55.5 ± 16.2	52.5 ± 18.1	0.063
Proteinuria (g/g creatinine)				
<0.3	155 (96.9%)	153 (95.6%)	140 (90.9%)	0.051
0.3–1	5 (3.1%)	5 (3.1%)	13 (8.4%)	
>1	0	2 (1.3%)	1 (0.6%)	
PTDM (cumulative incidence)	15 (9.4%)	27 (16.9%)	35 (21.9%)	<0.001
Selected histology				
Number of available protocol biopsies	160	146	160	
CTGFti area				
<1%	18 (11.3%)	4 (2.8%)	–	0.001
1–10%	36 (22.5%)	28 (19.6%)	–	
11–25%	58 (36.3%)	38 (26.6%)	–	
>25%	30 (30.0%)	73 (51%)	–	
BPAR (cumulative incidence)	28 (17.5%)	31 (19.4%)	34 (21.3%)	0.332
PVAN (cumulative incidence)	2 (1.3%)	5 (3.1%)	8 (5%)	0.031
IF/TA score	1.0 ± 1.0	1.6 ± 1.1	2.5 ± 1.8	<0.001
Subclinical acute rejection	1 (0.6%)	3 (2.1%)	3 (1.9%)	0.625
Borderline acute rejection	20 (12.5%)	9 (6.2%)	10 (6.3%)	0.078
Fibrosis with inflammation	3 (1.9%)	7 (2.7%)	14 (8.8%)	0.013
Urinary markers				
Urinary CTGF (pmol/g creatinine)	270 (178–470)	238 (141–389)	–	0.039
Plasma CTGF (pmol)	18.7 (14.2–25.7)	18.0 (13.9–25.5)	–	0.981
Fractional excretion of CTGF (%)	4.4 ± 3.2	4.6 ± 4.2	–	0.601
Urinary A1M (mg/g creatinine)	2.4 (0–4.6)	–	–	NA

A1M, α1-microglobulin; BPAR, biopsy-proven acute rejection; CTGF, connective tissue growth factor; CTGFti, tubulointerstitial CTGF-positive surface area; eGFR, estimated glomerular filtration rate; NA, not available; PTDM, post-transplant diabetes mellitus; and PVAN, polyomavirus-associated nephropathy.

IF/TA score is the sum of ci and ct scores. Fibrosis with inflammation is defined as ci > 0 and i > 0. CTGF and A1M values are median (interquartile range).

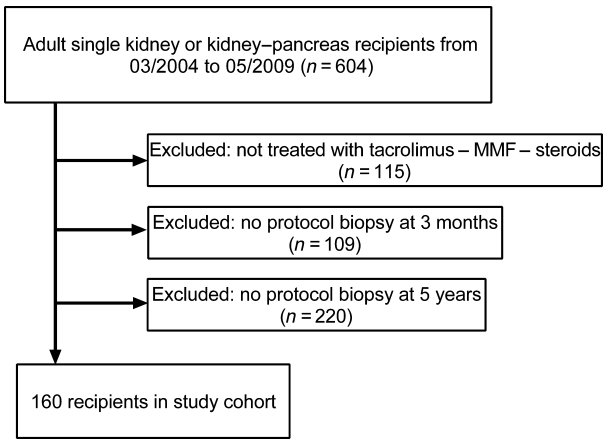

Figure 1 Flowchart of patient selection.

Prediction of fibrosis at 5 years

First, predictive models were constructed using only predictor variables available at 3 months. Uni- and multivariable predictors of IF/TA score ≥2 at 5 years (which occurred in 104/160 or 65% of patients) are presented in Table 3. Independent predictors of IF/TA were donor age and CTGFti >10%; CTGFu was not ($P = 0.066$). Donor age and CTGFti >10% were also independent predictors of the individual ci and ct scores (Tables S2 and S3). The relative value of CTGFti and CTGFu when added to donor age to predict development of fibrosis and/or tubular atrophy was evaluated in several subgroups of patients with favorable histologic features at 3 months, namely either no fibrosis (ci score 0), very low IF/TA score (≤1) or absence of any fibrosis, tubular atrophy, tubulitis, or interstitial inflammation, as shown in Tables 4 and S4–S6. Donor age, use of induction therapy, CTGFti >10%, and CTGFu were independently predictive of progression of fibrosis or IF/TA score in all three of these subgroups. Figure 3 illustrates the fact that in patients with IF/TA score ≤1 at 3 months, progression of IF/TA by 5 years was more pronounced in those who had 3-month CTGF >10%. A

