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Visual interstitial fibrosis assessment as continuous variable in protocol renal transplant biopsies

Anke Keijbeck,[1] Anniek Raaijmaakers,[1] Lisa Hillen,[2] Marielle Gelens,[3] Sander van Kuijk,[4] Jack P M Cleutjens,[1] Carine Peutz-Kootstra[1] & Maarten Christiaans[3]

[1]*Department of Pathology, Cardiovascular Research Institute Maastricht (CARIM), Maastricht University Medical Centre,* [2]*Department of Pathology, GROW School for Oncology and Developmental Biology, Maastricht University Medical Centre,* [3]*Department of Internal Medicine, Division of Nephrology, School of Nutrition and Translational Research in Metabolism University Maastricht (NUTRIM), Maastricht University Medical Centre and* [4]*Department of Clinical Epidemiology and Medical Technology Assessment (KEMTA), Maastricht University Medical Centre, Maastricht, the Netherlands*

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Aims: In current renal transplant pathology practice, interstitial fibrosis is visually assessed in categories according to the Banff classification. As this has a moderate reproducibility, which is little ameliorated by morphometric analysis, we investigated whether visual renal fibrosis assessment is feasible on a continuous scale, i.e. as a percentage of affected area of the cortex.

Methods and results: Protocol renal biopsies taken at transplantation (n = 125), three (n = 73) and 12 months (n = 88) after transplantation were visually scored in categories (Banff) and percentages for interstitial fibrosis (ci). Interobserver variation (ICC and weighted κ) was assessed, and morphometric analysis on Sirius red-stained sections was performed. Correlations between the different methods and their association with donor age and eGFR 1 and 5 years post-transplant were analysed using Pearson's or Spearman's rho. Interobserver agreement was equivalent for Banff and %ci (κ = 0.713 versus ICC = 0.792), and for Banff IF/TA and %IF/TA (κ = 0.615 versus ICC = 0.743). Both Banff and %ci were associated with Sirius red morphometry in 3 and 12 months. With all three methods, a significant correlation was found between donor age and fibrosis in the implantation biopsy and between fibrosis in the 12 months' biopsy and eGFR at 1 and 5 years (eGFR at 1 year: Sirius red ρ = 0.487, %ci ρ = 0.393, Banff ρ = 0.413, all $P < 0.01$, eGFR at 5 years: Sirius red ρ = 0.392, %ci ρ = 0.333, Banff ρ = 0.435, all $P < 0.01$).

Conclusion: Interstitial fibrosis assessment on a continuous scale can be used next to scoring in categories according to the Banff classification in protocol renal transplant biopsies.

Keywords: biopsy, fibrosis, kidney transplantation, Sirius red

Introduction

Kidney transplantation is the treatment of choice for patients with end-stage renal disease; however, allograft survival is limited because of the development of chronic transplant dysfunction.[1,2] Interstitial fibrosis and tubular atrophy (IF/TA) is the histological hallmark lesion of chronic transplant dysfunction, and IF/TA in protocol renal biopsies correlates with renal function and long-term outcome.[3,4] In the Banff classification,[5] severity of IF/TA is scored in categories according to the extent of the cortex affected (see

Address for correspondence: A Keijbeck, Department of Pathology, Cardiovascular Research Instilute Maastricht (CARIM), Maastricht University Medical Center, Maastricht, the Netherlands. e-mail: ankekeijbeck@gmail.com

Table 1), which is also conducted for the separate parameters interstitial fibrosis (Banff ci) and tubular atrophy (Banff cut). Interobserver agreement for Banff ci is fair to substantial,[6–11] and differs in relation to biopsy type (wedge versus core and frozen versus paraffin)[7,8] and the pathologist's experience.[11] As interstitial fibrosis assessment in renal transplant biopsies is used on a daily basis in clinical practice and may even serve as a surrogate endpoint in clinical trials,[3,12] there is a need to enhance its accuracy and reproducibility.

