Original Paper

Impaired Microcirculation in Children After Kidney Transplantation: Everolimus Versus Mycophenolate Based Immunosuppression Regimen

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Key Words

Everolimus • Skin microcirculation • Endothelial dysfunction • Children • Kidney transplantation • Atherosclerosis

Abstract

Background/Aims: Whether the immunosuppressive regimen is associated with micro- and macro-vascular status in pediatric kidney transplant recipients (KTx) is unknown. **Methods:** We performed a cross-sectional, case-control study in 44 pediatric KTx patients on either everolimus (EVR) plus calcineurin inhibitor or standard treatment, i.e. mycophenolate mofetil plus calcineurin inhibitor. Measurement of carotid intima-media thickness (cIMT) via ultrasound, central pulse wave velocity (PWV) by a cuff-based oscillometric technique, and skin microvascular blood flow during local heating via laser-Doppler-fluximetry (LDF) served as marker of subclinical vascular disease. Serum concentrations of angiotensin-1 and -2, fibroblast-growth factor 23 (FGF23) and soluble klotho were measured. **Results:** EVR-treated patients exhibited a similar degree of hypertension, increased cIMT, elevated pro-inflammatory angiotensin-2, and diminished endothelial survival factor angiotensin-1 compared to healthy children but presented with a twofold more reduced skin micro-vascular function compared to standard treatment (each $p < 0.001$). By contrast, PWV and soluble klotho levels were normal in both groups. **Conclusion:** Endothelial dysfunction seems more frequent in KTx patients on EVR-based immunosuppressive regimen compared to standard immunosuppression.

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Introduction

Cardiovascular complications are the major cause of the considerably increased mortality in pediatric patients with chronic kidney disease (CKD) [1]. Kidney transplantation (KTx) is the treatment of choice in these patients but mortality rates remain markedly elevated compared to the general population [1]. Impaired microcirculation was noted in pediatric patients with CKD stage 2-3 lacking arterial hypertension and was shown to be associated with mortality rates in adult CKD patients [2-5]. In addition changes in macro-circulation e.g. elevated carotid intima-media thickness (cIMT) and pulse wave velocity (PWV) were found in 40% and 20% of children with CKD stage 3, respectively [4]. Although, endothelial function usually improves after KTx, endothelial dysfunction can be detected in up to 72% of pediatric and adult renal transplant recipients [6-10]. This may be related to pre-transplantation, prevalent, traditional and nontraditional cardiovascular risk factors that can be aggravated by the effects of steroid use after transplantation [8, 11, 12].

Optical assessment of skin microvascular function has been used for diagnostic purposes in adults for more than 20 years [13-16], and in recent years cutaneous laser-Doppler fluximetry (LDF) has become accepted as an easy and noninvasive method to investigate microcirculation and/or the neurogenic vasodilation response secondary to physical or pharmacological stimulation in children [17, 18]. A study of both cross-sectional and longitudinal design in adults with end-stage renal disease revealed that an impaired cutaneous local thermal hyperemic response was associated with increased cardiovascular mortality [5]. An increasing number of studies suggest that cutaneous microcirculation can serve as surrogate parameter for generalized microvascular dysfunction [16, 19-21].

Materials and Methods

Objective

Everolimus (EVR) is an inhibitor of mammalian targets of rapamycin (mTOR) and a potent immunosuppressant inhibiting the interleukin-2 stimulated T-cell proliferation pathway [22]. In addition, it has direct inhibitory effects on proliferation of smooth muscle cells, endothelial cells and circulating vascular progenitor cells [23, 24]. In heart transplant recipients, EVR has been associated with a diminished progression of cardiac allograft vasculopathy [25].

In the absence of studies confirming comparable benefits in kidney transplant recipients we performed a cross-sectional, multicenter, pilot study in stable pediatric kidney transplant recipients comparing patients treated with EVR plus calcineurin inhibitor (CNI) with patients receiving standard treatment, i.e. mycophenolate mofetil (MMF) plus CNI regimen. Clinical evaluation consisted of a comprehensive assessment of the macro- and micro-vascular system.

Study design

The local ethics committees approved the study and written informed consent was obtained from patients, healthy controls and/or their guardians. In pediatric kidney transplantation our center at Hannover Medical School has acquired substantial knowledge using mTOR (Everolimus) based immunosuppressive regimen over the past years [26].

Step one inclusion criteria. Patients at Hannover Medical School, aged 6 to 18 years, with a functioning first or second transplant for at least 6 months, on stable Everolimus (EVR) based immunosuppressive regimen for at least 6 months and lack of rejection within this time.