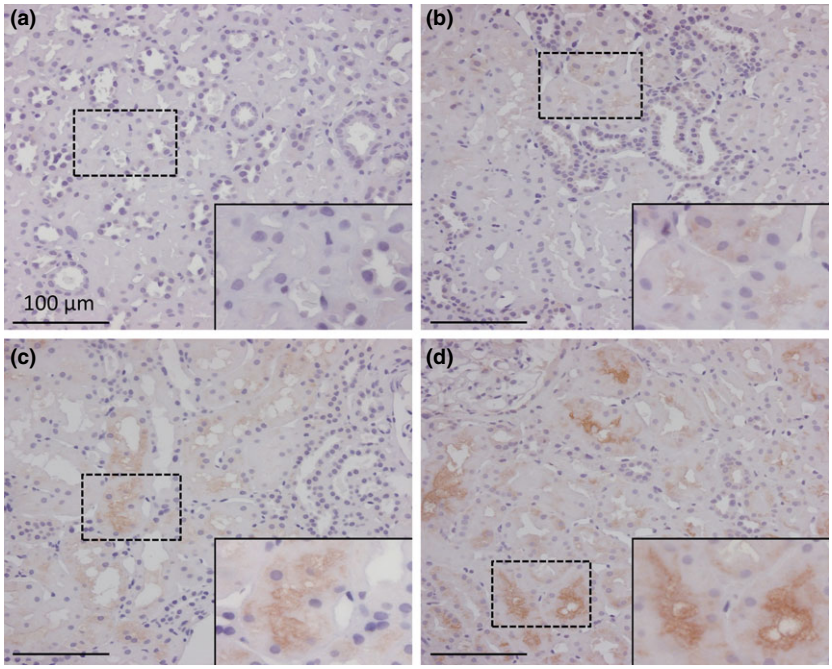

Figure 2 Representative tubulointerstitial connective tissue growth factor (CTGF) staining pattern. The four categories of staining intensity are (a) absent (<1%), (b) minimal (1–10%), (c) moderate (11–25%), and (d) extensive (>25%).

model combining donor age, CTGFti, and CTGFu had a positive predictive value (PPV) of 89.3% and negative predictive value (NPV) of 53.5% for development of *de novo* fibrosis, with receiver operating characteristic (ROC) curve area under the curve (AUC) of 0.76. Leave-one-out cross-validation of this model showed similar results [AUC 0.74 (0.65–0.84)]. When only donor age was included in this model, PPV was 84.0%, NPV was 46.5%, and ROC AUC was 0.70. Model performance of other combinations of predictor variables for these three subgroups is reported in Tables S7–S9. ROC curves for development of *de novo* fibrosis and IF/TA progression are shown in Fig. 4.

All of the above analyses were repeated using 1-year histologic lesions, clinical variables, and CTGFti and CTGFu values (available for 146 patients) as predictors of histology at 5 years. Independent predictors of ci, ct, and IF/TA ≥2 are presented in Tables S10–S15. At his time point, CTGFti and CTGFu were not predictive of 5-year histology. This remained true if the cut-off for "positive" CTGF staining was raised from 11% to 25% (data not shown).

Renal function and graft loss

Average renal function remained stable over the 5-year follow-up period, as shown in Table 2. Renal function declined >10 ml/min/1.73 m² in 16.2% of patients. In cross-sectional analysis at month 3, independent predictors of eGFR were ECD kidney, DGF, IF/TA score, and fractional excretion of CTGF (Table S16). Independent (month 3) predictors of eGFR at 5 years were eGFR at 3 months and donor age (Table S17).

Reduction in eGFR (%) between month 3 and year 5 was independently predicted by 3-month eGFR [B = 0.895 (0.566–1.224), $P < 0.001$], donor age [B = 0.703 (0.376–1.030), $P < 0.001$], and 3-month IF/TA score [B = 4.861 (0.229–9.492), P = 0.040]. Increase in IF/TA score over time was correlated with decrease in eGFR, but only to a limited degree (r = 0.167, P = 0.039). In the subgroup of patients with IF/TA score ≤1 at month 3 (n = 123), average change in eGFR did not differ between patients who progressed to IF/TA score ≥2 and patients who demonstrated no IF/TA score progression (P = 0.412). CTGFti or CTGFu at month 3 or 12 and the degree of change (delta) in CTGFti or CTGFu between month 3 and 12 did not correlate with eGFR at any time point and did not predict change in eGFR over time.

