Chapter 6

Improvement of microvascular damage after living donor kidney-transplantation

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Abstract

Background: Chronic kidney disease (CKD) is associated with endothelial damage and microvascular destabilization. Recently, sidestream dark-field (SDF) imaging has emerged as a noninvasive tool to visualize the microcirculation. In this study, we investigated whether CKD is associated with systemic microvascular damage using SDF imaging. In addition, the effects of kidney transplantation (KTx) on microvascular alterations was studied.

Methods: Mean capillary density and microvascular morphology were visualized with SDF imaging of the oral mucosa. Twenty-eight CKD patients were studied longitudinally before (CKD), 1, 6 and 12 months after living donor KTx. Furthermore, circulating levels of growth factors that control microvascular structure, including Angiopoietin-2 (Ang-2) and soluble Thrombomodulin (sTM) were measured using ELISA.

Results: We found increased capillary tortuosity in CKD patients compared to controls and reversibility starting at 6 months after KTx (respectively mean 1.9±0, SEM and 1.6±0, $p<0.05$). In line with these findings, endothelial destabilization markers Ang-2 (4084±612 pg/ml) and sTM (19±6 ng/ml) were increased in CKD patients and showed an improvement starting at 1 month after KTx (2263±316 pg/ml and 6.1±1 ng/ml respectively, $p<0.05$).

Conclusion: The microcirculation, as assessed by SDF, was disturbed in CKD patients compared to controls. Interestingly, KTx resulted in an improvement of microvascular tortuosity and normalization of angiogenic growth factors. Our findings indicate a clinical implication of SDF imaging to assess microvascular alterations in CKD patients before and after KTx.

Introduction

Progressive renal disease is characterized by a loss of the microvasculature, which is associated with the development of glomerular and tubulointerstitial scarring. The development of tubulointerstitial fibrosis is characteristic of chronic kidney disease (CKD) of diverse etiologies, and inhibition of its progression has been proposed to be of major importance in the preservation of renal function. In both experimental animal models and in humans, it has been shown that a significant loss of peritubular capillaries as well as defective capillary repair, triggers development of fibrotic changes and ultimately scar formation with progressive renal dysfunction (1;2). Besides renal microvascular abnormalities, patients with CKD demonstrate abnormalities which may represent manifestations of ongoing systemic microvascular damage, including a dysbalance of angiogenic factors and an increased incidence of atherosclerosis (3).

The microvasculature is relatively inaccessible to direct examination. Therefore investigators have concentrated on various surrogate markers of endothelial function which include the measurement of specific plasma markers including, circulating endothelial cells (CECs), angiopoietin-1 (Ang-1), Angiopoietin-2 (Ang-2) and soluble Thrombomodulin (sTM) (4-8). Competitive inhibition of Ang-2 to the Tie-2 receptor, destabilizes quiescent endothelium and leads to altered endothelial/pericyte interaction with subsequently abnormal microvascular remodeling (9-11). Different studies have assessed the expression of these factors in CKD and after kidney transplantation (KTx). Indeed, elevated circulating levels of Ang-2 and sTM were observed in CKD patients, which normalized after kidney transplantation (5;7).

An additional method for the assessment of endothelial function in CKD patients includes monitoring of the systemic microcirculation. Recently, SDF imaging has been used to visualize the human circulation (10;12). Using this non-invasive technique, we could previously demonstrate that patients with diabetes mellitus (DM) had significantly more microvascular damage compared to healthy controls and simultanous pancreas-kidney transplantation (SPK) resulted in restoration of microvascular damage (10). However, there are no studies using SDF imaging in CKD patients, to monitor microvascular alterations before and after KTx. The aim of our study was to use this validated SDF imaging technique to compare the labial mucosal capillary tortuosity, as markers for microvascular disease, in patients with CKD. In addition, we investigated whether KTx improves the microvasculature, in a prospective longitudinal study up to 1 year after transplantation. Furthermore, we assessed whether microvascular alterations were associated with increase in endothelial dysfunction markers. We hypothesize that CKD patients have a disturbed systemic microvasculature and that KTx will reverse microvascular damage.