Step two inclusion criteria. Patients at all participating centers with a functioning first or second transplant for at least 6 months, on stable calcineurin-inhibitor (CNI) based immunosuppressive regimen for at least 6 months and lack of rejection within this time, matched to step one patients by: (i) age, (ii) sex, (iii) first or second transplant, (iv) time since KTx and (v) estimated glomerular filtration rate (eGFR) (Table 1).

Exclusion criteria. Clinically overt inflammatory disease at the time of investigation or pregnancy.

Patients were identified by via chart review at the centers outpatient transplant clinics.

Participants

In pre-screening at our center 51 patients with EVR-based immunosuppressive regimen were eligible for this study; 26 patients eventually agreed to take part. Four patients had to be excluded due to technical problems and incomplete data. Twenty-two patients with a full data set were included in final data analysis.

Twenty-two patients with CNI-based (standard) immunosuppressive regimen matched according to criteria mentioned above were recruited at all participating centers: Hannover (EVR n=22; CNI n=8), Essen (CNI n=7), Berlin (CNI n=4), and Rostock (CNI n=3). All patients were transplanted between 1998 and 2012.

Setting

Patients were investigated between 9 am and 12 pm in a period from 2012 to 2015. This included review of the history, a full physical examination, and collection of blood and urine samples, which were stored at -80°C. Thereafter assessment of the vascular status was performed including determination of (i) skin microvascular endothelial function by use of cutaneous LDF (ii) stiffness of the conduit arteries by measurement of pulse wave velocity (PWV), (iii) ultrasound assessment of the carotid intima-media thickness (cIMT), (iv) ambulatory blood pressure monitoring (ABPM) for the following 24 hours. In addition, Data concerning endothelial function (skin microcirculation, serum biomarkers) were compared with a group of healthy children ("healthy controls").

Data sources and measurements

Immunosuppression regimens. All patients received 300 mg prednisolone/m² body surface area (BSA) intravenously at the time of engrafting. Prednisolone was discontinued in 19/22 (86.4%) and 10/22 (45.5%) at 1.1 years and 1.8 years after KTx in the EVR and standard group, respectively (each p>0.05). In the EVR group, patients were started on low dose CsA with target trough levels at 50-100 µg/L in the first 6 months post KTx and 25-75 µg/L thereafter [27, 28]. EVR target trough levels were 3-6 ng/ml in the first 6 months post KTx and 2-5 µg/L thereafter [29]. Low dose CsA had been switched to full dose Tacrolimus (TAC) in 5 patients in the EVR group due to biopsy proven rejection. The standard treatment group received

Table 1. Patient characteristics of 44 kidney transplant recipients on everolimus-based immunosuppression and standard immunosuppression. EVR, everolimus; KTx, kidney transplantation; eGFR, estimated glomerular filtration rate; CAKUT, congenital abnormalities of the kidney and urinary tract; HUS, hemolytic uremic syndrome; SDS, SD score; BMI, body mass index; PD, peritoneal dialysis; HD, hemodialysis; Tac, tacrolimus; CsA, cyclosporine A; *p<0.05 vs. healthy children; n.a., not applicable

Parameter	EVR (n=22)	Standard (n=22)	P value
Male sex [%]	59.1	54.6	0.9999
Age [years]	13.47±0.6	14.92±0.5	0.0689
Age at KTx [years]	9.05 (1.42-15.08)	10.38 (1.42-16.17)	0.7848
Time since KTx [months]	42 (6-168)	66 (6-169)	0.3481
eGFR [ml/min/1.73m ²]	64±4.00	60.13±5.91	0.5898
Height [SDS]	-0.99±0.22*	-1.38±0.20*	0.1900
Body weight [SDS]	-0.40±0.30	-0.74±0.31*	0.4338
BMI [SDS]	0.13±0.29	-0.06±0.31	0.6551
Underlying renal diseases			0.9000
CAKUT [%]	31.8	36.4	
glomerular [%]	18.2	13.6	
HUS [%]	4.6	4.6	
hereditary [%]	22.7	18.2	
others [%]	22.7	27.3	
Transplantation modalities			
pre-emptive KTx [%]	64.3	35.7	0.3319
living donor transplantation [%]	56.6	43.8	0.7546
previous acute transplant rejection [%]	27.3	22.7	0.9999
Dialysis modalities			
mode of dialysis			0.9999
PD [%]	84.6	70.6	
HD [%]	15.4	29.4	
time on dialysis [days]	215 (0-1205)	188 (0-1059)	0.9530
Immunosuppressive therapy			
current steroid treatment [%]	13.6	54.6	0.0097
prednisolone dosage [mg/m ² /day]	0 (0-3.4)	1 (0-4.6)	0.0091
cumulative prednisolone dosage [g]	3.92 (1.09-14.88)	6.80 (1.27-65.71)	0.0291
Tac treatment [%]	22.7	68.2	0.0058
Tac dosage [mg/m ² /day]	3.27 (1.31-4.09)	2.46 (0.81-5.44)	0.8660
Tac trough level [ng/ml]	3.9 (1.3-6.4)	5.7 (4.6-11.2)	0.1181
CsA treatment [%]	77.3	31.8	0.0058
CsA dosage [mg/m ² /day]	80.4 (49.36-128.1)	127.2 (67.27-188.9)	0.0085
CsA trough level [µg/l]	49 (26-87)	80.5 (63-129)	0.0094
EVR trough level [µg/l]	3.9±1.26	n.a.	

an immunosuppressive regimen, which represents the current standard of care for most pediatric KTx patients in Europe, i.e. a CNI-based regimen consisting of CsA or TAC in conjunction with MMF.