After the 5-year protocol biopsy, there were seven cases of death with a functioning graft and zero cases of graft loss censored for death over an average follow-up period of 7.6 years (range 5.6–10.8). As a result, whether CTGFti and CTGFu predict death-censored graft loss could not be assessed.

Transplant International 2017; 30: 695–705

Table 3. Uni- and multivariable predictors of IF/TA score ≥2 at 5 years.

	Univariable analysis			Multivariable analysis		
Predictors	OR	CI	*P* value	OR	CI	*P* value
Donor characteristics						
Age (years)	1.04	1.02–1.07	<0.001	1.05	1.02–1.08	0.001*
Gender (female)	0.62	0.32–1.22	0.167	0.40	0.18–0.90	0.026*
Donor type (ECD versus other)	3.49	1.14–10.70	0.029			
Cold ischemia time (h)	0.99	0.94–1.05	0.773			
Recipient characteristics						
Age (years)	0.99	0.97–1.02	0.515			
Gender (female)	2.14	1.07–4.30	0.032			
PRA > 20%†	3.88	0.34–526.37	0.301			
Diabetes mellitus pretransplant	0.88	0.36–2.16	0.781			
Transplant characteristics						
Repeat transplant	0.66	0.23–1.89	0.442	0.38	0.10–1.36	0.136
Number of HLA mismatches	1.27	0.97–1.65	0.082			
Induction therapy	1.78	0.87–3.62	0.112	2.76	1.10–6.89	0.030*
Delayed graft function	1.09	0.35–3.35	0.887			
Variables at 3 months						
eGFR (ml/min/1.73 m²)	0.99	0.97–1.02	0.687			
Proteinuria >300 mg/g‡	0.80	0.13–4.95	0.812			
Urinary A1M	0.61	0.33–1.12	0.113	0.57	0.30–1.10	0.089
BPAR	1.34	0.55–3.30	0.522			
Subclinical/borderline acute rejection	1.86	0.64–5.36	0.254			
Fibrosis with inflammation§	3.90	0.37–527.42	0.297			
CTGFti >10%	3.00	1.51–5.96	0.002	2.72	1.20–6.15	0.016*
CTGFu	1.50	1.03–2.18	0.034	2.00	0.70–5.73	0.109
IF/TA score ≥2	2.52	0.69–9.26	0.163	1.20	0.43–3.32	0.731

A1M, α1-microglobulin; BPAR, biopsy-proven acute rejection; CI, 95% confidence interval; CTGF, connective tissue growth factor; CTGFti, tubulointerstitial CTGF-positive surface area; CTGFu, urinary CTGF concentration; ECD, extended criteria donor; eGFR, estimated glomerular filtration rate; OR, odds ratio; and PRA, panel reactive antibody titer.

Fibrosis with inflammation is defined as ci > 0 and i > 0. IF/TA score ≥2 at 5 years occurred in 104/160 patients (65%).

*Significant at $P < 0.05$ in final model.

†Complete separation of data: all three patients with PRA > 20% had IF/TA score ≥2 at 5 years.

‡At 3 months, no patients had proteinuria >1 g/g creatinine.

§Complete separation of data: all three patients with fibrosis with inflammation at 3 months had IF/TA score ≥2 at 3 months and 5 years.

Discussion

In this study, tubulointerstitial CTGF expression was an independent predictor of interstitial fibrosis and tubular atrophy at 5 years after transplantation in a cohort of low-risk, stable renal recipients. These findings are in agreement with some [8] but not all [9] earlier studies assessing the prognostic value of early (mRNA level) CTGF gene expression. Particularly in patients with only minimal chronic histologic damage (either ci score 0 or IF/TA score ≤1) at 3 months, development of fibrosis and progression of IF/TA were determined by donor age, use of induction therapy, CTGFti, and CTGFu. The relationship between induction therapy and accelerated progression of fibrosis was likely confounded because induction therapy was reserved for patients at high immunological risk. Even though CTGF expression was higher in the presence of subclinical inflammation and in ECD kidneys, only CTGFti independently predicted allograft fibrosis in multivariable analysis. This could be due to the fact that CTGF expression might reflect early fibrogenic processes across the full spectrum of renal allografts, including non-ECD kidneys without any histologic evidence of subclinical inflammation. This is illustrated by the fact that in the subgroup of patients with ci, ct, i, and t scores of 0 at 3 months, 71% ($n = 32$) had CTGFti >10%, which was associated with a strongly increased risk of progressing to IF/TA score

Table 4. Analyses for progression of fibrosis and tubular atrophy.