Material and Methods

Patients

All procedures were approved by the institutions Medical Ethical Committee. Written informed consent was obtained from all the patients and healthy controls. A total of 48 persons (30 males and 18 females) were enrolled in the current longitudinal study after giving informed consent. We included 28 CKD patients with different dialysis modalities who were receiving a living donor KTx including 11 patients on hemodialysis (HD), 4 peritoneal dialysis (PD) patients and 13 preemptive kidney transplant recipients. Biochemical markers for endothelial dysfunction including Ang-2 and sTM, together with mucosal capillary density and morphology were compared to healthy and age matched volunteers who served as control group. None of the control subjects was taking medication. Patients with active infection, liver failure, active auto-immune disease, epilepsy or malignancy in the last 5 years (except patients treated for basal cell carcinoma that were in full remission) were excluded from the study. SDF measurements and the analysis of endothelial dysfunction markers were assessed prior to transplantation (CKD), and 1 (M1), 6 (M6) and 12 (M12) months after transplantation.

Transplantation aspects

All kidney transplantations were performed at the Leiden University Medical Center (LUMC) between 2010 and 2012 in the Netherlands. Kidney transplantation was performed as described previously (10). Patients were treated with basiliximab as induction therapy (20 mg on the day of transplantation and 4 days after transplantation) followed by triple therapy with prednisone (tapered to a dose of 10 mg by 6 weeks), tacrolimus (area under the curve (AUC) 210 ng.h/ml first 6 weeks, then 125 ng.h/ml) or cyclosporine (AUC 5400 ng.h/ml first 6 weeks then 3250 ng.h/ml) and mycophenolate mofetil (MMF) (AUC 30-60 ng.h/ml). Patients were treated routinely with oral valganciclovir prophylaxis for 3 months, except for cytomegalovirus (CMV) negative recipients receiving a CMV-negative graft.

Microcirculatory imaging and analysis of SDF measurements

The SDF microscan (MicroVision Medical Inc., Wallingford, PA, U.S.A) and analyses were performed as described earlier (10;13).

Laboratory assessment and endothelial structure evaluation

All persons enrolled in this study underwent routine venous blood sampling before the morning intake of immunosuppression. The following data were evaluated: creatinin, urea, HbA1c, glucose, hemoglobin and proteinuria in 24 hours urine. Glomerular filtration rate (eGFR) was calculated with creatinin concentration using the Modification of Diet in Renal Disease (MDRD) formula. Simultaneously, blood was collected for analysis of serum Ang-2

and sTM. Blood collection tubes were centrifuged for 10 minutes at 3000 rpm after which serum was stored in microcentrifuge tubes at -20oC until required for analysis. Ang-2 and sTM concentrations were measured by enzyme-linked immuno sorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA and Diaclone Research, Besançon, France) according to the manufacturer supplied protocol. The intra-and inter-assay coefficients of variation were 6.5% and 9.1% for Ang-2 levels and 3.9% and 9.8%, respectively, for sTM levels.

Statistical analyses

Continuous normally distributed data are presented as mean ± SEM, unless stated otherwise. Comparisons of mean differences between the four time points in the longitudinal study were performed using repeated measures ANOVA. Correlations between interval variables were calculated using the Spearman rank correlation. Differences between 2 groups were analyzed using the unpaired two-sample T-test. When criteria for parametric testing were not met, median and interquartile range (IQR) are presented and tested with the Mann-Whitney test. Categorical variables were analyzed by a Chi-square test. In addition, multivariable linear regression was used to adjust for possible confounders. Differences were considered statistically significant with $p<0.05$. Data analysis was performed using SPSS version 17.0 (SPSS Inc, Chicago, IL) and GraphPad Prism, version 5.0 (GraphPad Prism Software Inc, San Diego, CA).

Results

Patient characteristics

Baseline characteristics are shown in Table 1. There were no significant differences between CKD patients and healthy controls, with exception for eGFR and hematocrit levels ($p<0.05$). As expected, KTx showed improvement of eGFR and proteinuria ($p<0.05$). After transplantation, 2 patients experienced interstitial rejection treated with methylprednisolone. In total 4 patient developed diabetes type II (1 patient at M1; 3 patients at M6) treated with oral antidiabetics or insulin. In 3 patients diabetes mellitus was resolved at 12 months after transplantation. HbA1c levels were not significantly different after KTx compared to before transplantation ($p>0.05$).

Table 1: Patient characteristics of controls and chronic kidney disease patients before (CKD), 1 month (M1), 6 months (M6) and 12 months (M12) after kidney transplantation.