Biochemical parameters. In addition to standard hematological and biochemical analyses, ELISA measurements were performed for c-terminal FGF23, sKlotho, angiopoietin-1 and -2 according to the manufactures advice (#60-6100, #JP27998: TECO Medical, Neufahrn, Germany; #DANG10, #DANG20: R&D Systems, Minneapolis, USA). Hyperphosphatemia, hypocalcemia, and elevated alkaline phosphatase (ALP) levels were defined according to normal values in the K/DOQI Clinical practice guidelines [30]. The revised Schwartz “bedside formula” was used for assessment of eGFR [31].

Healthy controls. As control group for microvascular function we used data from 82 healthy children, which was obtained previously for another study by our workgroup [18]. Furthermore, normal plasma levels for Ang 1 and Ang 2 were obtained from a group of 38 age-matched healthy children for this study. Age and gender related SD scores (SDS) for sKlotho and FGF23 were calculated by use of published normative values (FGF23) and 80 pediatric in house controls (sKlotho) [32].

Assessment of skin microcirculation and definitions. Skin blood flow was assessed by LDF as previously described [18]. In brief, two laser probes were used with a dual channel laser duplex monitor (VMS-LDF2, Moor Instruments, Axminster, UK) to simultaneously record skin microcirculation at different sites and conditions. A heating device with a central hole for placing the laser probe (SH02 Heater, Moor Instruments, Axminster, UK) to the right volar forearm was used to produce a thermal stimulus at one site, the other probe was used as control. After a rest period of minimum 5 minutes, thermal stimulation started at 31°C for 90 seconds. Temperature was gradually increased to 40°C and then held for a further 30 minutes. Skin blood flow was recorded, converted and displayed as a graph with 10 points per minute. Mean baseline flow was used to normalize the data of both probes. Response of flow was expressed as x-fold of baseline flow. Data from the control probe was subtracted from the data of thermal stimulation to eliminate motion artefacts. Thereafter, the complete area under the curve (AUC), the AUC of axon reflex (AR) (first peak) from start of heating to first nadir of the graph and the peak height of AR were calculated. Total AUC is the best reproducible and least interference-prone parameter [18, 33]. Therefore, it was chosen as main indicator for endothelial dysfunction, whereas time to AR and AR height were used to assess neurogenic vasodilation response. As in previous studies we defined AUC below 10th percentile as endothelial dysfunction [18, 33].

Assessment of arterial stiffness, carotid intima-media thickness, and blood pressure. Carotic-femoral PWV was assessed by use of a portable cardiovascular laboratory “Vicorder” [SMT medical, Würzburg, Germany] as published previously [34]. cIMT was measured by high-resolution B-mode ultrasound using a “Linear TH small parts/musc probe PLT-805AT” (6.2-12 mhz) with a Toshiba Xario XG console (Toshiba Medical Systems GmbH, Neuss, Germany) according to the Mannheim cIMT consensus [35]. Systolic and diastolic office BP (median of three oscillometric measurements) were assessed by using locally available devices. For assessment of ABPM, the same device type (Spacelabs 90207-2Q) was used as previously described [36]. Published normative values in children were used to calculate age- and gender- related SD scores for PWV, cIMT, and office as well as 24h BP values [34, 36, 37]. The diagnosis of hypertension was made when office or ABPM BP values exceeded the 95th percentile. Increased PWV or cIMT was defined when the respective values exceeded the 95th percentile.

Bias

EVR-based regimen was the prevalent used regimen at Hannover Children’s Hospital at time of study-implementation [26]. Unfortunately, only half of potential participants agreed to the time consuming study procedure. So, the number of patients was limited. Given the limited number of pediatric patients on EVR-based immunosuppressive regimen in Germany (one center). Although, we tried to minimize this by carefully matching participants as mentioned above, this could only be a pilot study and may be limited by a potential selection bias. In order to prevent a bias in measurement of skin-microcirculation and assessment of arterial stiffness, carotid intima-media thickness and blood pressure standardized procedures were used, e.g. given daytime of participation in the study, predetermined positions of laser-Doppler probes and

applied heating-protocols. Due to the overall time consuming protocol (approximately 2 hours), repeated measurements with the same patient were not feasible.