Automated morphometric methods using Sirius red-stained sections have been applied in renal transplant pathology (summarised in Table 2). With this technique a percentage of affected cortex is given, and there is substantial interassay[10,13,14] and interobserver[10] correlation. Sirius red positivity is correlated with renal function[10,13–15] and with Banff ci in some,[10,13,14] but not all,[16] studies. Despite these advantages, morphometric fibrosis assessment has not yet widely reached clinical practice, as additional stains, digitalisation of images and morphometry software are needed. We hypothesised that visual assessment of interstitial fibrosis as a percentage (i.e. on a continuous scale) can combine the benefits of visual assessment with the quantitative approach of morphometry. Visual assessment of fibrosis was performed on a categorical and continuous scale, and compared with Sirius red morphometry in protocol renal transplant biopsies.

Material and methods

PATIENTS AND CLINICAL DATA

Patients transplanted in Maastricht UMC+ between April 2003 and December 2009, who received a tacrolimus-based immunosuppressive regimen, and from whom a Sirius red stain of a representative protocol biopsy at reperfusion, and/or 3 and 12 months after transplantation was available, were included in this study. Donor characteristics and follow-up data were retrieved from patient files. As additional immunosuppression, all patients received either sirolimus or mycophenolate mofetil, with an early withdrawal of steroids.[17] Estimated glomerular filtration rate (eGFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-epi) formula.[18]

Collection, storage and use of tissue and patient data were performed in agreement with the code of conduct for 'Proper Secondary Use of Human Tissue', as described by 'the Federation of Dutch medical scientific societies' (http://www.federa.org). Permission for this study was obtained from the Medical Ethical Committee of the MUMC+ (MEC 09-4-002).

RENAL BIOPSIES AND ANALYSIS

Paraffin tissue for light microscopy was cut at 3 μm and stained with haematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and methenamine silver periodic acid-Schiff (Jones), according to standard staining protocols. Sirius red staining was performed on archival paraffin tissue. In short, deparaffinised tissue was incubated in 0.2% molybdatophosphoric acid (Merck, Kennilworth, NJ, USA) for 5 min at room temperature followed by 90 min incubation at room temperature in 0.1% Sirius red in picric acid solution (both Sirius red and picric acid from Klinipath, Duiven, the Netherlands). Slides were then rinsed in 0.01 M hydrochloric acid, and tissue was dehydrated using subsequent ethanol steps.

For this study, renal biopsies were considered representative if they contained at least seven glomeruli and one interlobular artery, according to Banff criteria.[19] All renal biopsies were rescored for interstitial fibrosis in accordance with the Banff classification, using the PAS- and Jones-stained sections[5] by a blinded renal pathologist (L.H.). Banff parameters were scored in percentages as well as in categories. For interobserver variability, a subset of biopsies was scored for the same parameters by a second, blinded renal pathologist (C.P.K.).

Computerised evaluation of non-polarised Sirius red-positive tissue was performed to assess interstitial fibrosis by one blinded observer (A.R.). Per biopsy, 10 images of

Table 1. Visual assessment of interstitial fibrosis (ci), tubular atrophy (ct) and interstitial fibrosis and tubular atrophy (IF/TA) according to the Banff classification

		Score			
Banff lesion	Abbreviation	0	1	2	3
Interstitial fibrosis	ci	0–5%	6–25%	26–50%	> 50%
Tubular atrophy	ct	0%	1–25%	26–50%	> 50%
Interstitial fibrosis and tubular atrophy	IF/TA	ci 0 and ct 0	ci1 and/or ct 1	ci 2 and/or ct 2	ci 3 and/or ct3

Table 2. Overview of studies using Sirius red morphometry for interstitial fibrosis assessment in renal biopsies