Patients characteristics	Controls (N=20)	CKD (N=28)	M1 (N=28)	M6 (N=28)	M12 (N=28)
Age (years)	44.8 ±1	47.1 ±14	47.3 ±14	47.8 ±14	48.8 ±14
Sex, male N (%)	10 (50%)	20 (71%)	-	-	-
Smoking, N (%)	0 (0%)	3 (11%)	-	-	-
Primary kidney disease, N (%)	-		-	-	-
Glomerulonephritis		7 (25%)			
Focal segmental glomerulosclerosis		5 (18%)			
Urologic		2 (7%)			
Polycytic kidney disease		7 (25%)			
Hypertension		2 (7%)			
Unknown		4 (14%)			
Other		1 (4%)			
BMI (kg/m^2)	25.3 ±4	26.4 ±4	25.9 ±4	26.9 ±4	27.0 ±4
Dialysis, N (%)	-		0 (0%)	0 (0%)	0 (0%)
HD		11 (40%)			
PD		4 (14%)			
Preemptive		13 (46%)			
Median dialysis duration (years)	-	1.0 (1.0-2.0)	-	-	-
Systolic BP (mmHg)	135 ±18	138 ±15	131 ±18	131 ±12	134 ±1
Diastolic BP (mmHg)	83 ±9	84 ±12	78 ±7	79 ±7	81 ±8
eGFR (ml/min/1,73 m^2)	92 ±16	8.8 ±4*	51.7 ±15*#	53.5 ±13*#	54.3 ±14*#
Median proteinuria (g/24hr) (IQR)	-	1.5 (0.8-2.4)	0.2 (0.2-0.3)#	0.2 (0.2-0.4)#	0.2 (0.2-0.3)#
HbA1c (%)	-	5.2 ±0	5.3 ±1	5.5 ±1	5.6 ±1
Glucose (mmol/L)	5.3 ±1	5.3 ±1	6.0 ±2	5.7 ±1	5.8 ±2
Hematocrit (L/L)	0.4 ±0	0.4 ±0	0.4 ±0	0.4 ±0	0.4 ±0
Anti-hypertensives, N (%)	-				
ACE inhibitor		13 (46%)	3 (11%)	6 (21%)	8 (29%)
Diuretics		18 (64%)	1 (4%)	5 (18%)	4 (14%)
β-blockers		10 (36%)	12 (43%)	9 (32%)	7 (25%)
Calcium antagonists		12 (43%)	21 (75%)	20 (71%)	16 (57%)
Angiotensin-II antagonists		10 (36%)	2 (7%)	3 (11%)	7 (25%)
Statines, N (%)	-	13 (46%)	4 (14%)	10 (36%)	10 (36%)
Acetylsalicylic acid, N (%)	-	6 (21%)	3 (10%)	2 (7%)	2 (7%)
Immunosuppressive, N (%)	-	-			
Cyclosporine			3 (11%)	6 (21%)	4 (14%)
Tacrolimus			24 (86%)	19 (68%)	19 (68%)
Prednisone			28 (100%)	28 (100%)	28 (100%)
Everolimus			1 (3%)	3 (11%)	5 (18%)
Mycophenolate mofetil			27 (96%)	26 (93%)	24 (86%)
Donor characteristics	-		-	-	-
Age (years)		56.0 ±13			
Sex, male N (%)		10 (36%)			
Warm ischemia time (minutes)		29.5 ±8			

All data are mean ±SD, unless otherwise specified. * $p<0.05$ vs controls. # $p<0.05$ vs CKD. BMI, body mass index; BP, blood pressure; ACE, angiotensin converting enzyme; eGFR,estimated glomerular filtration rate; IQR, interquartile range;

CKD patients have increased capillary tortuosity and angiogenic factors compared with controls

Our study demonstrated a markedly disturbed microvasculature with increased capillary tortuosity in CKD patients (mean 1.9 ±0, SEM) compared with healthy controls (mean 1.6 ±0, SEM, $p<0.05$) (Fig 1A). Dialysis modalities did not influence capillary tortuosity. Mean vessel density was not significantly different between CKD (mean 19.9 ±1, SEM) and controls (mean 21.4 ±1, SEM, $p>0.05$).