Outcome parameter	Predictors at month 3	Odds ratio (CI)	*P* value
Subgroup with IF/TA score ≤1 at month 3 (*n* = 123)			
IF/TA ≥2 at 5 years (*n* = 77)	Donor age	1.08 (1.03–1.12)	0.001
	CTGFti >10%	4.43 (1.63–12.04)	0.004
	CTGFu	7.68 (1.68–35.16)	0.009
	Induction therapy	4.12 (1.35–12.59)	0.016
Subgroup with ci score 0 at month 3 (*n* = 119)			
ci score ≥1 at 5 years (*n* = 75)	Donor age	1.08 (1.04–1.13)	<0.001
	CTGFti >10%	3.27 (1.19–8.95)	0.021
	CTGFu	9.19 (1.87–45.27)	0.006
	Induction therapy	3.16 (1.06–9.46)	0.039
Subgroup with IF/TA, t and i scores of 0 at month 3 (*n* = 45)			
IF/TA ≥2 at 5 years (*n* = 32)	Donor age	1.09 (1.02–1.16)	0.014
	CTGFti >10%	20.34 (2.23–185.73)	0.008
	CTGFu	19.94 (1.59–180.90)	0.019
	Induction therapy	12.43 (1.25–123.30)	0.031
	Donor gender (female)	0.20 (0.02–1.66)	0.136

CI, 95% confidence interval; and IF/TA, interstitial fibrosis and tubular atrophy (sum of ci and ct scores).
CTGFu is 10-log transformed.

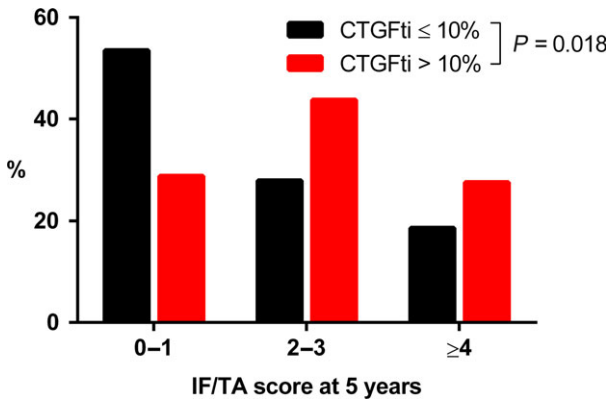

Figure 3 Differences in 5-year IF/TA score (sum of Banff ci and ct scores) by 3-month connective tissue growth factor (CTGF) staining intensity in the subgroup of patients with IF/TA score ≤1 at 3 months.

≥2 by 5 years, after correction for donor age, use of induction therapy and CTGFu. These findings are compatible with microarray data, which have demonstrated frequent and significant upregulation of genes related to immunity, inflammation, remodeling, and fibrosis in histologically normal protocol biopsies [8,16].

Our results confirm that donor age is not only of the dominant predictors of baseline histologic damage [17,18], but is also associated with an accelerated progression of IF/TA regardless of baseline histology [19–21]. The predictive model for *de novo* development of fibrosis was only modestly improved by adding CTGFti and CTGFu to donor age, which is not surprising given that 5-year fibrosis reflects the cumulative burden of injury sustained over the entire follow-up period. Many other factors may have contributed after the 3 month point.

CTGF undergoes glomerular filtration, tubular reabsorption (which is quasi-complete under normal circumstances) and can be produced by tubular epithelial cells, immune cells, and mesangial cells (including fibroblasts) [3,22,23]. The presence of CTGF in urine can theoretically indicate (i) intrarenal production, (ii) tubular dysfunction, and (iii) saturation of tubular reabsorption, either resulting from increased filtration due to glomerular damage or because of high plasma concentrations. In this analysis, however, CTGFu did not correlate with A1M, an established marker of tubular proteinuria. Proteinuria and CTGFp did correlate with CTGFu, and the presence of an interaction between proteinuria and CTGFp indicates that they magnified each other's effect: CTGF was particularly prone to be present in urine if high systemic CTGF concentrations were combined with (presumed) glomerular damage. However, the fact that CTGFp and proteinuria only explained 17% of interindividual variability in CTGFu seems to indicate that filtration and glomerular damage are not dominant factors. It is likely that intrarenal fibrogenesis is another key factor. Ideally, quantification of CTGF mRNA expression on biopsies would clarify the relative contributions of these mechanisms but,