Study	Patients	Results
Diaz Encarnacion *et al*. 2004[13]	CAN $n = 49$	Interassay correlation SR: ICC 0.84 SR is associated with Banff ci ($\rho = 0.57$, $P < 0.01$), and eGFR ($\rho = -0.29$, $P = 0.05$).
Farris *et al*. 2011[14]	Native kidney disease ($n = 14$) and postTx ($n = 1$)	Inter assay correlation SR: $R^2 = 0.96$, $P < 0.001$ SR associated with: ci% ($R^2 = 0.86$, $P < 0.001$), and eGFR ($R^2 = 0.45$, $P < 0.05$)
Nara *et al*. 2017[16]	$N = 144$ protocol post-transplant biopsies at M0 and M12 (mainly living donors)	No association Banff ci and SR at M12 ($\rho = -0.112$, $P = 0.927$)
Scholten *et al*. 2006[20]	Protocol post-transplant M6 ($n = 94$), M12 ($n = 97$)	SR is associated with IF/TA score (ρ not given, $P < 0.001$)
Rowshani *et al*. 2006[15]	Protocol post-transplant M6 ($n = 94$) and M12 ($n = 97$)	SR M6 and M12 associated with renal function M6 and M12 (ρ not given, $P = 0.03$ and $P = 0.05$, respectively)
Dao *et al*. 2020[10]	Post-transplant biopsies M0 ($n = 43$), d15–20 ($n = 20$), M3 ($n = 28$) and M12 ($n = 28$) DCD type 2	SR Interobserver correlation ICC = 0.75 (95% CI = 0.67–0.81) ($n = 151$) SR Intraobserver correlation ICC 0.88 (95% CI = 0.72–0.95) ($n = 21$) SR is associated with Banff ci ($\rho = 0.62$, $P < 0.001$) SR and serum creatinine are correlated at 1 year ($R^2 = 0.32$, $P = 0.013$)

CAN, chronic allograft nephropathy; ci, interstitial fibrosis; eGFR, estimated glomerular filtration rate; SR, Sirius red; TIF, tubulointerstitial fibrosis.

the renal cortex were taken in a serpentine manner. All pictures were taken with a non-polarised light microscope (Leica DM3000) at objective ×40. Medullary tissue, blood vessels and glomeruli were excluded when images were acquired. All images were processed by an image processing and analysis system (QWin, Leica's Windows-based image analysis tool kit; Leica, Cambridge, UK); Sirius red-positive tissue area was quantified by a custom-made macro. The software identified Sirius red-positive tissue and Sirius red-positive tissue was expressed as percentage of total analysed cortical tissue. See Figure 1 for representative examples.

STATISTICS

Continuous data are presented as mean with standard deviation or median and range, where appropriate. Categorical data are given as number with percentage. Interobserver agreement of continuous parameters was tested using intraclass correlation coefficient (ICC), with two-way random-effects model with absolute agreement definition. ICC with 95% confidence interval (95% CI) is presented. Interobserver agreement of ordered categorical parameters was tested using weighted kappa with squared weights; kappa values are given. Association between Sirius red and ci% or IF/TA% and between Sirius red and renal function was tested by Pearson's rho test. Association between Sirius red and Banff IF/TA or Banff ci score and the association between Banff ci score and renal function was tested using the Spearman's rho test. $P < 0.05$ was considered statistically significant. All analyses were executed using SPSS version 25.0 (IBM SPSS, Chicago, IL, USA).

Results

PATIENTS

A total of 144 renal transplant recipients were included for analysis. Of these patients 286 biopsies were scored: 125 implantation biopsies, 73 biopsies taken at 3 months and 88 biopsies taken at 12 months. As shown in Table 3, grafts were from living (21.5%), deceased after brain death (37.5%) and deceased after cardiac death (41%) donors. Mean donor age was 51.2 ± 13.9 years, and 81 (56.3%) of donors were male.