Statistical methods

Statistical analysis was performed using Prism 7.0 (GraphPad Software, Inc., La Jolla, USA) and IBM SPSS Statistics 24.0 (International Business Machines Corp., Armonk, USA). The level of significance was set at a $p < 0.05$. Normal distribution was tested with the Kolmogorov Smirnov Test and normally distributed data are given as mean $\pm$ SD whereas median and range is given for non-normally distributed data. In testing for significant differences, the t-test was used for normally distributed data, otherwise the Mann Whitney U-test was applied. Differences in subgroups were tested using Fisher's exact test. Potential predictors of LDF AUC, endothelial dysfunction (LDF AUC $< 10^{\text{th}}$ percentile), PWV, and cIMT were identified by using Spearman's rank correlation. Variables from this single regression analysis with a p-value < 0.1 were transferred to a multiple linear regression analyses model using a stepwise technique to identify independent predictors of vascular status.

Results

Patient characteristics

Patients were investigated after a mean period of 5.2 years post KTx with a mean age of 14.8 years, and a mean eGFR of 62 ml/min per 1.73m² (Table 1). Mean time on dialysis and mode of dialysis treatment prior to KTx, the number of previous transplant rejections, and underlying renal diseases did not differ between groups (each $p > 0.05$) (Table 1). The proportion of living-related and preemptive KTx was higher in the EVR group compared to the standard treatment group, although this did not reach statistical significance. The percentage of patients on daily glucocorticoid therapy and the cumulative steroid exposure was significantly lower in the EVR group compared to the standard treatment group (each $p < 0.01$).

Main results

Hypertension. The vast majority of patients (87%) received antihypertensive drugs. Its prevalence and the use of angiotensin-converting enzyme inhibitors (ACEI) or betablockers were comparable in both groups (Table 2). Mean daytime and nighttime 24-hour MAP values were similarly elevated in both patient groups compared to healthy children (each $p > 0.05$ EVR vs. standard) (Table 2). Using the 95th percentile of 24-hour MAP values, 6 patients (27.3 %) in the EVR group and 3 patients (14.3 %) in the standard treatment group were hypertensive ($p = 0.456$). Using the 95th percentile of office BP values, 7 patients (33.3 %) in the EVR group and 6 patients (35.3 %) in the standard treatment group were hypertensive ($p = 0.993$).

Endothelial function. All parameters of skin microcirculation were significantly diminished in KTx patients compared to healthy controls. The mean skin blood flow was reduced by approximately 50 - 60% compared to healthy children with respect to total AUC (196 (28-583) vs. 489 (165-990) rPU*min), AUC of axon reflex (18 (2.5-81) vs. 49 (10-55) rPU*min), and axon reflex amplitude (6.6 (2-24) vs. 13 (6-30) rPU), respectively (each $p < 0.001$). Endothelial dysfunction (LDF AUC $< 10^{\text{th}}$ perc.) was seen in 77% of patients. Moreover, there were significant differences in microcirculation measurements between groups of KTx patients (Table 2). In the EVR group, mean total AUC, AUC axon reflex, and axon reflex amplitude were significantly lower compared to the standard group (each $p < 0.01$; Fig. 1A-1C). All EVR patients showed endothelial dysfunction (LDF AUC $< 10^{\text{th}}$ perc.), whereas this was observed in only 12 patients (55 %) in the standard treatment group ($p < 0.001$).

Table 2. Endothelial function and vascular status of 44 kidney transplant recipients on everolimus-based immunosuppression versus standard immunosuppression. LDF, laser-Doppler fluximetry; AUC, area under the curve; AR, axon reflex; cIMT, carotid intima-media thickness; SDS, SD score; MAP, mean arterial pressure; BP, blood pressure; ACE, angiotensin converting enzyme; ARB, angiotensin receptor blocker; #p<0.001 vs. healthy children; *p<0.05 vs. healthy children