Consistently, Ang-2 and sTM levels were elevated in CKD patients (mean 4084 ±612 pg/ml and 19 ±6 ng/ml, SEM, respectively) compared with healthy controls (mean 2291 ±326 pg/ml and 6.6 ±0 ng/ml, SEM, $p<0.05$ and $p<0.0001$, respectively) (Fig 1B-C).

The difference in capillary tortuosity, Ang-2 and sTM between controls and CKD patients remained significant after adjustment for age, sex, body mass index (BMI), blood pressure and glucose levels.

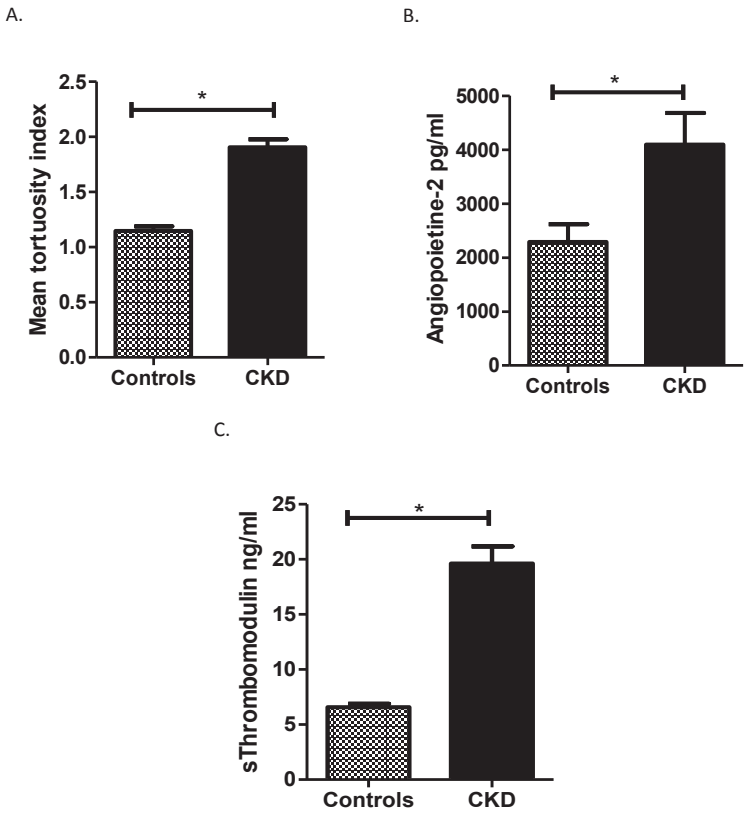


Figure 1. A. Mean tortuosity index of microvascular capillaries in the control and CKD group. Serum levels of Angiopoietin-2 (B) and Soluble thrombomodulin (C) levels in CKD patients and healthy controls. Data shown are mean±SEM. *P<0.05 compared to controls.

Kidney transplantation leads to reversal of microvascular tortuosity and decrease in circulating levels of Ang-2 and sTM

After KTx a significant decrease in capillary tortuosity was observed as early as 6 months (mean 1.6 ±0, SEM, p<0.001) following transplantation (Fig 2A). Mean vessel density did not show significant changes between the different time points after KTx (Fig 2B).

In line with the observed decrease of capillary tortuosity after KTx, Ang-2 levels showed a significant decrease already at 1 month (mean 2263 ±316 pg/ml, SEM, p<0.05) after KTx and remained decreased up to 1 year (mean 2080 ±281 pg/ml, SEM, p<0.01) after transplantation (Fig 2C). sTM levels started to decrease at 1 month after transplantation (mean 6 ±1 ng/ml, SEM, p<0.001) compared with before KTx (Fig 2D). After correction for age, sex, BMI, blood pressure, glucose levels and smoking the differences remained significant for capillary tortuosity, Ang-2 and sTM.