INTEROBSERVER AGREEMENT OF VISUAL INTERSTITIAL FIBROSIS ASSESSMENT AS CATEGORICAL AND CONTINUOUS VARIABLE

To address interobserver variability, a subset of 147 biopsies was available ($n = 48$ implantation, $n = 43$ at

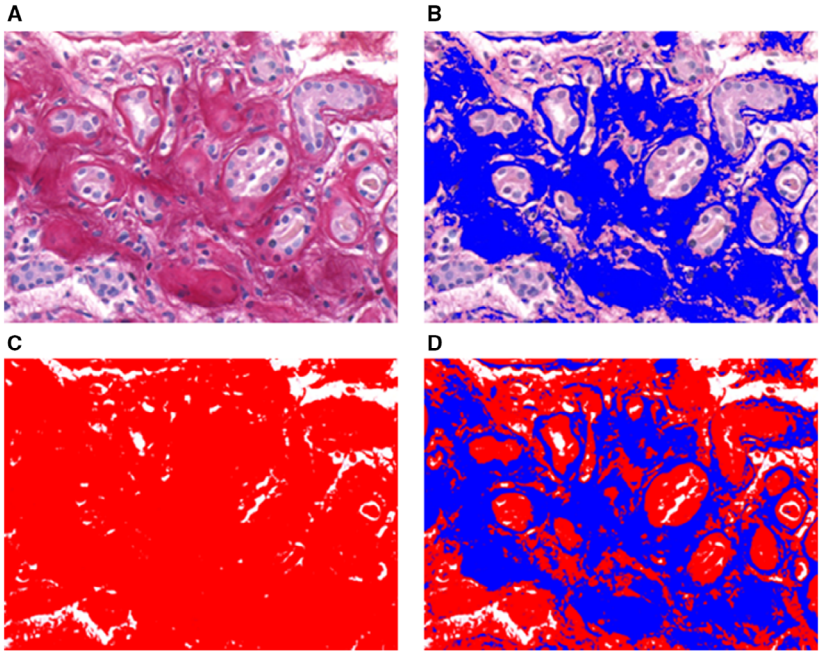

Figure 1. A, A representative example of a picture at objective 40× of a Sirius red-stained renal biopsy taken at 12 months with moderate fibrosis (35% in total biopsy). **B,** The step in which the Quin software detects the Sirius red-stained tissue. **C,** The detection of all renal tissue by the software and (**D**) is the overlay generated by the Leica Qwin software of the image shown in (**A**). The all blue area is classified as Sirius red-positive tissue.

3 months and $n = 56$ at 12 months), and data are given in Table 4. For the categorical assessment on a 0–3 scale, overall interobserver agreement for Banff ci was κ0.713 and for Banff IF/TA κ0.615. For fibrosis as continuous parameter (ci% and IF/TA%), ICCs also showed substantial agreement. For both categorical Banff scorings and continuous % scoring methods, ICCs for Banff ci were higher than for Banff IF/TA, which is especially apparent in the 12 months' protocol biopsy.

COMPARISON OF VISUAL FIBROSIS ASSESSMENT ON A CONTINUOUS SCALE WITH SIRIUS RED MORPHOMETRY

As Sirius red morphometry gives interstitial fibrosis assessment as a percentage, a comparison of Sirius red with %ci and Banff ci was made (Table 5). As expected, higher %ci was associated with more Sirius red ($\rho = 0.437$, $P < 0.01$). Fibrosis measurements correlated better in biopsies taken 3 and 12 months after transplantation than in the implantation biopsies (implantation $\rho = 0.198$, $P = 0.027$, 3 months $\rho = 0.602$ $P < 0.001$ and 12 months $\rho = 0.506$ $P < 0.001$) (Table 5, Figure 2). As can be seen from Figure 2, in the implantation biopsies % Sirius red was higher than %ci.