Parameter	EVR (n=22)	Standard (n=22)	P value
LDF			
AUC [rpU*min]	118.2 ± 13.84*	274.4 ± 34.45*	0.0001
AUC AR [rpU * min]	14.3 (3.52 - 35.1)*	22.6 (2.53 - 81.0)*	0.0063
AR Amplitude [rpU]	5.79 ± 0.53*	9.93 ± 1.22*	0.0035
cIMT			
[mm]	0.48 (0.39 - 0.58)	0.47 (0.30 - 0.65)	0.4715
[SDS]	1.72 (0.38 - 3.86)*	1.43 (-1.4 - 4.39)*	0.5830
PWV			
[m/s]	4.83 ± 0.46	5.11 ± 0.91	0.1076
[SDS]	-0.01 ± 0.26	0.61 ± 0.42	0.2162
24h MAP			
daytime [mmHg]	90 (81 - 105)	88.5 (82 - 108)	0.3594
[SDS]	0.79 (-0.48 - 2.89)*	0.26 (-0.46 - 4.38)*	0.3076
nighttime [mmHg]	76 (64 - 88)	77 (66 - 107)	0.8325
[SDS]	0.91 (-1.46 - 2.42)*	0.76 (-0.96 - 7.19)*	0.4444
> 95 th perc. [%]	14.3	27.3	0.4566
Office BP			
systolic [mmHg]	118.5 ± 2.1	122.8 ± 3.9	0.3162
[SDS]	0.90 ± 0.23*	0.60 ± 0.57*	0.5805
diastolic [mmHg]	70.1 ± 1.7	75.2 ± 3.1	0.1339
[SDS]	0.57 ± 0.25*	0.98 ± 0.54	0.4382
> 95 th perc. [%]	33.3	35.3	0.9998
Antihypertensive therapy			
patients treated [%]	86.4	86.4	0.9999
number of drugs	1.91 ± 0.28	1.96 ± 0.28	0.9040
ACE or ARB inhibitors [%]	68.2	54.6	0.5365
betablockers [%]	40.9	54.6	0.5467

Pulse wave velocity and cIMT. Mean PWV SDS of KTx patients did not significantly differ from healthy children irrespective of the immunosuppressive regimen (Fig. 2 and Table 2). Only 1 patient (4.5%) in the EVR group and 4 patients (18%) in the standard group demonstrated elevated PWV (p=0.175). Mean cIMT SDS was comparably elevated in both patient groups compared to controls (each p<0.01 vs. healthy children). Increased cIMT was noted in 7 patients (36.8%) and 6 patients (35.3 %) in the EVR group and the standard group, respectively (p=0.999; Fig. 2).

Serum markers of vascular health. Mean serum angiotensin-1 levels were reduced and angiotensin-2 levels were elevated in both treatment groups, resulting in a markedly increased angiotensin-2/angiotensin-1

Fig. 1. A-C: Skin microcirculation as assessed by area under the curve (AUC), axon reflex (AR) AUC, and axon reflex amplitude during local heating induced vasodilation in kidney transplant recipients on everolimus-based immunosuppression (EVR, grey circles) versus standard immunosuppression (Standard, dark grey circles), and healthy controls (Controls, light grey circles); rpU, relative perfusion unit; broken horizontal lines indicate the 10th percentile in healthy controls.

Fig. 2. Pulse wave velocity (PWV) and carotid intima-media thickness (cIMT) in kidney transplant recipients on everolimus-based immunosuppression (EVR, red circles) versus standard immunosuppression (Standard, blue circles). Broken horizontal lines indicate the upper (+2SD) and lower (-2SD) normal range, respectively. * $p < 0.001$ compared to healthy controls. Mean PWV SDS and cIMT SDS did not significantly differ between the EVR and standard treatment group.

Fig. 3. A-C: Serum angiopoietin 1, angiopoietin 2, and angiopoietin-2/angiopoietin-1 ratio (Ang2-/Ang-1 ratio) in kidney transplant recipients on everolimus based-immunosuppression (EVR, grey circles) versus standard immunosuppression (Standard, dark grey circles), and healthy controls (Controls, light grey circles).

ratio compared to healthy controls (Fig. 3A-3C, Table 3). Angiopoietin-1 levels were lower in EVR than in the standard treatment group, although this did not reach statistical significance ($P > 0.05$). Compared to healthy children, mean serum FGF23 SDS levels were significantly elevated in the standard treatment group only. Total and albumin-adjusted serum calcium levels were significantly higher in the standard treatment group compared to the EVR group. Likewise, patients in the EVR group were more frequently hypocalcemic (40.1% vs. 0%, $p < 0.01$), whereas hyperphosphatemia was observed more frequently in the standard treatment group (50.0% vs. 18.2%, $p < 0.01$). Serum ALP levels were increased above the normal range in 50.0% and 38.1% in the EVR and standard treatment group, respectively ($p = 0.751$). Mean serum cholesterol levels were significantly higher and hemoglobin levels were lower in EVR compared to the standard treatment group (each $p < 0.05$). By contrast, mean serum levels of sKlotho, and 25-hydroxyvitamin D were normal irrespective of treatment. (Table 3).