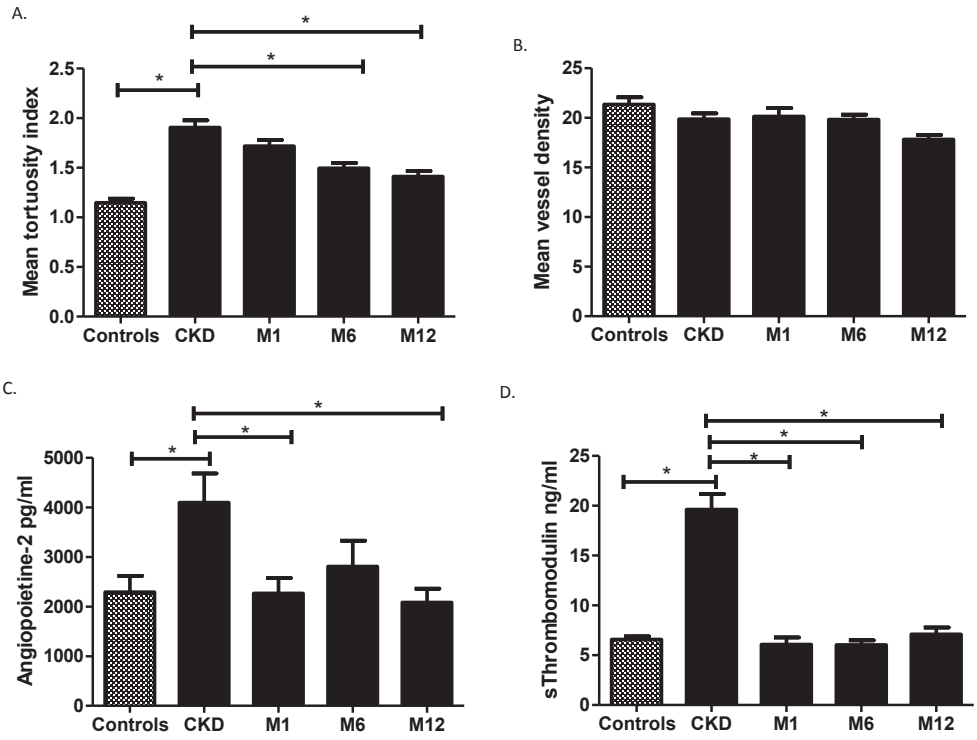
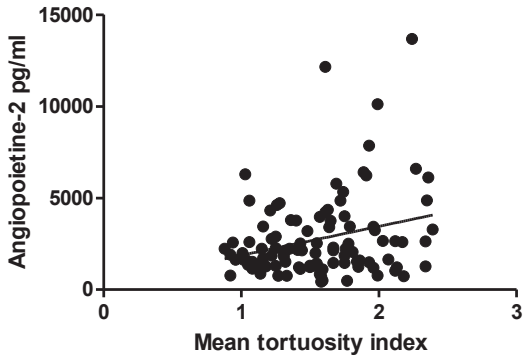


Figure 2. A. Longitudinal course of mean tortuosity index (A) and vessel density (B) before (CKD), 1 (M1), 6 (M6) and 12 (M12) months after KTx. Longitudinal course of serum levels Angiopoietin-2 (C) and soluble thrombomodulin (D). Data shown are mean±SEM. *P<0.05.

Correlation analyses

Spearmans correlation analyses were performed between Ang-2, sTM serum levels and capillary tortuosity, glucose levels, calcineurin inhibitor use (CNI) and proteinuria. The Ang-2 (r=0.1893, p=0.05; Fig 3A), sTM (r=0.2917, p=0.0014) and proteinuria (r=0.3036, p=0.0025) were positively correlated with microvascular tortuosity. In addition, a negative correlation was observed between eGFR, tortuosity (r=-0.5762, p<0.0001; Fig 3B), Ang-2 (r=-0.2352, p=0.0114) and sTM (r=-0.4959, p<0.0001). Moreover, sTM correlated positively with proteinuria levels (r=0.4188, p<0.001) and no correlation between Ang-2 with proteinuria was observed. No correlation was found between the different markers and tortuosity with glucose levels and CNI use.

A.



B.

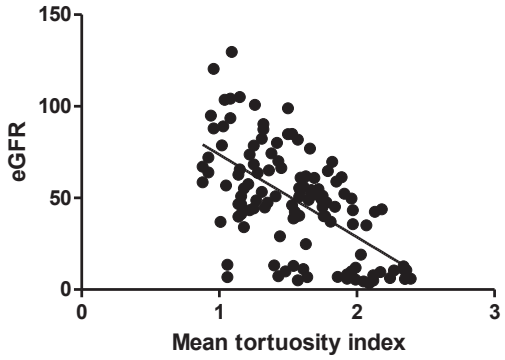


Figure 3. A. Scatter plots with correlation analyses between Angiopoietin-2 (A) and eGFR (B) and mean tortuosity index.