A significant correlation with donor age is found with both assessment methods for interstitial fibrosis in the implantation biopsy (ci% $\rho = 0.290$, $P = 0.001$, Sirius red $\rho = 0.220$, $P = 0.013$). Banff ci correlated with eGFR at 3 months ($\rho = 0.432$, $P < 0.001$) and 12 months (ρ 0.413, $P < 0.001$). Also for ci% and Sirius red there was a significant negative correlation between fibrosis in the 3 months' biopsy and eGFR at 3 months (ci% $\rho = 0.404$, $P < 0.01$; Sirius red $\rho = 0.468$, $P < 0.01$, Figure 3A, B). Both methods showed a significant negative correlation with eGFR after 1 year, i.e. for the 3 months biopsy (ci% $\rho = 0.337$, $P = 0.004$; Sirius red $\rho = 0.446$, $P < 0.001$) as well as at 12 months (ci%: $\rho = 0.393$, $P < 0.001$, Sirius red: $\rho = 0.487$, $P < 0.001$). The correlation between Banff ci M3 and eGFR M12 is rho – 0.408, $P < 0.001$. After 5 years eGFR measurements of 67 patients with a functioning graft were available. The mean eGFR in this group after 5 years was 48.7 ± 20.6 ml/min/1.73 m^2. Fibrosis in the 12 months biopsy correlated with eGFR after 5 years with all three methods (Banff ci $\rho = 0.435$, $P < 0.001$; Sirius red $\rho = 0.392$, $P = 0.001$; %ci $\rho = 0.333$, P 0.006).

For 51 patients biopsies from all three time-points were available, and progression of fibrosis within the

Table 3. Cohort characteristics

	n = 144
Donor characteristics	
Donor age (years)	51.2 ± 13.9
Donor sex male (%)	81 (56.3%)
Donor type	
Living donor (%)	31 (21.5%)
DBD (%)	54 (37.5%)
DCD (%)	59 (41.0%)
Recipient characteristics	
Recipient age (years)	54.4 ± 13.0
Recipient sex male (%)	86 (59.7%)
Cause of ESRD	
Glomerular disease	45 (31.3%)
Tubulointerstitial	18 (12.5%)
Diabetes mellitus	12 (8.3%)
Hypertension/renal vascular disease	18 (12.5%)
Systemic diseases affecting the kidney	4 (2.8%)
Familial/hereditary	26 (18.1%)
Miscellaneous	21 (14.6%)
Any acute rejection in first year (%)	33 (22.9%)

DBD, donation after brain death; DCD, donation after circulatory death; ESRD, end stage renal disease.

first year post-transplant was studied. The median increase in fibrosis assessed by %ci was 10.0% (range = −5.0 to 65.0%), while with Sirius red this increase was less pronounced median = +1.4% (range = −15.0 to 19.4%). The progression of %ci significantly correlated with Sirius red progression ($\rho = 0.435$, $P = 0.001$) during the first year after transplantation.

Discussion

We confirm that interstitial fibrosis in protocol post-transplant biopsies correlates with renal function.[3,4] Furthermore, interstitial fibrosis in the implantation biopsy correlates with donor age, in line with the literature.[20–22] Visual scoring of the percentage of the affected cortical area might give a more precise estimate of interstitial fibrosis compared to current categorical Banff scoring; however, for clinical application it needs to be reproducible and representative. Our comparison of three different methods shows that visual assessment of interstitial fibrosis as continuous parameter (%ci) performs equal to the categorical Banff scoring and morphometric analysis of Sirius red-stained sections.

Interobserver variation for ci% and IF/TA% is at least equivalent to categorical scoring according to Banff. In our setting of retrospectively scored paraffin-embedded needle biopsies, interobserver agreement for ci (scored on PAS and Jones) is relatively high when comparing with data from literature.[6–11] This relatively high interobserver agreement might be explained by the fact that we compared the scores of only two pathologists, while some studies included more pathologists.[6,23] Furthermore, the two pathologists in this study worked in the same institution. In addition, in literature pathologists use a wide range of different stains to score ci;[23] to the best of our knowledge, the effect of using different/other stains on the ICC has not yet been studied. Therefore, multicentre studies regarding interobserver agreement of ci %, preferably with standardised use of ancillary

Table 4. Interobserver agreement of two blinded pathologists for visual interstitial fibrosis (ci) and interstitial fibrosis and tubular atrophy (IF/TA) assessment

	Overall (*n* = 147)	0 months (*n* = 48)	3 months (*n* = 43)	12 months (*n* = 56)
Banff ci*	0.713	0.522	0.555	0.684
Banff IF/TA*	0.615	0.448	0.521	0.546
% ci†	0.792 (0.720–0.846)	0.810 (0.684–0.889)	0.751 (0.586–0.857)	0.723 (0.514–0.843)
% IF/TA†	0.743 (0.623–0.823)	0.763 (0.602–0.862)	0.770 (0.615–0.869)	0.620 (0.280–0.794)

*Weighted kappa.
†ICC (95% CI).