Transplant International 2017; 30: 695–705

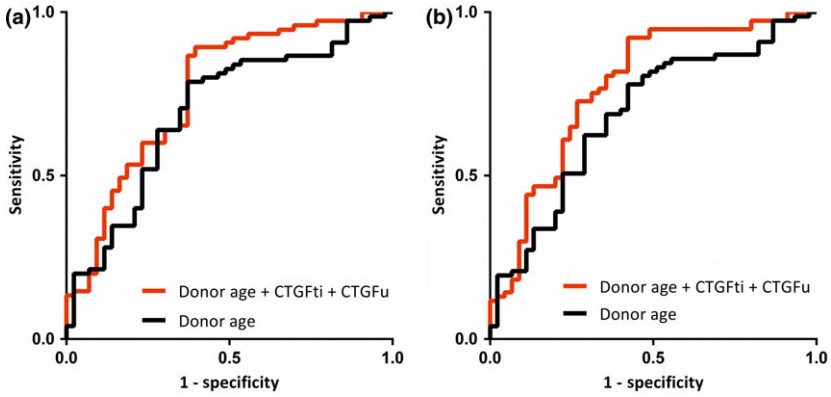

Figure 4 ROC analysis. The combination of donor age, tubulointerstitial CTGF-positive surface area (CTGFti), and urine CTGF concentration (CTGFu) outperformed donor age alone in (a) predicting *de novo* fibrosis (ci > 0) by year 5 in the subgroup without fibrosis at 3 months (AUC = 0.76 vs. 0.70) and in (b) predicting progression to IF/TA score ≥2 in the subgroup with IF/TA score ≤1 at 3 months (AUC = 0.78 vs. 0.68).

unfortunately, this was not possible on the current cohort (discussed under limitations). Previous research suggests that the relative importance of intrarenal fibrogenesis, filtration, and tubular dysfunction depends on study context and underlying pathology. Gerritsen *et al.* [24] performed a study in which recombinant CTGF was infused in diabetic mice, which led them to estimate that 60% of CTGFu was produced intrarenally and 40% originated from plasma. The same authors reported a strong correlation ($r = 0.85$) between CTGFu and B2M in a variety of human glomerular diseases, suggesting that, in this population, it mainly reflected tubular dysfunction [22], contrasting with our population of renal recipients. Regardless of the origins and biology of CTGF in renal recipients, these results indicate that it is an attractive candidate biomarker to include in future studies examining early prediction of long-term allograft outcome. It would almost certainly perform best when combined with other markers, as no single predictor can be expected to capture the complexities and temporal dynamics of renal inflammation and fibrogenesis.

This study has several limitations. First, study design was based on availability of paired protocol biopsies, which allowed for an analysis of factors that predict evolution of histology in individual recipients. However, the resulting cohort was selected for graft survival and compliance (as patients often refuse their 5-year protocol biopsy) and, therefore, biased with regard to clinical endpoints. This precludes a reliable analysis of the predictive performance of CTGFti and CTGFu concerning renal function and graft loss, particularly since no death-censored graft loss occurred during the extended follow-up period. Renal function was stable in most patients and correlated cross-sectionally with the degree of IF/TA, but progression of IF/TA and decrease in eGFR were only weakly correlated. CTGF expression was not predictive of renal function at 5 years. As it is known that chronic histologic damage is a risk factor for graft loss [25], it is likely that the accelerated progression of IF/TA related to high early CTGF expression will eventually translate into renal functional decline. However, this will need to be addressed separately in future studies correlating CTGFti at 3 months with hard outcome parameters in an unselected population. Second, protocol biopsies have limitations, such as interobserver variability and sampling error. The former does not apply to this study because a single pathologist (EL) scored all biopsies. Sampling error was partly offset by the use of paired biopsies, where every patient was his own historic control. Third, CTGF expression could not be assessed on the mRNA level because, at the time, biopsies were not yet stored in an RNA stabilization solution, and *in situ* hybridization of mRNA on paraffin slides was not successful, possibly because of mRNA degradation over time. We cannot exclude that part of the tubulointerstitial CTGF protein originated from tubular reabsorption of circulating CTGF. However, CTGF protein and mRNA expression have been shown to correlate well in the tubulointerstitium of renal allografts in mice [2] and the glomeruli of adult humans [26]. Fourth, DSA was not systematically determined during this period and could not be included as covariable.