Predictors of endothelial function, PWV, and cIMT

Results of univariate correlation analysis of variables associated with endothelial function, PWV, and cIMT is shown in Table 4. Multiple linear regression analysis revealed EVR

Table 3. Markers of vascular health in kidney transplant recipients on everolimus-based immunosuppression versus standard immunosuppression. Ang, angiotensin; FGF23, fibroblast growth factor 23; sKlotho, soluble Klotho; SDS, SD score; *p<0.05 vs. healthy controls

Parameter	EVR (n=22)	Standard (n=22)	P value
Angiotensin-1 [pg/ml]	2838 (1159-43485)*	4746 (1027-30212)*	0.5553
Angiotensin-2 [pg/ml]	1658 (1036-3294)*	2035 (900-4688)*	0.3070
Ang-1/Ang-2 ratio	0.5 (0-1.3)*	0.1 (0-2.9)*	0.8757
sKlotho			
[pg/ml]	2829 (503-8598)	2192 (278-14739)	0.0653
[SDS]	0.28 (-1.3-2.54)	-0.43 (-2.1-3.27)	0.0601
FGF23			
[RU/ml]	50.45 (5.73-476)	117.4 (27.67-260)	0.0099
[SDS]	1.03 (-4.1-7.17)	1.33 (-2.1-4.32)*	0.0128
Calcium			
[mmol/l]	2.37 ± 0.02	2.47 ± 0.02	0.0038
adjusted [mmol/l]	2.29 (2.1-5.44)	2.36 (2.21-2.66)	0.0096
Albumin [g/l]	43.64 ± 0.53	43.67 ± 0.53	0.9614
Phosphate [mmol/l]	1.36 ± 0.27	1.36 ± 0.24	0.9763
Alkaline phosphatase [U/l]	234.3 ± 25.07	194 ± 22.71	0.2400
25-hydroxyvitamin D [µg/l]	25.4 (10.51-37.54)	25.36 (5.92-43.8)	0.3730
25-hydroxyvitamin D > 30 µg/l	54.6 %	45.5 %	0.7195
iPTH [ng/l]	68.35 (20.9-215)	58.95 (32.9-457)	0.6672
Creatinine [µmol/l]	84.15 (55.7-293)	103.4 (46.9-396)	0.3022
Blood urea [mmol/l]	8.35 (4-19.4)	7.6 (3.7-25.3)	0.3421
Cholesterol [mg/dl]	198.7 ± 9.64	164.2 ± 10.18	0.0181
Triglycerides [mg/dl]	151 (45-831)	121 (48-411)	0.4924
Hemoglobin [mg/dl]	11.63 ± 0.26	12.55 ± 0.30	0.0259
Albuminuria [g/mmol]	1.98 (0.54-33)	4.41 (0.34-347)	0.1969

Table 4. Correlation analysis of variables associated with endothelial function, pulse wave velocity, and carotid intima-media thickness. LDF, laser-Doppler fluximetry; AUC, area under the curve; PWV, pulse wave velocity; cIMT, carotid intima-media thickness; SDS, SD score; BP, blood pressure; EVR, everolimus; KTx, kidney transplantation; Tac, tacrolimus; CsA, cyclosporine A; ALP, alkaline phosphatase; ULN, upper limit of normal level; FGF23, fibroblast growth factor-23

Parameter	LDF AUC	LDF AUC < 10 th perc.	PWV SDS	cIMT SDS
Age [years]	r = 0.378; p = 0.011	r = -0.339; p = 0.013		
Sex [female]	r = 0.428; p = 0.004	r = -0.289; p = 0.030		
Age at KTx [years]	r = 0.287; p = 0.009			
Diastolic office BP [SDS]			r = 0.497; p = 0.020	
EVR treatment		r = 0.640; p < 0.001		
EVR dosage [mg/m ² /day]	r = -0.523; p < 0.001	r = 0.491; p < 0.001		
EVR trough level [µg/dl]	r = -0.468; p = 0.001	r = 0.578; p < 0.001		
CsA treatment	r = -0.334; p = 0.027	r = 0.384; p = 0.005		
MMF treatment	r = 0.503; p < 0.001	r = -0.586; p < 0.001		
MMF dosage [mg/m ² /day]		r = -0.625; p < 0.001		
TAC treatment		r = -0.384; p = 0.005		
TAC dosage [mg/m ² /day]		r = -0.303; p = 0.024		
TAC trough level [ng/ml]				r = 0.315; p = 0.061
Prednisolone treatment		r = -0.368; p = 0.008		
Adjusted calcium [mmol/l]	r = 0.319; p = 0.035	r = -0.325; p = 0.017		
ALP [U/l]	r = -0.436; p = 0.005			
ALP > ULN	r = -0.153; p = 0.174			
iPTH [ng/l]				r = -0.287; p = 0.090
FGF23 [SDS]			r = 0.430; p = 0.002	

Table 5. Linear regression models of variables associated with endothelial function, pulse wave velocity and carotid intima-media thickness after stepwise variable selection. LDF, laser-Doppler fluximetry; AUC, area under the curve; EVR, everolimus; TAC, tacrolimus; ALP, alkaline phosphatase; UNL, upper limit of normal level; PWV, puls wave velocity; cIMT, carotid intima-media thickness; SDS, SD score; BP, blood pressure