Table 5. Associations by Pearson's or Spearman's rho, between Sirius red (SR) morphometry and visual interstitial fibrosis (ci) and interstitial fibrosis and tubular atrophy (IF/TA) assessment

	0 months	3 months	12 months
Banff ci and SR†	0.164	0.451**	0.491**
Banff IF/TA and SR†	0.106	0.441**	0.441**
ci% and SR‡	0.198*	0.602**	0.506**
IF/TA% and SR‡	0.203*	0.594**	0.500**

†Spearman''s rho.
‡Pearson's rho.
*$P < 0.05$.
**$P < 0.001$.

histopathological stains, are needed. At all timepoints, there was more agreement between the two pathologists for ci than for IF/TA. This may be explained by the differing cut-offs and definitions, as ci is scored as 1 if 5–25% of the cortex is affected by interstitial fibrosis, while IF/TA is already scored as 1 if 1–25% of the cortex in the biopsy is affected.[5] Particularly in the 12 months' protocol biopsy, scoring of Banff ci may be preferable over scoring Banff IF/TA if interobserver variation needs minimisation. Of note, we scored percentages as a number with differences on a 5% scale, performed by eyeballing. Some pathologists prefer to use an ocular grid to quantify the extent of fibrosis, while both methods have not yet been compared.[23]

In this study we compared visual assessment of fibrosis with computerised assessment on unpolarised Sirius red. Sirius red can also be assessed on polarised light; however, several studies showed stronger correlations between unpolarised Sirius red with ci and eGFR than with polarised Sirius red.[13,14] We show that morphometric interstitial fibrosis assessment by Sirius red is associated with visual % ci and Banff ci scoring in protocol transplant biopsies, ranging from a weak association in the implantation biopsies to a moderate association at 3 and 12 months. This study therefore extends the earlier observation by Farris *et al.*, who investigated predominantly native kidney biopsies from 15 patients.[14] The weak association in the implantation biopsy may be explained by the presence of oedema in the implantation biopsy, which may give an overestimation of the Sirius red-positive tissue. In months 3 and 12 biopsies, ci% is often higher than Sirius red. This difference may