In conclusion, early tubulointerstitial expression and urinary excretion of CTGF are independent predictors of IF/TA at 5 years after transplantation in low-risk

renal recipients with a relatively benign clinical course, even in those with very favorable histology at 3 months.

Authorship

TV: researched the data and wrote the article. HK: performed the CTF scoring. EL: performed the Banff histological assessments. KP: provided analytical techniques. TV, DK, RG and TN: designed the study. HK, MN, TN, CM, KP, EL, RG and DK: reviewed the article.

Funding

The authors have declared no funding.

Conflict of interest

Roel Goldschmeding has received research support from FibroGen Inc. (San Francisco, CA, USA) and spent a sabbatical year as a senior research fellow at this company. FibroGen develops anti-CTGF drugs. The other authors declare no conflict of interest.

Acknowledgements

The authors thank R. Broekhuizen, T. Coopmans, J. de Loor, and M. Dekens for their technical assistance and M. Coemans for his statistical advice.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Evolution of histologic lesions over time.

Table S2. Uni- and multivariable predictors of ci score at 5 years.

Table S3. Uni- and multivariable predictors of ct score at 5 years.

Table S4. Subgroup with ci score 0 at month 3 (n = 119): predictors of progression to ci score ≥1 at 5 years (n = 75).

Table S5. Subgroup with IF/TA score ≤1 at month 3 (n = 123): predictors of progression to IF/TA ≥2 at 5 years (n = 77).

Table S6. Subgroup with IF/TA, t and i scores of 0 at month 3 (n = 45): predictors of progression to IF/TA score ≥2 at 5 years (n = 32).

Table S7. Performance of different models in predicting *de novo* ci score ≥1 at 5 years in patients with ci score 0 at month 3 (n = 119).

Table S8. Performance of different models in predicting *de novo* IF/TA score ≥2 at 5 years in patients with IF/TA score ≤1 at month 3 (n = 123).

Table S9. Performance of different models in predicting *de novo* IF/TA score ≥2 at 5 years in patients with IF/TA, t and i scores of 0 at month 3 (n = 45).

Table S10. Twelve month predictors of ci score at 5 years.

Table S11. Twelve month predictors of ct score at 5 years.

Table S12. Twelve month predictors of IF/TA ≥2 at 5 years.

Table S13. Subgroup with ci score 0 at month 12 (n = 88): predictors of progression to ci score ≥1 at 5 years.

Table S14. Subgroup with IF/TA score ≤1 at month 12 (n = 76): predictors of progression to IF/TA score ≥2 at 5 years.

Table S15. Subgroup with IF/TA score, t and I scores of 0 at month 12 (n = 16): predictors of progression to IF/TA score ≥2 at 5 years.

Table S16. Month 3 predictors of renal function at month 3.

Table S17. Month 3 predictors of renal function at 5 years.

REFERENCES

1. Okada H, Kikuta T, Kobayashi T, *et al.* Connective tissue growth factor expressed in tubular epithelium plays a pivotal role in renal fibrogenesis. *J Am Soc Nephrol* 2005; **16**: 133.
2. Cheng O, Thuillier R, Sampson E, *et al.* Connective tissue growth factor is a biomarker and mediator of kidney allograft fibrosis. *Am J Transplant* 2006; **6**: 2292.
3. Ito Y, Aten J, Bende RJ, *et al.* Expression of connective tissue growth factor in human renal fibrosis. *Kidney Int* 1998; **53**: 853.
4. Metalidis C, van Vuuren SH, Broekhuizen R, *et al.* Urinary connective tissue growth factor is associated with human renal allograft fibrogenesis. *Transplantation* 2013; **96**: 494.
5. Yokoi H, Mukoyama M, Nagae T, *et al.* Reduction in connective tissue growth factor by antisense treatment ameliorates renal tubulointerstitial fibrosis. *J Am Soc Nephrol* 2004; **15**: 1430.
6. Luo GH, Lu YP, Song J, *et al.* Inhibition of connective tissue growth factor by small interfering RNA prevents renal fibrosis in rats undergoing chronic