Outcome	Predictor	Beta (95% CI)	P value	Adjusted r ²
LDF AUC	EVR treatment	-0.424 (-185.9; -58.5)	<0.001	0.586
	Sex [female]	0.421 (61.2; 182.7)	<0.001	
	Age [years]	0.395 (8.7; 33.8)	0.001	
	ALP > ULN	-0.321 (-157.2; -28.7)	0.006	
LDF AUC < 10. perc.	EVR treatment	0.625 (0.398; 0.852)	<0.001	0.459
	Sex [female]	-0.271 (-0.499; -0.043)	0.021	
PWV SDS	Diastolic office BP [SDS]	0.552 (0.3; 0.8)	0.001	0.284
cIMT SDS	TAC trough level [ng/ml]	0.341 (0.1; 0.4)	0.042	0.117

treatment, gender, age, and ALP levels above the upper limit of normal as independent predictors of skin microcirculation as measured by the LDF-AUC (Table 5). Neither TAC nor CsA were significantly associated with LDF-AUC and were therefore omitted from the final regression model. Likewise, EVR treatment and male gender were independent predictors of endothelial dysfunction (AUC < 10th percentile), whereas TAC and CsA were not included in the model. PWV SDS was positively associated with diastolic office BP SDS and FGF23 SDS. The TAC trough serum level was the only independent factor associated with cIMT in the multivariable analysis (Table 5).

Discussion

This is the first pilot study investigating micro-circulation in pediatric patients after kidney transplant (KTx) and comparing different immunosuppressive regimens. The sample size of this pilot study represents about 10% of children aged 6-18 with working kidney transplant in Germany [38, 39]. In this matched cross-sectional study we found significant differences in micro-vascular parameters between patients treated with either an EVR-based regime or a standard immunosuppressive treatment. Against our expectation, EVR-treated patients showed a profound decrease of skin microvascular function parameters compared to standard treatment patients. Indeed, all patients on EVR showed decreased thermal hyperemic response, whereas this was observed in only half of patients in the standard treatment group. Of note, both groups of KTx patients exhibited a comparable degree of hypertension, increased cIMT, as well as elevated proinflammatory angiotensin-2 and diminished endothelial survival factor angiotensin-1 compared to healthy children. Of note EVR treated patients received less CNI and glucocorticoid medication compared to those on standard treatment. Neither TAC nor CsA was associated with skin micro-circulation and endothelial function. Since both, CNI and glucocorticoid medication, are known to impair endothelial function [40] it is unlikely that these discrepancies contributed to the observed higher frequency of endothelial dysfunction in the EVR group.

With Laser-Doppler fluximetry relative changes in skin microcirculation occurring in a small volume (approx. 1mm³) of tissue in response to an exogenous stimulus, i.e. heat, are accurately detected and quantified [15, 17, 41]. Thermal hyperemia response is mediated by two independent phases: The initial rapid phase is predominantly mediated by local sensory nerves and can be significantly attenuated in the presence of local anesthesia [16]. In contrast, the following plateau is mediated by NO, but also by sympathetic neurotransmitters i.e. norepinephrine and neuropeptide Y [42]. Since the development of this method, microvascular dysfunction has been constantly associated with several

vascular diseases as well as with aging [16, 17, 41]. A correlation between abnormalities in cutaneous microcirculation and microvascular alterations in other organs has been reported for coronary heart disease, retinal vascular damage in diabetes and CKD, among others, suggesting that human cutaneous circulation may be a surrogate marker of systemic microvascular dysfunction in various diseases [17]. One of the most remarkable studies on LDF evaluated thermal hyperemic response using LDF in end-stage renal adults in both cross-sectional and longitudinal design [5]. Patients with abnormal cutaneous thermal responses showed significantly increased cardiovascular mortality. This high-risk population was characterized by a markedly diminished local thermal hyperemic response at initial heat peak, nadir and plateau. The most robust parameter in this study was the total area under the curve.