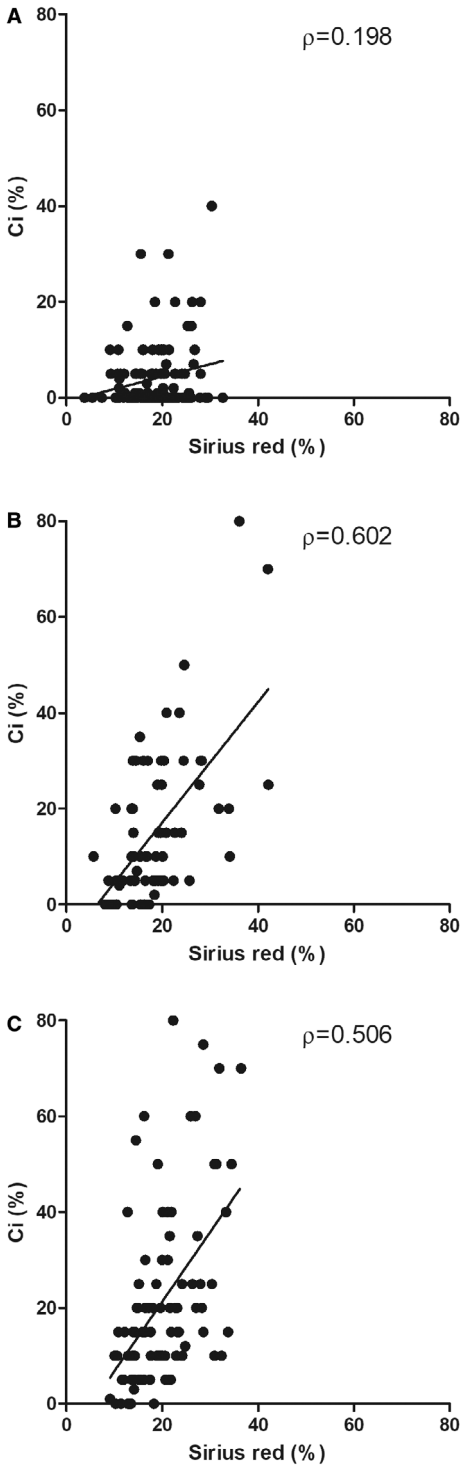

Figure 2. Association between interstitial fibrosis (ci%) and Sirius red association between ci% and Sirius red at (**A**) time of transplantation ($\rho = 0.198$, $P = 0.027$), (**B**) 3 months after transplantation ($\rho = 0.602$, $P < 0.001$) and (**C**) 12 months after transplantation ($\rho = 0.506$, $P < 0.001$).