Discussion

The present study demonstrated increased capillary tortuosity in CKD patients using SDF imaging, and elevated levels of markers for endothelial destabilization Ang-2 and sTM. Interestingly, KTx showed reversal of microvascular damage and normalization of Ang-2 and sTM levels, within 1 year after transplantation. Furthermore, we found a correlation between capillary tortuosity and Ang-2 and sTM. These data suggest that KTx is effective in reversing microvascular damage early after transplantation. Early non-invasive monitoring of the microvasculature may be of great clinical value to assess progression and treatment efficacy of microvascular disease in CKD patients before and after KTx.

A major contributor to microvascular damage in CKD patients is the accumulation of uremic toxins, which leads to changes in renal EC structure and function that favor microvascular injury, which may play a role as a trigger for the inflammatory response (14). Microvascular destabilization induces local areas of interstitial hypoxia and is associated with a dysbalance of angiogenic growth factors (9;11). Increased expression or release of Ang-2 after EC injury has been shown to destabilize capillaries and to increase inflammation and vessel leakage, by promoting the weakening of pericyte-EC interaction (9). Activation of pericytes will lead to differentiation of pericytes into collagen producing myofibroblasts and detachement from ECs (1;15-17). This process is accompanied by failure of reparative angiogenesis and consequently formation of unstable and tortuous vessels (1;10).

In the current study a significantly disturbed systemic microvasculature was observed in patients with CKD compared to healthy controls using SDF imaging. Using this imaging technique, we could previously demonstrate increased capillary tortuosity in patients with diabetic nephropathy (10). To date, there are no studies investigating the systemic changes or abnormalities in non-diabetic CKD patients before and after KTx using SDF imaging and correlated this with angiogenic growth factors and renal function. In a study of Snoeijs et al, SDF imaging was used to study the human renal microcirculation after KTx by direct visualization of cortical peritubular capillaries. In this study, ischemic acute kidney injury was associated with reduced cortical microvascular blood flow (12). Moreover, Edwards et al. showed an association between retinal microvascular alterations and renal dysfunction (3).
In our recent study in diabetic nephropathy patients, KTx alone did not result in reversal of capillary tortuosity and angiogenic growth factors (10). Previous studies have demonstrated that improvement in glucose control with pancreas transplantation, performed simultaneously or after KTx, is the most important therapeutic approach of microvascular disease in diabetes mellitus patients (18;19). These findings suggest, in concordance with our previous study, that in diabetic nephropathy patients, normalization of renal function alone is not sufficient to

restore microvascular damage, but glycemic control is also necessary (10). However, In this study, KTx resulted in reversal of microvascular tortuosity within 1 year after transplantation and renal function correlated significantly with capillary tortuosity. Thus, in non-diabetic CKD patients, normalization of uremic state is able to improve microvascular damage evident in these patients.

Another interesting finding is that the observed increase in capillary tortuosity coincided with increase in Ang-2 and sTM levels in CKD and showed the same course after KTx. Moreover, there was also a correlation between Ang-2, sTM and capillary tortuosity. Our data suggest that these factors might participate in the pathogenesis of microvascular tortuosity in CKD. This is consistent with previous reports which demonstrated an association between microvascular damage and these angiogenic markers (4;6;10;29). Consistent with our observation, David et al demonstrated increased Ang-2 levels in serum of CKD patients and normalization after KTx (5). Recently, we reported on the relation between endothelial activation and increased renal expression of Ang-2 in rats subjected to renal ischemia reperfusion injury (I/R), which was accompanied by proliferation of pericytes, endothelial cell loss and development of fibrosis (16). Moreover, de Vries et al demonstrated Ang-2 release in the circulation from human kidneys grafts shortly after reperfusion (30).

In conclusion, this study demonstrates systemic microvascular damage in CKD patients, reflected by increased capillary tortuosity and angiogenic growth factors. Kidney transplantation resulted in reversal of capillary tortuosity and normalization of angiogenic growth factor levels. Given the central role of microvascular damage in CKD, therapies aiming at restoring the renal microvasculature might be an effective strategy to prevent profibrotic responses in CKD. Our findings indicate a clinical implication of SDF imaging to assess microvascular alterations after in CKD patients before and after KTx.

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