Endothelial dysfunction is characterized by an imbalance between nitric oxide (NO) bioavailability and oxidative stress [43]. Overall, 77% of patients showed endothelial dysfunction, which is well in line with previous studies in children and adult KTx patients [8-11]. Several *in vitro* studies suggest that mTOR inhibitors may impair endothelial cell function by inhibition of endothelial NO synthase (eNOS) activity [44-46]. Moreover, a negative effect of mTOR inhibitors on endothelial-dependent vasodilation was previously described in humans undergoing coronary mTOR-inhibitor-eluting-stent implantation. While use of this type of drug-eluting stents is intended to prevent neo-intimal proliferation [47] a paradoxical, vasoconstrictive response to acetylcholine in the vascular area next to the stent was observed 6 months after implantation [47]. Endothelial senescence has been proposed to be involved in endothelial dysfunction, atherogenesis, and thrombosis. Treatment of human umbilical vein endothelial cells (HUVEC) with sirolimus and everolimus significantly caused a senescent phenotype and plasminogen activator inhibitor 1 up-regulation, associated with a decrease in endothelial eNOS and sirtuin 1 (Sirt1) expression [48]. Sirt1 has been recognized as a key regulator of vascular endothelial homeostasis controlling angiogenesis, endothelial senescence, and dysfunction [49, 50]. Though, it remains unclear, if the effects of mTOR inhibitors on endothelial cells *in-vitro* are easily transferable to the complex situation of the whole living body.

The cIMT exceeded the 95th percentile in approximately one third of patients, which is well in line with previous reports in pediatric CKD patients prior to and after KTx [4, 37, 51]. Interestingly, TAC trough levels appeared as the only independent factor associated with cIMT in the present study. However, one has to bear in mind that several other factors were independently associated with cIMT in pediatric CKD patients in larger studies, i.e. physical activity, obesity, and serum phosphorus, which were not fully controlled for in the present study [4]. Aortic stiffness was shown to be an independent predictor of cardiovascular mortality in adult CKD patients [52]. In line with a recent study in a large cohort of pre-dialysis pediatric CKD patients, PWV was independently associated with diastolic BP SDS [4].

Angiopietin-1 (Ang1) is an endothelial survival factor, whereas angiotensin-2 (Ang2) shows proinflammatory and anti-angiogenic actions [53, 54]. An Ang1/Ang2 imbalance is thought to be a serum marker for endothelial dysfunction [33, 55, 56]. Reduced serum levels of Ang1 and/or increased Ang2/Ang1 ratios have been reported in pediatric patients with pre-dialysis CKD and hemolytic uremic syndrome and were shown to be significantly associated with cardiovascular measures, e.g. BP and cIMT [33, 56]. In the present study Ang1/Ang2 imbalance was frequently present in KTx patients irrespective of immunosuppressive regimen. Comparable studies are missing, but our data suggest, that some serum markers for generalized endothelial dysfunction are still present after kidney transplantation. Further studies are necessary to elucidate this finding.

A recent *in vitro* study using cultured human coronary artery endothelial cells demonstrated that, in the presence of klotho, FGF23 induces NO release and its stimulating effects on oxidative stress are counterbalanced by increased reactive oxygen degradation [57]. This may at least partly explain the lack of association between endothelial function

and FGF23 levels in the present study, as sklotho levels were normal in the majority of our patients.

Several limitations of this pilot study must be noted. Patient numbers were low and the follow-up time after KTx was comparatively short (5.2 years) short and thus, within the pediatric age span. Admittedly, even matched carefully, both groups remain heterogenous in many regards and the cross-sectional study design does not allow for drawing conclusions on the progression of vascular damage. Therefore, our findings need further confirmation by longitudinal follow-up in larger patient cohorts to further assess the predictive value of a diminished endothelial function in KTx patients.

Conclusion

Contrary to our expectations, the patient group treated with EVR showed more hints of endothelial dysfunction in this study of pediatric KTx recipients compared to children with standard immunosuppression regimen. By contrast, the prevalence of increased cIMT and PWV as a measure of premature atherosclerosis and arterial stiffness, respectively, were similar in both groups. Also, there was no significant difference in serum markers for endothelial dysfunction (Ang1/Ang2 ration) between groups. This pilot study highlights the need for subsequent longitudinal studies with larger sample size investigating endothelial (dys-)function and influencing factors in pediatric kidney transplant recipients.

Abbreviations

EVR (everolimus); KTx (kidney transplantation); eGFR (estimated glomerular filtration rate); CAKUT (congenital abnormalities of the kidney and urinary tract); HUS (hemolytic uremic syndrome); SDS (SD score); BMI (body mass index); PD (peritoneal dialysis); HD (hemodialysis); Tac (tacrolimus); CsA (cyclosporine A); cIMT (carotid intima media thickness); PWV (pulse wave velocity)

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D.H. and M.K. designed the study. D.H. and M.K. drafted the study protocol. S.R. performed the laser Doppler fluximetry measurements in patients. D.C.F. provided data of healthy controls for vascular parameters. M.L.N. performed the biomarker assays and provided data of healthy controls. A.M. performed measurements for cIMT. S.R., M.K., and D.H. evaluated the data and produced the statistics. S.R., M.K., L.P. and D.H. wrote the first draft of the paper. S.R., M.K., A.B, R.B., J.T., U.Q., L.P., T.A. and H.S. recruited patients for this study. All authors read, revised and approved the manuscript.

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