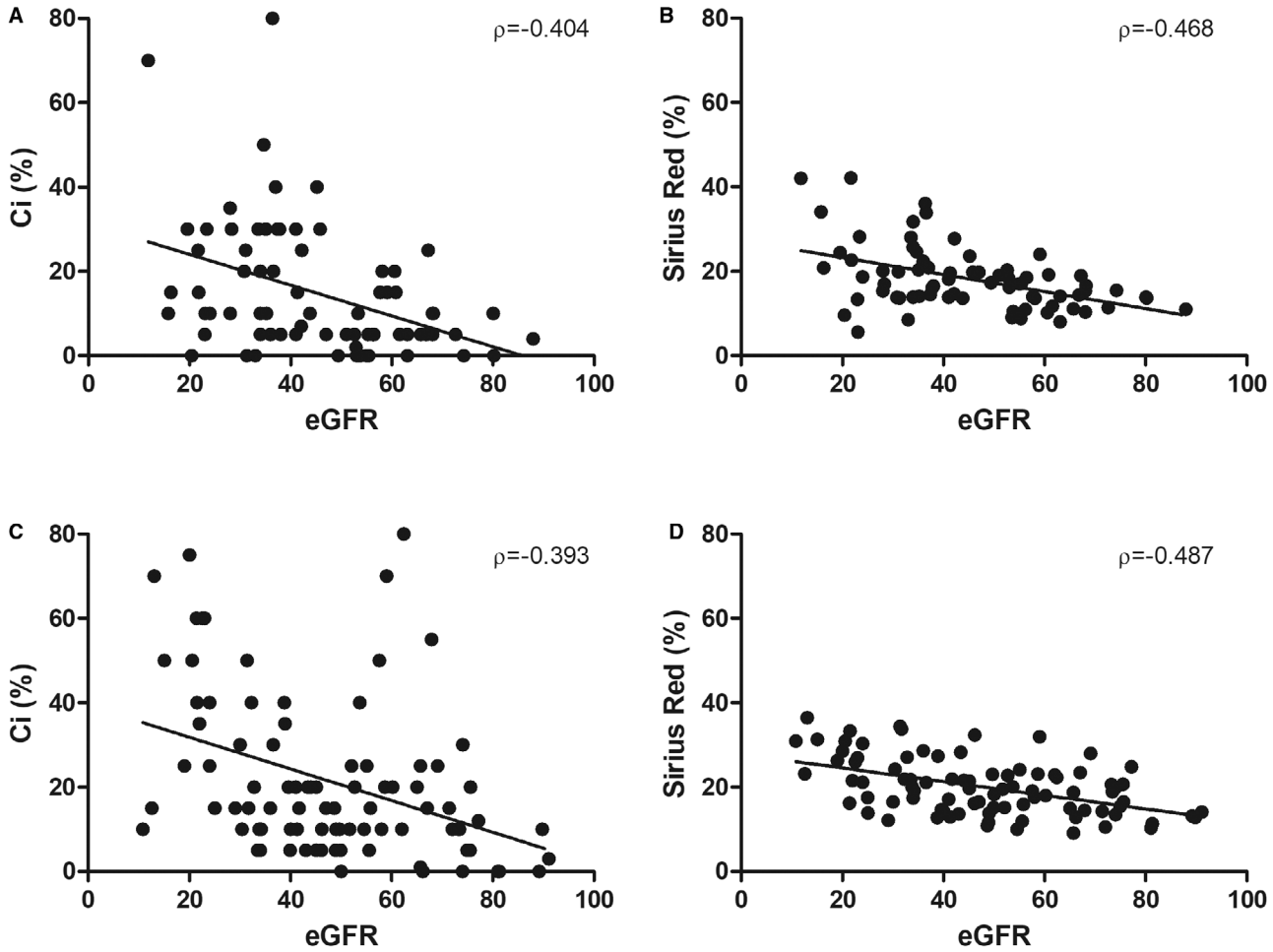

Figure 3. Association of interstitial fibrosis (ci%) and Sirius red with eGFR. **A,** Association between ci (%) and renal function (eGFR) 3 months after transplantation ($\rho = -0.404$, $P < 0.01$; slope = −0.36. **B,** Association between Sirius red (%) and renal function (eGFR) 3 months after transplantation ($\rho = -0.468$, $P < 0.01$; slope = −0.20). **C,** Association between ci (%) and renal function (eGFR) 12 months after transplantation ($\rho = -0.393$, $P < 0.001$; slope = −0.38). **D,** Association of Sirius red (%) and renal function (eGFR) at 12 months after transplantation ($\rho = -0.487$, $P < 0.001$; slope = −0.16).

be explained by the way that ci% is scored. Roufousse *et al.* describes that the Banff ci was meant to purely reflect the cortex composed of fibrous tissue.[5] However, in practice, tubular atrophy and interstitial fibrosis progress concomitantly. It may therefore be possible that the pathologist overestimates fibrosis when there is tubular atrophy. This may also contribute to the difference in fibrosis progression. We observed more fibrosis progression when assessed by %ci than by Sirius red, which is in line with findings of Sund *et al.* in a cohort of 32 transplants.[22] The observation that ci correlates with Sirius red morphometry is in line with most studies performed on renal transplant biopsies (summarised in Table 2). Only Nara *et al.*[16] did not observe a correlation between Banff ci and Sirius red morphometry; however, they only studied grafts from living donors, who have less extensive IF/TA than grafts from deceased donors.[17,20]

Furthermore, there was a negative association of both %ci, Banff ci and Sirius red with renal function (eGFR), as has been described previously.[13–15] Future studies are needed to study whether %ci adds prognostic value for the prediction of eGFR decline. At present, morphometric fibrosis assessment is not feasible in day-to-day clinical practice, which may alter with the ongoing introduction of digital pathology and artificial intelligence software applications.[24,25] Therefore, it may be beneficial to compare visual fibrosis assessment as a continuous parameter with these innovative techniques in future studies. Artificial intelligence approaches may also aid in standardising fibrosis assessment by pathologists in different centres, but

development and validation of algorithms in large and publicly available data sets is needed.[26]

It remains to be established whether our findings can be extrapolated to biopsies taken because of clinical deterioration of graft function, and/or to kidney biopsies with primary renal disease, as we only studied protocol renal biopsies. In tumour nephrectomy specimens, scoring of IF/TA density (number of IF/TA foci per area cortex) predicts progression of chronic kidney disease, independent of IF/TA percentage.[27] Whether scoring of IF/TA foci in renal transplant biopsies is feasible and useful also remains to be established. We could only study %ci and Sirius red progression in a subgroup of 51 patients in our cohort. Further studies, with multivariable models, are needed to investigate whether IF/TA or ci as continuous variable has added value over current Banff classification in the prediction of renal function decline and graft failure. In summary, our data support the introduction of scoring ci and IF/TA on a continuous scale in clinical practice.

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Conflicts of interest

The authors declare no conflicts of interest.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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