allograft nephropathy. *Transplant Proc* 2008; **40**: 2365.
7. Adler SG, Schwartz S, Williams ME, *et al.* Phase 1 study of anti-CTGF monoclonal antibody in patients with diabetes and microalbuminuria. *Clin J Am Soc Nephrol* 2010; **5**: 1420.
8. Vitalone MJ, O'Connell PJ, Wavamunno M, *et al.* Transcriptome changes of chronic tubulointerstitial damage in early kidney transplantation. *Transplantation* 2010; **89**: 537.
9. Menon MC, Chuang PY, Li Z, *et al.* Intronic locus determines SHROOM3 expression and potentiates renal allograft fibrosis. *J Clin Invest* 2015; **125**: 208.
10. Racusen LC, Colvin RB, Solez K, *et al.* Antibody-mediated rejection criteria – an addition to the Banff 97 classification of renal allograft rejection. *Am J Transplant* 2003; **3**: 708.
11. Levey AS, Greene T, Schluchter MD, *et al.* Glomerular filtration rate measurements in clinical trials. Modification of Diet in Renal Disease Study Group and the Diabetes Control and Complications Trial Research Group. *J Am Soc Nephrol* 1993; **4**: 1159.
12. Nguyen TQT, Tarnow L, Andersen S, *et al.* Urinary connective tissue growth factor excretion correlates with clinical markers of renal disease in a large population of type 1 diabetic patients with diabetic nephropathy. *Diabetes Care* 2006; **29**: 83.
13. Falke LL, Dendooven A, Leeuwis JW, *et al.* Hemizygous deletion of CTGF/CCN2 does not suffice to prevent fibrosis of the severely injured kidney. *Matrix Biol* 2012; **31**: 421.
14. Heinze G, Dunkler D. Five myths about variable selection. *Transpl Int* 2017; **30**: 6.
15. Heinze G, Schemper M. A solution to the problem of separation in logistic regression. *Stat Med* 2002; **21**: 2409.
16. Park W, Griffin M, Grande JP, *et al.* Molecular evidence of injury and inflammation in normal and fibrotic renal allografts one year posttransplant. *Transplantation* 2007; **83**: 1466.
17. De Vusser K, Lerut E, Kuypers D, *et al.* The predictive value of kidney allograft baseline biopsies for long-term graft survival. *J Am Soc Nephrol* 2013; **24**: 1913.
18. Cosio FG, Grande JP, Wadei H, *et al.* Predicting subsequent decline in kidney allograft function from early surveillance biopsies. *Am J Transplant* 2005; **5**: 2464.
19. Nankivell BJ, Borrows RJ, Fung CL-S, *et al.* The natural history of chronic allograft nephropathy. *N Engl J Med* 2003; **349**: 2326.
20. Naesens M, Lerut E, de Jonge H, *et al.* Donor age and renal P-glycoprotein expression associate with chronic histological damage in renal allografts. *J Am Soc Nephrol* 2009; **20**: 2468.
21. Vanhove T, Vermeulen T, Annaert P, *et al.* High intrapatient variability of tacrolimus concentrations predicts accelerated progression of chronic histologic lesions in renal recipients. *Am J Transplant* 2016; pub ahead of print doi: 10.1111/ajt.13803: 1.
22. Gerritsen KG, Peters HP, Nguyen TQ, *et al.* Renal proximal tubular dysfunction is a major determinant of urinary connective tissue growth factor excretion. *Am J Physiol Renal Physiol* 2010; **298**: F1457.
23. Gerritsen KG, Abrahams AC, Peters HP, *et al.* Effect of GFR on plasma N-terminal connective tissue growth factor (CTGF) concentrations. *Am J Kidney Dis* 2012; **59**: 619.
24. Gerritsen KGF, Leeuwis JW, Koeners MP, *et al.* Elevated urinary connective tissue growth factor in diabetic nephropathy is caused by local production and tubular dysfunction. *J Diabetes Res* 2015; **2015**: 539787.
25. Naesens M, Kuypers DRJ, De Vusser K, *et al.* Chronic histological damage in early indication biopsies is an independent risk factor for late renal allograft failure. *Am J Transplant* 2013; **13**: 86.
26. Ito Y, Goldschmeding R, Kasuga H, *et al.* Expression patterns of connective tissue growth factor and of TGF-beta isoforms during glomerular injury recapitulate glomerulogenesis. *Am J Physiol Renal Physiol* 2010; **299**: F545.